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Clinical peer review in the United States: History, legal development and subsequent abuse

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Core tip: This article will highlight progress and drawbacks of the current clinician's peer review system prevailing in the United States.

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Abstract

The Joint Commission on Accreditation requires hospitals to conduct peer review to retain accreditation. Despite the intended purpose of improving quality medical care, the peer review process has suffered several setbacks throughout its tenure. In the 1980s, abuse of peer review for personal economic interest led to a highly publicized multimillion-dollar verdict by the United States Supreme Court against the perpetrating physicians and hospital. The verdict led to decreased physician participation for fear of possible litigation. Believing that peer review was critical to quality medical care, Congress subsequently enacted the Health Care Quality Improvement Act (HCQIA) granting comprehensive legal immunity for peer reviewers to increase participation. While serving its intended goal, HCQIA has also granted peer reviewers significant immunity likely emboldening abuses resulting in Sham Peer Reviews. While legal reform of HCQIA is necessary to reduce sham peer reviews, further measures including the need for standardization of the peer review process alongside external organizational monitoring are critical to improving peer review and reducing the prevalence of sham peer reviews.

INTRODUCTION

In 1952 the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) began requiring physician peer review at all United States hospitals^[1]. However, economic abuse of the review process and a subsequent court ruling in 1986 lead many physicians to fear the possible consequences in participating in peer reviews^[2]. In order to legislatively solidify the role of peer review as a means of physician quality improvement across the United States, Congress enacted the Health Care Quality Improvement Act (HCQIA) in 1986^[2,3]. Despite its intended role of physician quality improvement, HCQIA has unintentionally led to significant abuse of the peer review system across the United States^[4]. This review focuses on the history and legal development of physician peer review in the United States, and addresses subsequent abuses resulting in what is known today as "Sham Peer Review".

What is peer review?

Peer review is the process whereby doctors evaluate the

quality of their colleagues' work in order to ensure that prevailing standards of care are being met^[5]. The process has its roots dating back to the early 20th century when the American College of Surgeons began using peer review as a means of defining minimum standard of care requirements for hospitals and their medical staff^[6,7]. Today, the majority of peer review conducted in the United States occurs exclusively through retrospective chart review *via* peer review committees. The ultimate decision making authority however often lies with the hospital board of directors, often which follows the recommendations of the review committees^[8]. The process has continued to grow in the 20th century and is now required by the JCAHO for hospital accreditation^[9].

Currently, there are three main reasons peer reviews are conducted throughout the United States. First, in order to maintain accreditation, hospitals are required to initiate peer reviews for all privileges requested for new physicians and any new requests by existing physicians for new privileges^[9,10]. Second, while initiation of peer reviews can often be triggered by substandard physician performance as required by JCAHO, physician colleague and hospital administrators can often request peer reviews of specific physicians that can be granted or denied by the hospital's peer review committee^[4,10-12]. Finally, some hospitals have used peer review to improve quality by randomly selecting cases or designing schemes looking at poor outcome cases in order to determine root causes^[8]. Nonetheless, despite being mandated by JCAHO, the manner in which peer reviews are conducted, analyzed, and utilized varies widely across institutions^[8].

History of peer review

Physician regulation was strongly opposed by both the public and physicians in the early 19th century^[10]. Despite the opposition, governmental and medical societies saw a critical need for the standardization of care in order to protect both the public and the medical profession. In turn, State Medical Licensure Boards were created in the late 19th century with an emphasis on creating peer review systems to monitor physician behavior^[10]. However, both the American Medical Association and the United States Department of Health and Human Services saw that efforts by these organizations did not meet standardized criteria for improving care and enforcing disciplinary action^[11,12]. This deficiency was attributed mainly to physician unwillingness to conduct peer reviews^[13].

To further exacerbate these concerns, disciplinary action handed down by either hospitals or State Medical Licensure committees was often circumvented by "State Hoppers", or, physicians who avoided disciplining actions by moving to another state or hospital which were not aware of their previous disciplinary action^[3,13]. In response, States developed a national data bank of disciplinary action to stop such actions. Unfortunately, the data bank was often found to be ineffective^[13].

Patrick vs Burget

The peer review process further suffered a major blow in

1986 when Dr. Timothy Patrick, a general and vascular surgeon, sued Columbia Memorial Hospital (CMH) after being unfairly subjected to a bad faith peer review for economic reasons^[14]. Upon starting practice in the small town of Astoria, Oregon, Dr. Patrick joined a group of established surgeons at the Astoria Clinic. After several years of employment Patrick was offered partnership at the clinic which he later refused in order to open his own, competing surgical practice in the same geographic area. In retaliation, Patrick's former colleagues at the Astoria Clinic reported Patrick to the hospital executive committee at CMH for peer review. The charges levied claimed that Patrick exhibited irresponsible behavior towards patient care. An executive peer review committee was formed and was chaired by Dr. Gary Boeling, a partner of the Astoria Clinic. After an investigation was conducted and subsequent false evidence concerning Patrick's care was presented, the committee voted to terminate Patrick's privileges at CMH. Fearing termination, Patrick instead chose to resign^[14].

A subsequent federal antitrust lawsuit filed by Patrick against partners of the Astoria Clinic, including Dr. William Burget, claimed that the defendants participated in a bad faith peer review in order to stifle competition. The United States Supreme court which later ruled in Patrick's favor awarded the plaintiff \$2.2 million and further disbanded the Astoria Clinic based on the clinic's violation of the Sherman Antitrust Act^[14,15].

Following the Patrick verdict many physicians became hesitant to participate in peer review activities as they feared possible involvement in future litigation. More concerning at the time was that malpractice lawsuits were at an all-time high during the same period. Viewing peer review as a critical means of decreasing the number of malpractice claims, then Rep. Ron Wyden (now Senator), brought forth legislation known as the HCQIA to expand reviewer immunity in order to encourage physician participation in the process^[16].

HCQIA and the national data bank

Five reasons were explicitly stated by congress for the enactment of HCQIA (Table 1). HCQIA consists of two parts. Part A of the law grants hospitals and reviewers immunity from litigation resulting from physicians aggrieved by the process. In order to qualify for this immunity however, congress set four minimum requirements that must be met when conducting peer reviews (Table 2)^[17]. Part B of the law tackled the issue of "state hoppers" by creating the National Practitioner Data Bank (NPDB). The NPDB was created to serve as a centralized repository given the authority to collect and release information relating to the competence and professionalism of physicians. Currently, in order to gain clinical privileges at hospitals, all practitioners are required by law to be screened through the NPDB^[18]. The NPDB receives three types of reports: adverse actions, malpractice payments, and Medicare/Medicaid exclusion reports. Table 3 further quantifies the types of reports in the NPDB. The NPDB can only be ac-

Table 1 Congressional reasons for law enactment

| |
|--|
| The increasing occurrence of medical malpractice and the need to improve the quality of medical care have become nationwide problems that warrant greater efforts than those that can be undertaken by any individual state |
| There is a national need to restrict the ability of incompetent physicians to move from State to State without disclosure or discovery of the physician's previous damaging or incompetent performance |
| This nationwide problem can be remedied through effective professional peer review |
| The threat of private money damage liability under Federal laws, including treble damage liability under Federal antitrust law, unreasonably discourages physicians from participating in effective professional peer review |
| There is an overriding national need to provide incentive and protection for physicians engaging in effective professional peer review |

Table 2 Part A Health Care Quality Improvement Act peer review immunity requirements

| |
|---|
| Peer review action is taken: |
| In the reasonable belief that the action was in furtherance of quality of care |
| After a reasonable effort to obtain the facts of the matter |
| After adequate notice and hearing procedures are afforded to the physician involved or after such other procedures as are fair to the physician under the circumstances |
| In the reasonable belief that the action was warranted by the facts known after such reasonable efforts to obtain the facts |

Table 3 Causes of reports to the National Practitioner Data Bank (Satiani 2004)

| |
|---|
| Adverse actions (17%) |
| Peer review findings adversely affect the clinical privileges of physicians or dentist for more than 30 d |
| Privileges are restricted or surrendered while under peer review investigation for possible incompetence or improper professional conduct |
| Privileges are restricted or surrendered in exchanged for peer reviewers not conducting an investigation |
| Physician's or Dentists' license are revoked, suspended, or surrendered |
| Physicians or Dentists are censured, reprimanded, or put on probation |
| Malpractice payments (82%) |
| Insurers settling claims or judgments relating medical malpractice on behalf of physicians |
| Medicare/medicaid exclusion reports (1%) |

Percentage refers to proportion of reports attributable to 132896 physicians in the National Practitioner Data Bank in 2002.

cessed by third parties directly involved in physician regulation including hospitals, state medical boards, and professional societies^[19]. Despite repeated efforts by public consumer groups to access the NPDB however, congress has kept the database confidential and closed to consumer review^[18,20].

SHAM PEER REVIEW

Sham peer review is characterized as a review called for by either a single, or group of physicians, conducted in order to lead to adverse action taken by the review committee^[21]. Prior to HCQIA, such bad faith cases could often be fought in court as in the Patrick case. However, the extraordinary levels of immunity granted to hospitals and peer reviewers under HCQIA have inhibited such successful endeavors. Currently the prevalence of such cases in the medical community is undefined due the dearth of published literature on the subject^[21,22]. As an estimate however, thirty three lawsuits were brought to United States courts claiming sham peer review between 2003-2007^[23]. Further estimates put the number of sham peer reviews occurring at upwards of 10% of cases reviewed^[24].

Legislative history of HCQIA

In the process of drafting HCQIA, the Patrick *vs* Burget

ruling was delivered by the Supreme Court and many members of congress saw further need to protect peer reviewers. However, congress was simultaneously well aware of the real potential for abuse the law had. In turn, original immunity provisions granted by the HCQIA were specifically scaled back in order to avoid misinterpretation of the law^[25]. In fact, Rep. Henry Waxman, floor manager of the bill at the time, stated that "Bad faith peer review activities permitted by the Patrick case could never obtain immunity under H.R. 5540"^[26]. Nevertheless, since its initiation in 1986, the congressionally written HCQIA has been transformed from a law granting hospitals and peer reviewers limited immunity provisions into a law that today grants nearly absolute immunity by the courts^[26].

HCQIA immunity and the courts

In one example of claimed peer review abuse, Dr. Susan Meyer, an emergency room physician at Sunrise Hospital, was required to undergo review after her treatment of Adolph Anguiano, a homeless patient who two hours after being seen by her in the ER, died in the parking lot of Sunrise Hospital^[27]. Upon entering the ER, Meyer performed a full physical exam, took vital signs, measured oxygenation levels of Mr. Anguiano and subsequently determined the patient did not require any acute medical care and later discharged the patient from the

ER. Upon discovering that Mr. Anguiano had died, Dr. Graham Wilson, Chair of the Department of Emergency Services advised Dr. Meyer to finish her shift in the ER and subsequently informed her that she was being suspended due to her substandard care. She was advised to obtain legal counsel in order to undergo a fair hearing process.

Meyer, who later lost an appeal of her case in the Nevada Supreme Court, was later informed by Dr. Rick Kilburn, the Chief Operating Officer of Sunrise Hospital, that she would be suspended regardless of the result of her peer review hearing. Despite knowing the final result beforehand, Meyer requested a formal peer review by the hospital in order to have her clinical judgment assessed by her colleagues. Despite several Emergency room physicians testifying that Meyer's treatment was "well within the standard of care", the review committee found otherwise and recommended her suspension. The recommendation was reaffirmed by the Appellate Review Committee of the hospital.

Meyer in turn filed a civil action lawsuit against Columbia Sunrise hospital alleging a breach of contract and breach of the covenant of good faith and fair dealing. The hospital, claiming immunity under HCQIA in turn succeeded in dismissing the case in district court. The case was met with the same decision at the Nevada Supreme Court. However, the Justices gave a rare glimpse into the reason for Meyer's loss and the extent of the powerful immunity granted to hospitals and peer reviewers in their concluding summary statement.

I must concur in the result reached in the majority opinion because HCQIA sets such a low threshold for granting immunity to a hospital's so-called peer review. Basically, as long as the hospitals provide procedural due process and state some minimal basis related to quality health care, whether legitimate or not, they are immune from liability. Unfortunately, this may leave the hospitals and review board members free to abuse the process for their own purposes without regard to quality medical care.... Unfortunately, the immunity provisions of HCQIA sometimes can be used, not to improve the quality of medical care, but to leave a doctor who is unfairly treated without any viable remedy [emphasis added]^[27].

In a second, similar sham review case, Dr. Carol Bender, an internist, brought a lawsuit against the Maryland Suburban Hospital to the Maryland Special Court of Appeals for a breach of contract and early termination alongside defamation *via* the peer review process^[28]. The court ruled against Bender despite having "legitimate gripe (with the hospital)" stating that the hospital was granted immunity under HCQIA despite how "reprehensible some of [the peer reviewers] actions may have been"^[28]. In another example of *Jenkins v. Methodist Hospital of Dallas*, United States District Court of the Northern District of Texas, held that the court was troubled that a statute exist under HCQIA granting immunity to individuals that are knowingly providing false information to the courts^[29].

Characteristics of sham peer review

Two types of physicians are targeted in sham peer review. The first are often competitors to an often larger, more powerful physician group^[21,22]. The second are often outspoken critics of patient quality of care or safety issues seen as whistleblowers by hospital leadership^[21,22]. William Parmley, currently the immediate past Editor-in-Chief of the *Journal of the American College of Cardiology*, has recently characterized three sham peer review cases he has recently been presented with^[21]. The cases describe either solo practitioners or practitioners working in small groups at private hospitals. Their accusers are often large groups that appear to be moving against them using peer review in order to stifle competition. The accusers often have positions on the executive hospital board or, are deeply connected to the board. In one case, Parmley describes a situation where an external peer review committee was hired by the hospital to give a bad faith review. The result was the loss of hospital privileges for two of three physicians and in turn their forced relocation. The third physician was cleared of any wrongdoing at the expense of severe financial loss. Parmley further describes these scenarios as being "far more common than is appreciated"^[21].

NPDB reporting

Hospitals are mandated by law to query practitioner's request of clinical privileges, or admission to the medical staff and re-queries are required every 2 years for any clinician on staff^[30,31]. Moreover, hospitals are required to report any adverse actions to the NPDB (Table 3)^[31]. Sham peer reviews rely heavily on the fear of physicians being reported to the NPDB^[4]. Physicians reported to the NPDB face significant hurdles when seeking employment, licensure, and credentialing^[4]. Physicians are often questioned about all previous reports to the NPDB prior to receiving any hospital credentialing activities^[4,31]. Furthermore, HMOs and insurance carriers are increasingly using the NPDB when choosing physicians to be covered under provider panels^[4]. Single transgressions in the NPDB or loss of medical privileges can often result in further negative consequences as physicians become progressively dropped from these provider panels^[4,32].

Consequences of sham peer review

In light of the immunity granted to peer reviewers and hospitals, many physicians find themselves victims of sham peer review without any timely legal recourse. Consequently, upon seeing the signs of an impending sham peer review, wrongly accused physicians will choose one of two dire possibilities. On one hand, practically all peer reviews meet the "reasonable belief" provision of HCQIA and in turn qualify for near absolute immunity. Moreover, proving malicious intent to the courts is almost practically impossible^[23]. Despite the odds, some physicians will choose to fight sham peer reviews in court often at substantial financial and reputational cost, mental stress, and time^[27-29,33,34]. On the other hand, as previously

stated, physicians acknowledge that being reported to the NPDB can negatively affect future employment and reputation. In this situation, many physicians will often instead decide to resign from their hospitals or retract statements seen as unfavorable by hospital executives in exchange for early termination of the investigation and subsequent failure to report to the NPDB.

Hospitals are required by law to report situation in where physicians resign in the midst of a peer review investigation^[31,35]. Nevertheless, several studies have shown that there is significant evidence of hospital underreporting to the NPDB every year^[9,36-38]. Furthermore, a five year study looking at hospital reporting to the NPDB showed that 67% of hospitals did not report a single adverse event to the NPDB^[39]. Another study showed that 75% of potentially reportable actions and 60% of unquestionable reportable actions were not reported to the NPDB by their respective hospitals. While ambiguous, such significant underreporting can likely account for such an arrangement.

FUTURE DIRECTION

Evidently legal immunity is necessary to protect hospitals and physicians conducting good faith peer review as not every review of a physician is unwarranted, abusive or malicious. These peer reviews serve to protect the public and the medical profession from poorly behaved, unethical, or incompetent physicians. However, such absolute immunity under HCQIA has evidently weakened the process and lead to significant abuse. In the case of Dr. Timothy Patrick, a direct competitor was able to chair the peer review committee and was able to maliciously affect the peer review outcome in order to gain economic advantage. In order to change this paradigm, a multifaceted approach must be employed focusing on standardization, external peer reviews and finally legislative reform.

Standardization of peer review

Lack of standardization of the peer review process at the majority of hospitals leaves the door open for abuse. Today, only 62% of hospitals consider their review process to be either highly, or greatly, standardized^[9]. The variation in structure in turn leaves two variants of peer review systems in place at most hospitals. The first is a highly standardized process involving several committees, revolving peer reviewers, and finally objective measures of quality assessment. The second is an unstandardized review process that can be significantly prone to exploitation due to the complete subjective nature of such committees.

Moreover, studies have shown that peer reviews are often unreliable measures of quality and have not served their intended role in quality improvement^[6,40]. Standardization of the review process stands to benefit from both significant quality improvement and likely decreased abuse of the process to allow for sham peer reviews^[41]. However, national standardization efforts of peer review remains difficult as the process is both costly and requires signifi-

cant resources. Nevertheless, several models implemented at both large and small United States hospitals have shown that standardization and structuring of the review process can significantly improve medical care^[42-48].

External peer reviews

Recognizing the concerns peer review has placed on hospitals and physicians, recent JCAHO reforms of the Medical Staff Standards for hospitals were released in 2007. These changes require mechanisms allowing for fair hearings and appeal process in decisions adversely affecting medical staff members^[49]. However, it is unclear how much these reforms have contributed to mitigating sham peer review. Furthermore, while hospitals are required to implement such reforms, these standards still do not provide for independent peer review or oversight of the review process to ensure proper implementation. One approach to solving this issue is the creation of a second layer of protection involving external peer reviewers to verify that actions are taken in compliance with HCQIA and JCAHO requirements. Another suggested approach requires the use of Quality Improvement Organizations (QIOs) to independently review and supervise peer reviews conducted across United States hospitals. QIOs are physician operated organizations contracted by the Centers for Medicare and Medicaid Services in order to conduct reviews and further improve quality of services provided to Medicare beneficiaries in all 50 states^[50]. These QIOs are currently accustomed to dealing with quality across United States hospitals and could be primed to serve as important, external supervisors of the peer review process.

Legislative reform of HCQIA

Despite countless physician lawsuits against sham peer reviews reaching high level United States federal courts, the United States Supreme Court has continually denied to preside over such appeals in order to rule certiorari over the legality of HCQIA immunity^[51-54]. Considering the extent of immunity granted, several legal commentators have argued that these antitrust immunities should be repealed^[40,41,55,56]. Nonetheless, considering the firm position for immunity in the medical community and congress, this is unlikely. In turn, several measures can be taken to ensure peer review fairness *via* HCQIA reform rather than repeal^[23]. While these recommended reforms have been described in extensive detail elsewhere, we will provide a short overview here^[23].

First, due process requirements under HCQIA are inadequate and must be reformed in order to inhibit partial or biased reviewers from passing judgments on physicians. Second, the “reasonable belief” standard under HCQIA is virtually impossible to challenge in court and often place a significant burden on the targeted physicians to overcome. In turn, Congress or the Department of Health and Human Services needs to narrowly clarify what is meant by “reasonable belief” in order to qualify for HCQIA immunity. Third, legislation reform should

effectively mandate umbrella oversight by outside institutions in order to ensure fair, evidence-based, and appropriately motivated peer reviews are conducted^[23]. Lastly, if congressional reform unlikely, advocacy at the state level, which cannot be preempted by HCQIA, should be sought to further protection against Sham peer reviews^[26].

CONCLUSION

Peer review serves to discipline incompetent or unethical physicians in order to protect the public. Immunity granted under HCQIA serves to protect hospitals and peer reviewers from litigations from appropriately sanctioned physicians. Unfortunately, HCQIA extends these immunities to sham peer reviews. In the hypercompetitive and highly political United States medical system, this immunity has been abused and has led to the devastating destruction of many physicians careers. Considering Congressional and Judicial forbearance on this crisis, significant leadership by physicians, professional societies, and hospital administrators is needed in order to remedy the faults of peer review. Furthermore, there is considerably need to study the precise prevalence of sham peer review across the United States. Moreover, further research is needed to show if the recent JCAHO reforms have decreased the prevalence of such cases. Lastly, further research is needed in order to determine the cause of NPDB underreporting of adverse events.

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori infection in older people

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Core tip: Gastritis, peptic ulcer and related complications occur more commonly in elderly people. *Helicobacter pylori* (*H. pylori*) testing and treatment should be regarded as an important goal in clinical practice in elderly people, but only a few studies have been published to date. This article presents an overview of the epidemiology, diagnosis, clinical manifestations and therapy of *H. pylori* infection with a focus on elderly people, based on a multidimensional approach and the clinical practice modifications (or not) aroused during the past three decades.

Abstract

Since the discovery of *Helicobacter pylori* (*H. pylori*) infection as the major cause of gastroduodenal disorders three decades ago, *H. pylori* has been the focus of active research and debate in the scientific community. Its linkage to several diseases, such as peptic ulcer disease, gastritis and gastric malignancy is incontestable. In particular, it has been noticed that, as the aged population is increasing worldwide, older people are at increased risk of developing several gastroduodenal diseases and related complications. At the same time, gastric cancer is definitely more frequent in elderly than in adult and young people. In addition, it has been showed that peptic ulcer and related complications occur much more commonly in aged individuals than in young people, resulting in a significantly higher mortality. Although this infection plays a crucial role in gastrointestinal disorders affecting all age groups and in particular older people, only a few studies have been published regarding the latter. This article presents an overview of the epidemiology, diagnosis, clinical manifestations and therapy of *H. pylori* infection in elderly people.

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INTRODUCTION

In 1983, *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterial pathogen that selectively colonizes the gastric epithelium, was isolated by culture from gastric biopsy specimens by Warren and Marshall^[1], who were then awarded the Nobel Prize in Physiology or Medicine for their imperative discovery. Their work started a new interest in the previously neglected field of gastric microbiology, and in 1994 the National Institutes of Health consensus conference in the United States declared an association between *H. pylori* and peptic ulcer disease^[2,3]. In the same year, *H. pylori* was identified as a carcinogen associated with gastric adenocarcinoma^[4] and gastric non-Hodgkin's lymphoma^[5,6]. Thus, only a decade after its first isolation, *H. pylori* became the most important microbiological agent in human upper gastrointestinal tract disorders and it was classified as a type I carcinogen group by the World

Health Organization and the International Agency for Research on Cancer (IARC)^[4].

Although *H. pylori* infection is important in gastrointestinal diseases affecting all age groups, only a few studies have been published regarding elderly people. In this review, papers published in English were searched in PubMed using the key words “*H. pylori*”, “-elderly”, “-geriatrics”, “-diagnosis” and “-treatment”, focusing on recent information and studies. The review sought to re-examine the role of *H. pylori* infection in peptic ulcer, gastric cancer (GC) and extra-digestive diseases, addressing also its diagnosis and options for treating the infection in elderly people.

EPIDEMIOLOGY

Approximately 50% of the global population worldwide is thought to be colonized with *H. pylori*, which is typically acquired within the first 5 years of life^[7]. The prevalence of the infection varies according to different ages, socioeconomic strata and geographical regions. In developing countries the prevalence of *H. pylori* is higher in children, likely due to lower socioeconomic status, poor hygiene, overpopulation and lack of safe drinking water^[8], whereas in developed countries the prevalence increases with age, probably as a cohort effect of an earlier generation exposed to poor sanitation. Prevalence of infection varies between 7% and 87% and was lower in European countries^[9].

About 10 years ago, most of the studies reported a worldwide increasing prevalence of *H. pylori* infection with age, reaching 40%-60% in asymptomatic elderly individuals and > 70% in elderly patients with gastroduodenal diseases^[10]. Studies conducted in the past decade have reported a high prevalence of *H. pylori* infection within the oldest population, especially in institutionalized old people, with a prevalence ranging from 70% to 85%^[11,12]. However, a marked reduction in the prevalence of infection is noticed in elderly people (> 85 years)^[9,13,14]. Chronic atrophic gastritis and the extensive use of current or previous treatment with antibiotics and antisecretory drugs may explain this observation^[14]. However, it has been shown that the prevalence of the infection has decreased in adults and children in many countries almost 25 years after the discovery of *H. pylori*^[15,16]. Indeed, the infection persists throughout life, unless treated. This finding suggests that attention should be paid to diagnosis in order to treat *H. pylori* infection in older people.

DIAGNOSIS OF *H. PYLORI* INFECTION

Diagnosis of *H. pylori* can be achieved with invasive or noninvasive techniques. Invasive tests (histology, culture, and rapid urease test) need upper gastrointestinal endoscopy and biopsy material for tests, whereas the noninvasive techniques [C-urea breath test (UBT), stool antigen test, and serological blood test] use other methods. Each test has advantages, disadvantages and limitations.

INVASIVE TESTS

Histology

Histological evaluation has traditionally been the gold standard method for diagnosing *H. pylori* infection. The disadvantage of this technique is the need for endoscopy to obtain tissue specimens. However, upper gastrointestinal endoscopy is always indicated for elderly people with different abdominal symptoms because of the high prevalence of severe gastric diseases in this age group^[17]. Histology has the benefit of evaluating the morphological parameters of the gastric mucosa in order to identify the presence and severity of histological gastritis^[18]. Recently, an international group of gastroenterologists and pathologists Operative Link on Gastritis Assessment (OLGA) has developed a new system of histologically reporting gastritis^[19]. The assessment/description of the elementary lesions (in each of the biopsy samples considered) represents the core element in the histology report. A semiquantitative score of some of the elementary lesions should be provided, that is: (1) lymphoid-monocytic inflammation; (2) polymorphs (*i.e.* activity); (3) atrophy (distinguished as metaplastic and nonmetaplastic); and (4) *H. pylori* status (positive *vs* negative). This system places the histological phenotype of gastritis on a scale of progressively increasing risk of GC, from the lowest (stage 0) to the highest (stage IV) stage.

In order to provide a proper evaluation of the presence of *H. pylori* in elderly patients, it is necessary to go through a bioptic sampling with two antral and two body gastric biopsies. The high level of atrophic gastritis in elderly people can, in fact, reduce the sensitivity of the test itself.

Rapid urease test

The rapid urease test [CLO (Campylobacter-like organism) test] can detect the presence of *H. pylori* within 1 h with satisfactory accuracy (90%)^[20]. However, as for the histology, the rapid urease test performed on antral biopsies has a lower sensitivity in 60-year-old (and older) compared to younger patients (57% *vs* 75%)^[21]. Moreover, false-negative results can occur in patients taking antisecretory drugs.

Bacterial culture

H. pylori is difficult to grow on culture media, thus, the role of culture in diagnosis of infection is limited mostly to research and epidemiological considerations. Its interest mainly lies in the possibility of performing antimicrobial susceptibility testing. The rationale relates to the fact that in the case of clarithromycin resistance, the rate of success of clarithromycin-containing triple therapy is low, ranging between 10% and 30%^[22]. After a second failure, it should be performed in all cases as recommended at the Maastricht III Conference^[23].

These findings suggest that in elderly people it is advisable to obtain gastric biopsies at least from both the antrum and corpus of the stomach, and to perform a

second test for *H. pylori* if a urease-based or histological test is negative, and it is mandatory to suspend antisecretory drugs at least 10 d prior to the tests.

NONINVASIVE TESTS

UBT

The C-UBT is a noninvasive test that relies on bacterial urease activity. The main principle involved is the conversion of urea into NH_3 and CO_2 by urease. Urea marked by ^{13}C and ^{14}C is hydrolyzed by *H. pylori* into NH_3 and CO_2 in the stomach and then labeled isotopes of carbon in the CO_2 exhaled by the lungs are measured. The greatest advantage of the C-UBT is that it samples the whole stomach, avoiding obstacles normally linked to invasive tests such as biopsy, which can hardly detect the bacteria if it is patchily distributed on the stomach mucosa.

The ^{13}C -UBT is an accurate, practical and easily available test^[24]. The diagnostic accuracy of the ^{13}C -UBT was 95% in several studies^[25]. In elderly people, the ^{13}C -UBT has demonstrated significantly higher sensitivity (100%), specificity (95.7%) and diagnostic accuracy (98%) compared to serology (IgG *H. pylori* antibodies)^[26]. Furthermore, the ^{13}C -UBT has shown to be unaffected by potential co-variables, such as cognitive function, disability, comorbidity and co-treatments^[27].

Recently, a study performed in 100 patients aged > 65 years, using ^{14}C -UBT, demonstrated a sensitivity of 91.4% and specificity of 93.8%^[28].

Serological IgG test

After *H. pylori* infection is acquired, the immune system typically reacts by producing IgG against organism-specific antigens. These antibodies can be detected in serum or whole-blood samples. The presence of IgG antibodies against *H. pylori* can be detected through a biochemical assay and several different ones are available. The sensitivity, specificity and diagnostic accuracy of serology in a study of elderly patients were 74.4%, 59% and 67%, respectively^[29]. It is important to remember that it does not effectively tell apart current from past infections. This method is not a useful means to confirm the eradication of *H. pylori*, but it may be used in the monitoring phases after eradication; indeed, it could be useful to observe and verify the former presence of *H. pylori* in patients with atrophic gastritis.

H. pylori stool antigen testing

H. pylori stool antigen (HpSA) testing involves an enzyme immunoassay to detect the presence of *H. pylori* antigen in stool specimens. The monoclonal HpSA test is an accurate noninvasive method for both the initial diagnosis of *H. pylori* infection and confirmation of its eradication after treatment^[30]. The sensitivity and specificity of the HpSA test compared with two tests (histology and UBT) were respectively 76% and 95% in 122 elderly hospitalized patients^[31]. Similar results were found in a study involving 85 hospitalized frail elderly people^[32]. According

to the Maastricht IV Conference, the diagnostic accuracy of the HpSA test is equivalent to the UBT if a validated laboratory-based monoclonal test is used with an evidence level “1a” and grade of recommendation “A”^[33]. In older people, however, some limitations of the HpSA test could be due to constipation, a frequent disorder in elderly people. Indeed, as previously suggested, the prolonged gastrointestinal transit time could slow down the passage of the bacteria into the colon, leading to degradation of *H. pylori* antigens and compromising their detection^[14].

Other noninvasive tests (GastroPanel blood)

GastroPanel is determined by four parameters measured in blood samples: serum pepsinogen (s-PG) I and s-PG II, gastrin-17 and anti-*H. pylori* IgG. Levels of s-PGI and s-PGII are known to increase in cases of *H. pylori*-related nonatrophic gastritis. s-PG II levels are higher in patients with peptic ulcer and are correlated with the severity of inflammation^[34]. *H. pylori* eradication induces improvement of histological gastritis activity. A study performed in elderly patients demonstrated that s-PG II levels decreased significantly in the case of successful eradication of *H. pylori*^[35]. Therefore, s-PG I /PGII ratio could be a useful marker for monitoring the outcome of *H. pylori* treatment^[29,36]. Moreover, the measurements of s-PGI or the ratio of s-PG I /s-PG II may be useful to identify atrophic gastritis of the gastric corpus, and assays for gastrin (particularly gastrin-17) could be an indicator of the morphological status of the antral mucosa^[37]. Further studies are needed to evaluate the clinical usefulness of the GastroPanel test in clinical practice, especially in older people.

CLINICAL FEATURES

Epidemiological and clinical studies suggest that with advancing age there is an increase in both the prevalence and severity of upper gastrointestinal diseases^[38]. As reported above, *H. pylori* infection is the major risk factor for developing chronic gastritis and peptic ulcer, and it is also related to gastric mucosa-associated lymphoid tissue lymphoma and GC^[2-4]. Moreover, interesting data suggest a clinical association between *H. pylori* infection and extra-digestive disorders, including some that are particularly frequent in older people.

CHRONIC ATROPHIC GASTRITIS

Although previous studies have reported that advancing age is independently related to chronic atrophic gastritis and a functional status of hypo/achlorhydria, more recent data suggest that atrophic changes of the gastric mucosa are associated with *H. pylori* infection rather than with aging^[4]. Eradication of *H. pylori* infection in elderly patients can lead to a significant decrease in gastritis activity as compared with no change in histology in patients with continuing chronic infection^[39]. Eradication of *H. pylori* infection in elderly patients with advanced atrophic

gastritis may also lead to significant improvement in the mean histological scores of inflammation, atrophy and intestinal metaplasia, after a mean follow-up of 2.5 years^[40] after eradication therapy. In a recent study performed in 84 elderly patients, the authors confirmed that eradication of *H. pylori* infection improved gastric atrophy and prevented the progression of intestinal metaplasia during long-term follow-up^[41]. Lastly, in patients with corpus atrophic gastritis, there is long-term improvement of physiological gastric function after *H. pylori* eradication, as suggested by the significant and continuous increment of s-PGI levels over a 4-year period^[42].

GASTROESOPHAGEAL REFLUX DISEASE

Gastroesophageal reflux disease (GERD) is a multifactorial disorder characterized by reflux of acidic gastric contents into the esophagus, leading to tissue damage and symptoms. Gastric acid secretion does not decrease with age, although factors leading to atrophic gastritis, such as *H. pylori* infection, reduce gastric acid secretion^[43].

Although it has been previously suggested that *H. pylori* eradication may cause both reflux symptoms and erosive esophagitis, the relationship between *H. pylori* infection and GERD has not been clarified yet, particularly in elderly people.

Epidemiological data do not seem to support a role for *H. pylori* in the pathogenesis of reflux disease, and suggest a negative association with the increasing incidence of esophageal diseases^[44,45]. A recent meta-analysis of 10 trials in which data of patients treated for *H. pylori* infection were compared to those receiving placebo concluded that the post-treatment incidence of reflux symptoms (17% *vs* 22.6%) and erosive esophagitis (5% *vs* 5.1%) were similar between both groups^[46]. However *H. pylori* testing should be considered in older patients affected by GERD and receiving long-term maintenance treatment with proton pump inhibitors (PPIs)^[39].

PEPTIC ULCER DISEASE

The correlation between *H. pylori* infection and peptic ulcer and peptic bleeding diseases has been widely studied. A meta-analysis reported that the prevalence of peptic ulcer disease ranged worldwide between 0.1% and 4.7%, with an annual incidence ranging from 0.19% to 0.3%^[47]. Epidemiological studies have indicated that the prevalence and incidence of peptic ulcer show a constant decline in the general population, but current studies report an increased rate of peptic ulcer disease, its complications and mortality in older patients^[48,49]. Two major factors that might explain the observed increasing incidence of peptic ulcer in elderly patients are the high prevalence of *H. pylori* infection and the large use of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or aspirin. An endoscopic study carried out in 520 peptic ulcer patients aged > 65 years (mean age: 81 years) reported that 67% of gastric ulcers and 69% of duodenal ulcers were *H. pylori* positive;

moreover, NSAID or aspirin use, alone or in combination with *H. pylori* infection, were reported by 39% of gastric ulcer and 25% of duodenal ulcer patients^[50].

NSAID use and *H. pylori* infection are independent risk factors for peptic ulcer and gastroduodenal bleeding in elderly patients. In *H. pylori*-positive older patients who are starting long-term treatment with NSAIDs, the treatment of *H. pylori* infection significantly reduces the 6-mo risk of peptic ulcer^[51]. In elderly high-risk patients, however, the use of PPIs concomitantly with NSAIDs reduces the occurrence of NSAID-related gastroduodenal damage more effectively than the eradication of *H. pylori* infection^[52].

Moreover, after eradication of *H. pylori*, maintenance treatment with a PPI is more effective than placebo in the prevention of ulcer bleeding in elderly patients^[53]. All these findings suggest that *H. pylori* eradication would surely be a useful strategy, but it is not sufficient for the prevention of severe gastroduodenal damage in elderly *H. pylori*-positive patients and NSAID and aspirin users^[39,54].

Indeed, the few short- and long-term studies performed in elderly patients with *H. pylori*-positive peptic ulcer have demonstrated that treatment of *H. pylori* infection results in ulcer healing in > 95% of patients. Moreover, it significantly improved clinical outcomes, including a reduction in recurrence^[55], but unfortunately the percentage of elderly patients with peptic ulcer who are treated for their *H. pylori* infection is still low^[56].

All the above-mentioned considerations suggest that, along with aging of the population, the risk of developing peptic ulcer and related complications, including gastrointestinal bleeding, is more frequent in elderly patients than in younger ones^[57]. Consequently, we suggest testing for and treating *H. pylori* infection in elderly people. Moreover, it has been proposed that in patients characterized by comorbidity or a history of peptic ulcer and requiring long-term NSAID or aspirin treatment, *H. pylori* infection should be eradicated before starting the above-mentioned therapy^[58,59].

GC

GC is the fourth commonest cancer in the world and accounts for 8% of the total cancer cases and 10% of total cancer-related deaths worldwide, with > 70% of new cases and deaths occurring in developing countries^[60]. GC is rare below the age of 30 years; thereafter, it increases rapidly and steadily to reach the highest rates in the oldest age groups, both in men and women. Although the incidence of GC has declined in the general population, the incidence in elderly people is increasing, most probably as a result of extended life expectancy.

It is now clear that *H. pylori* infection induces a cascade of events that could ultimately lead to gastric neoplasia in genetically predisposed hosts^[61]. The key pathophysiological events include the onset of gastric atrophy and hypochlorhydria. The increased proliferation induced by inflammation creates a genetically unstable gastric

mucosa, which is further compromised by the presence of genotoxic substances generated by inflammatory and bacterial products^[62]. The hypochlorhydria contributes to bacterial overgrowth, which further exacerbates the inflammation and leads to generation of carcinogenic nitrogenous products. Elderly people have a higher prevalence of *H. pylori* infection and those who are infected develop a gradual decline in gastric acid secretory function, which is induced by the chronic gastritis and atrophy. In the presence of other environmental factors such as poor diet and smoking, the neoplastic process is even accelerated.

Several animal studies have confirmed that gastric carcinogenesis originates from *H. pylori*^[63,64]. Meta-analyses that included additional epidemiological studies have confirmed the association between the bacterium and GC^[65]. To prevent the development of GC, eradication therapy should be ideally administered early, before premalignant gastric lesions (*i.e.* atrophy) develop^[66,67]. In fact, a prospective observational study in 1526 Japanese patients revealed that 2.9% of the *H. pylori* infected patients developed GC after 7.8 years, whereas none of the noninfected individuals developed GC^[68]. Furthermore, an interventional study in China has shown a risk reduction of 37% after 7.5 years in patients who received *H. pylori* eradication therapy. *H. pylori* eradication reduces the incidence of gastric adenocarcinoma, therefore, a deeper screening activity and calculated treatment strategy for this infection in the general population in high-risk areas are suggested.

EXTRA-DIGESTIVE DISEASES

The list of diseases associated with *H. pylori* seropositivity is long^[69]. We consider only those extra-digestive diseases that can represent an interest for the treatment of elderly patients and/or that have a significant social impact.

The proinflammatory nature of *H. pylori* infection could be the common factor in the pathogenesis of those diseases in which inflammation is an important feature in the pathogenesis. Thus, it is possible that *H. pylori* infection triggers or aggravates a systemic inflammatory response that acts concurrently with the key triggering factors in many diseases. The apparent association of so many different diseases with *H. pylori* infection also suggests that a final common pathway could exist for all these conditions. The effects of this local inflammation may not be confined only to the digestive tract, but may also spread to involve extraintestinal tissues and/or organs.

Cardiovascular and Alzheimer's diseases

Several studies have demonstrated a relationship between *H. pylori* seroprevalence and coronary artery disease^[70,71]. However, it has not yet been determined if *H. pylori* infection is associated with increased risk of coronary heart disease. The few studies performed in elderly populations have failed to find any association between *H. pylori*

infection and coronary heart disease^[72,73] or extracardiac atherosclerosis^[74].

Recent data have demonstrated that *H. pylori* chronic infection can play a role in mild cognitive impairment^[75] and Alzheimer's disease^[76-78], but further studies are needed to attest to the impact of *H. pylori* infection on disease course, especially on cerebrovascular lesions and neuroinflammation.

Appetite regulation

The possible role of *H. pylori* infection in appetite regulation in elderly people is an interesting new area of research. *H. pylori* has an influence on the release of gastric hormones and therefore it plays a significant role in the regulation of body weight, hunger and satiety^[79,80]. The main hormones involved are leptin and ghrelin, which are multifunctional hormones that co-operate in order to balance different metabolic functions. They both act at the hypothalamic level, but with opposite effects. Ghrelin increases appetite, and decreases the waste of energy and catabolism in adipose tissue and plasma glucose, improving body weight. Leptin acts in the opposite way. *H. pylori* infection leads to a decrease of circulating ghrelin through a reduction of ghrelin-producing cells in the gastric mucosa and increases the amount of gastric leptin with no effect on circulating leptin levels^[68]. One study has reported that treatment of *H. pylori* infection increases the level of plasma ghrelin, leading to a more intense appetite and weight gain^[81]. Another study concerning frail elderly patients aged > 80 years has shown that the presence of *H. pylori* chronic gastritis induces a decrease in both leptin and ghrelin gastric production, probably due to the high prevalence of atrophic lesions observed in this particular population^[82].

Iron-deficiency anemia

Iron-deficiency is a common cause of anemia in elderly people. Several studies have shown a relationship between *H. pylori* and iron-deficiency anemia (IDA)^[83,84]. Possible pathogenetic mechanisms involved in IDA in patients with *H. pylori* infection include: occult blood loss secondary to chronic erosive gastritis; decreased iron absorption secondary to chronic gastritis of the corpus causing hypo- or achlorhydria; and increased iron uptake and use by bacteria.

Cobalamin deficiency

Cobalamin (vitamin B12) deficiency is particularly common in elderly people but is often unrecognized because its clinical manifestations are subtle and nearly undetectable^[85,86]. Vitamin B12 is a water-soluble vitamin needed for DNA replication, production of S-adenosyl-L-methionine, normal nerve cell activity, and above all, it acts together with folic acid to control homocysteine levels. An excess of homocysteine is associated with an increased risk of heart disease, stroke and potentially other diseases such as osteoporosis and Alzheimer's disease^[87].

Cobalamin malabsorption could be due to several

factors including pernicious anemia, gastrectomy, histamine-2 antagonists, PPIs, and *H. pylori* infection itself^[88]. Food cobalamin malabsorption is caused primarily by chronic atrophic gastritis^[89]. This theory has been supported by two different studies evaluating the effect of eradication treatment of *H. pylori* infection on the improvement of vitamin B12 deficiency in patient groups with atrophic^[90] and non-atrophic gastric mucosa^[91].

All these findings suggest that in presence of IDA and cobalamin deficiency, *H. pylori* should be sought and eradicated.

TREATMENT

The triple therapy regimens including a PPI, clarithromycin and amoxicillin or metronidazole for *H. pylori* infection have been universally accepted since they were recommended at the first Maastricht Consensus. This regimen has also been reported as effective and safe for the treatment of *H. pylori* infection in older people^[92]. The most recent data, however, show that this combination has lost some efficacy and often allows the treatment of only a maximum of 70% of the patients, which is less than the initial target rate of 80% and far below what should be expected for an infectious disease. Consequently, the recommended first-line therapy regimens are dependent on the prevalence of antibiotic resistance^[22]. In a recent study performed in 2204 patients in 18 European countries, the resistance rate of *H. pylori* was 17.5% for clarithromycin, 14.1% for levofloxacin and 34.9% for metronidazole, while the prevalence was $\leq 1\%$ for other antibiotics tested^[93]. Actually, no data on antibiotic resistance in elderly patients are available.

The recommended treatment strategy in the Maastricht Consensus Report was triple therapy with PPI, clarithromycin and metronidazole in areas with clarithromycin resistance rates $< 15\%$ - 20% and metronidazole resistance rate $< 40\%$, and triple therapy with PPI, clarithromycin and amoxicillin in areas with clarithromycin resistance rates $< 15\%$ - 20% and metronidazole resistance rate $> 40\%$. Bismuth-containing quadruple therapy (10 or 14 d) is an option for first-line treatment. It leads to satisfactory eradication rates despite the increased resistance to both clarithromycin and metronidazole.

Although it is now known that the increase in duration of triple therapy (from 7 to 14 d), as well as quadruple treatments, may be associated with more successful eradication, few studies have evaluated the clinical utility of these regimens in elderly patients. Only one study in 95 dyspeptic elderly patients (aged 65-81 years) demonstrated that the eradication rate of quadruple therapy including 20 mg esomeprazole, 500 mg tetracycline, 500 mg metronidazole and 240 mg bismuth subcitrate tablets twice daily for 10 d was 91% (intention-to-treat analysis) and 95% (per-protocol analysis); the compliance was excellent, but mild-to-moderate side effects occurred in 27 patients (28%)^[94]. Prolonging the duration of treatment may increase the risk of side effects, which tend to be-

come more marked after the first week of therapy^[95].

Recently, sequential treatment consisting of 5 d of PPI plus amoxicillin followed by five additional days with PPI plus clarithromycin and tinidazole has revealed a better solution than the combination of a PPI plus amoxicillin and clarithromycin for 7 d^[96,97]. In a study designed to assess the eradication rate of this 10-d sequential regimen in geriatric patients with peptic ulcer, the authors confirmed that the 10-d sequential treatment regimen achieved significantly higher eradication rates in comparison with standard triple therapy^[98].

Eradication of *H. pylori* infection is more difficult when a first treatment attempt has failed^[99]. The optimal strategy for retreatment after failure of eradication has not yet been established yet in elderly patients. Thus, the choice of a second-line treatment depends on which treatment was used initially. Despite retreatment ideally being guided by data on susceptibility, the updated Maastricht Consensus Report^[23,33] recommends that culture and antimicrobial sensitivity testing should be routinely performed only after two treatment failures with different antibiotics.

The leading second-line therapy regimens are quadruple therapies, in which a PPI is added to a bismuth-based triple regimen. A different approach of retreatment without susceptibility testing is to prescribe a second course of PPI-based triple therapy avoiding antimicrobial agents against which prior therapy may have induced resistance, such as clarithromycin-based and/or metronidazole-based regimens. It has recently been suggested^[100] that levofloxacin-based rescue therapy constitutes an encouraging second-line strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure, with the advantage of efficacy, simplicity, and safety. However the rapid acquisition of resistance may compromise its future efficacy, and levofloxacin should not be used in patients with chronic infectious bronchopneumopathy, who may have received fluoroquinolones^[33].

CONCLUSION

H. pylori testing and treatment in elderly people should be regarded as an important goal in clinical practice due to its crucial role in gastrointestinal disorders in that age group. The current recommendations confirm that the standard methods for diagnosis and treatment of *H. pylori* infection could be safe and effective in elderly patients. However, because of the impact of underlying conditions, that is, multi-morbidity and functional impairments that may influence the outcome in older people^[101], a multidimensional approach including the evaluation of functional, cognitive, nutritional and social conditions in addition to comorbidity and concomitant treatments is required in clinical practice and research to manage older patients better^[102], in accordance with the high epidemiological and clinical impact that *H. pylori* infection has aroused during the past three decades in older people.

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori infection and inflammatory bowel disease: Is there a link?

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Abstract

Helicobacter pylori (*H. pylori*) infection is one of the most widely spread infectious diseases in humans. It can cause chronic gastritis, peptic ulcer disease and gastric malignancies and has been associated with extra-gastric disorders. *H. pylori* elicit a chronic systemic inflammatory response which, under certain conditions, may trigger autoimmune reactions and may be implicated in the pathogenesis of autoimmune diseases. Although the pathogenesis of inflammatory bowel disease (IBD) is unknown, it is thought to result from complex interactions between environmental factors and microbiota in the gut of individuals who are genetically susceptible. Several bacterial and viral agents have been implicated in the aetiology of IBD. In theory, *H. pylori* infection could be involved in the pathogenesis of IBD by inducing alterations in gastric and/or intestinal permeability or by causing immunological derangements resulting in absorption of antigenic material and autoimmunity via various immunological pathways. Similar mechanisms may also be responsible for the co-existence of IBD with other autoimmune diseases and/or extra-intestinal manifestations. However, the epidemiological data fail to support this association. In

fact, various studies indicate that the prevalence of *H. pylori* infection is low in patients with IBD, suggesting a protective role for this infection in the development of IBD. In this report, we aim to shed light on proposed mechanisms and confounding factors underlying the potential link between *H. pylori* infection and IBD.

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Key words: *Helicobacter pylori*; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Colorectal cancer

Core tip: By gathering a large volume of published data, this review attempts to shed light on the mechanisms and confounding factors underlying the potential link between *Helicobacter pylori* (*H. pylori*) infection and Inflammatory Bowel Disease (IBD). However, whether the link between *H. pylori* and IBD is coincidental, epiphenomenal or mechanistic remains to be elucidated as there are contradictory data regarding both the causative and the protective role of *H. pylori* infection against IBD. This review provides a tool for researchers in this field to use as they perform further research to find the missing links.

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INTRODUCTION

Inflammatory bowel diseases (IBDs), which includes Crohn's disease (CD) and ulcerative colitis (UC), are chronic, relapsing-remitting diseases that constitute a growing worldwide health burden^[1-3]. Over time, these

diseases may lead to intestinal damage, complications, surgical interventions, gut failure and/or disability^[4-7]. IBD is thought to result from complex and unidentified interactions between environmental factors (such as infections, medicines, tobacco, food particles) and genetic factors of the host, resulting in abnormal and/or inappropriate immunological reactions to elements of the intestinal flora. For example, Gradel *et al.*^[8] demonstrated that infection with either *Campylobacter* or *Salmonella* species predisposed individuals to subsequent development of IBD.

Helicobacter species easily colonize the gastrointestinal surface due to microaerophilic metabolism, spiral shape, and peculiar motility^[9]. Based on their location within the gastrointestinal system, they are divided into gastric *Helicobacters*, such as the *Helicobacter pylori* (*H. pylori*), and enterohepatic *Helicobacters* (EHH), which predominantly colonize the intestine and the hepato-biliary system and have been linked to chronic liver and intestinal diseases^[9]. *H. pylori* usually resides in the surface epithelium of the stomach, but *H. pylori* DNA has also been identified in both the colon^[10] and stool of infected patients^[11-13].

H. pylori is a gram-negative, spiral-shaped pathogenic bacterium that causes chronic gastritis. Peptic ulcer disease and/or gastric malignancies may develop in a small number of individuals infected with the bacterium^[9,14]. The inflammatory response of the gastric mucosa to *H. pylori* most likely reflects the combined effects of a cellular immune response that is driven by an on-going stimulation of the host's immune system by the bacterium. This results in high production of interleukin (IL)-12, leading to a T helper type 1 (Th1)-polarized response and elevated levels of Th1 cytokines^[15-18]. Products of the local immune reactions may travel to extra-gastric sites, thus linking *H. pylori* infection to the pathophysiology of a variety of extra-gastric diseases, including autoimmune disorders^[19-21]. Interestingly, however, *H. pylori* has been proposed to play a protective role against the development of certain autoimmune disorders^[21] such as asthma^[22] and type 1 diabetes mellitus^[23]. The mechanisms underlying this protective role of *H. pylori* infection is thought to be differential expression of an acute and/or chronic local mucosal inflammatory response, which may elicit a systemic release of cytokines^[24], which in turn may down-regulate systemic immune responses and suppress autoimmunity.

In IBD, dysregulation of the immune response of the host to commensal bacteria has been proposed as an important underlying pathogenetic mechanism. Increased attachment of gut bacteria to the intestinal epithelium has been documented in IBD. A Th1 immune reaction and secretion of pro-inflammatory cytokines is implicated in the pathogenesis of IBD, especially CD^[5]. Up-regulation of cell signalling molecules, such as the macrophage inflammatory protein 3a (MIP-3a), has also been documented in IBD^[25]. Some IBD patients suffer from concomitant autoimmune diseases and autoimmune-type extra-intestinal manifestations. The similarities between the immunobiology of IBD and that of *H. pylori* infec-

tion provides background for the hypothesis that *H. pylori* infection may be implicated in the pathogenesis of IBD.

Nevertheless, there is epidemiological evidence that contradicts the association between *H. pylori* and IBD. *H. pylori* infection is an infection that occurs in underprivileged societies and its prevalence declines when environmental hygienic conditions improve. In contrast the prevalence of IBD increases in societies adapting a Western life style^[26]. Thus, it appears that there is an inverse relation between the prevalence of IBD and *H. pylori* infection. IBD is highly prevalent in the United States^[27], an area with low rates of *H. pylori* infection whereas, a steady rise in the incidence of IBD has been observed in *H. pylori* endemic regions following widespread use of therapeutic regimens to treat *H. pylori*^[28]. Although environmental changes may be the confounding factor underlying this inverse relationship, many (but not all) epidemiological studies have shown a low incidence of *H. pylori* infection in patients with IBD^[29-60]. This has led to the hypothesis that *H. pylori* infection may exert a protective role against IBD. However one can argue that it is the medication used to treat IBD that eradicates *H. pylori* and /or the IBD associated mucosal alterations that may prevent colonization of the stomach by *H. pylori*. The latter may be true, especially in IBD patients with focally enhanced gastritis (FEG) who have a particularly low incidence of *H. pylori* infection, even if they live in *H. pylori* endemic areas^[33,34,61-64].

Possible mechanisms of the potential protective role of *H. pylori* infection against the development of IBD may be alteration of the host immunologic response away from the pro-inflammatory Th1/Th17 response towards an increased T-regulatory cell immune response^[65,66]. Moreover, *H. pylori* may induce the production of antibacterial peptides that counteract potentially harmful bacteria implicated in the pathogenesis of IBD^[67] or compete with bacteria for the same ecologic niche in the upper gastrointestinal tract^[68].

CAUSAL ASSOCIATION OF *HELICOBACTER* SPECIES AND *H. PYLORI* WITH IBD

In animal models, EHH such as *Helicobacter hepaticus* (*H. hepaticus*) and *Helicobacter bilis* (*H. bilis*) have been shown to induce a persistent inflammation in the colon and cecum in immuno-deficient rodents^[69,70]. *Helicobacter hepaticus* triggers colitis in a specific pathogen-free IL-10-deficient mice through an IL-12 and interferon-gamma (IFN- γ) dependent mechanism^[69]. *Helicobacter muridarum* increases disease activity and inflammation in an acute colitis model^[71] and provokes a CD-like inflammation in severe combined immunodeficiency mice upon receipt of T cells^[72]. Accumulating evidence from gene knockout rodents also indicate that the presence of EHH worsens the severity or hastens the development of colitis^[73,74].

However, observations from human studies are conflicting. Several EHH species have been identified in the

large intestine of patients with enteritis and / or proctitis^[75]. *Helicobacter macacae* has been linked with chronic idiopathic colitis in young rhesus monkeys^[76]. Similarly, Laharie *et al*^[77] found that *Helicobacter pullorum* (*H. pullorum*) or *Helicobacter canadensis* infection was significantly associated with CD in adults. *Helicobacter* species were found either in faecal specimens^[78] or in colonic biopsy samples^[79] of children with CD, and the prevalence of the *Helicobacteraceae* was significantly higher in children with CD (32/77, 41.5%) compared to controls (23/102, 22.5%)^[80]. A German group found *Helicobacter fennelliae* and *H. pullorum* in colonic samples of 12% of CD patients^[81]. *Helicobacter* genus PCR positivity was also significantly higher in UC than in controls (32/77 *vs* 11/59, $P = 0.004$)^[82]. *H. pylori* was isolated and detected by PCR in the intestinal mucosa of patients with UC-like CD and UC^[53,54,83]. Moreover, in another study *H. pylori* was found in faecal specimens in the majority of children with CD^[78]. In contrast, *Helicobacter* species were not detected in colonic biopsies of IBD patients in various studies^[84-88]. Additionally, no significant difference was observed in the rate of detection of *Helicobacter* species in intestinal biopsy specimens from 160 Chinese IBD patients (10%) and 80 controls (6.3%)^[57]. Furthermore, in an earlier study assessing gastrointestinal mucosal lesions in children with IBD, infection with *H. pylori* was found in only 2 of 41 children with CD (4.8%) and in 5 of 47 with UC (10.6%)^[89].

H. PYLORI AND THE NATURAL HISTORY OF IBD

It is conceivable that *H. pylori* infection may influence the clinical course of CD by triggering both specific and nonspecific immune responses in the human intestine. Phenotype modification of CD was identified in a study in which seropositive non-smoking CD patients had significantly fewer relapses and a lower risk of bowel resection compared to seronegative non-smoking patients^[90]. Moreover, serum anti-*H. pylori* IgG levels were significantly lower in subgroups of patients with fibro-stenotic and fistulising CD^[54].

There are several hypotheses regarding how *H. pylori* may influence the host immune response and thus alter the clinical course of CD. *H. pylori* infection may exert a direct damaging effect via urease and cytotoxins on the ileal or colonic mucosa^[91]. Moreover, *H. pylori* may induce an autoimmune-like reaction in the stomach with the production of anti-Lewis X and/or Y antibodies that have systemic auto reactive properties, thereby influencing the course of the disease^[92]. Another mechanism could be the induction of platelet activation and aggregation as shown in murine gastric venules which can cause the formation of microthrombi in gastric and intestinal epithelium and lead to infarction and development of ulcers^[93]. Another possibility is that *H. pylori* influences the host immune response via activation of the mucosa-associated lymphoid tissue (MALT), which may lead to a more generalized

immune response to *H. pylori* infection in IBD, contributing to the initiation or perpetuation of inflammation. In fact, Duchmann *et al*^[94] showed that bacteria-specific T cell clones are increased in inflamed intestinal mucosa of patients with IBD.

It appears that in *H. pylori* infected patients, CD is more often confined to the terminal ileum, a location that is frequently affected by complications, yet may be associated with a lower clinical disease activity^[95]. *H. pylori* infection usually occurs early in life, before the onset of CD, so it is possible that this early infection may influence disease location in these patients^[96]. As a result, *H. pylori* infection may not influence the course of the disease primarily, but may influence the location of the disease and thus secondarily alters its course.

PROTECTIVE ROLE OF H. PYLORI AGAINST IBD

Many studies have reported that the prevalence of *H. pylori* infection is lower in patients with IBD compared to controls, demonstrating an inverse relationship between IBD and *H. pylori* infection that suggests a protective role of *H. pylori* infection against the development of IBD (Table 1)^[32-34,38,39,41-46,48,49,52,55,57-59]. However this has not been confirmed by other studies (Table 1)^[30,35-37,53,56,60]. Väre *et al*^[41] found that seropositive CD patients presented at a significantly later age (40 years) compared to seronegative patients (30 years, $P < 0.001$), suggesting that the higher age of disease onset in seropositive IBD patients is the result of a protective modifier effect that *H. pylori* infection exerts on the development of IBD^[41], although this has not been confirmed by other studies^[34,42]. Furthermore, a meta-analysis of 23 studies suggested a protective role of *H. pylori* infection in CD pathogenesis, but the heterogeneity among enrolled studies and the possibility of publication bias limited the reliability of these results^[97]. The published literature on the prevalence of *H. pylori* infection in UC and CD is diverse. Various studies have found a lower prevalence of this infection in CD compared to UC^[29,31,34,41-43], whereas others have found exactly the opposite^[34,35,55]; still others have reported no difference in the occurrence of *H. pylori* between the two diseases^[30,32,33,36,37,39,48,56,57].

Moreover, the increased occurrence of *H. pylori*-negative FEG among IBD patients also confirmed the inverse association between the prevalence of *H. pylori* infection and IBD (Table 2). For example, *H. pylori*-negative chronic active gastritis was found in only 2% of patients without IBD compared to 20% of patients with IBD (CD 26%, UC 13%)^[98]. Furthermore, permanent colonization of the stomach by *H. pylori* is unusual in children with IBD^[40].

Heterogeneity among studies regarding the method of IBD and *H. pylori* diagnosis differences in study population, ethnicity and age across studies, and the possibility of publication bias may limit the certainty of the above findings. As environmental hygiene and intestinal

Table 1 Prevalence of *Helicobacter pylori* infection in patients with inflammatory bowel disease in different populations

| CD | UC | C | Control group | Method | Positive (%) | Country | Ref. |
|-----|-----|-------|--|---|---|----------------|---------------------|
| 42 | 51 | 40 | Patients with irritable bowel syndrome | UBT, <i>H. pylori</i> IgG (+) | IBD: 17.2, C: 25 CD: 11.9, UC: 21.6 | United Kingdom | [29] |
| 110 | 213 | 337 | Non-IBD patients with elective surgery ¹ | <i>H. pylori</i> IgG (+) | IBD: 34.2, C: 36.2 CD: 33.3, UC: 34.7 | United Kingdom | [30] |
| 139 | 137 | 139 | patients with functional GI disorders ¹ | <i>H. pylori</i> IgG (+) | IBD: 9.4, C: 16 CD: 5, UC: 14 | United Kingdom | [31] ² |
| 47 | 63 | 100 | Blood donors ¹ | <i>H. pylori</i> IgG (+), UBT, histology | IBD: 21.8, C: 52 CD: 14.9, UC: 27, | United Kingdom | [32] ² |
| 67 | 41 | 43 | Non-IBD patients | Biopsies | IBD: 28.7, C: 39.5 CD: 28.4, UC: 29.3, | Italy | [33] ² |
| 123 | 93 | 216 | Blood donors ¹ | <i>H. pylori</i> IgG (+), histology | IBD: 48.1, C: 58.8 CD: 40.7, UC: 55.9 | Italy | [34] ² |
| 32 | 40 | 72 | Healthy subjects ¹ | UBT | IBD: 47.2, C: 61.1 CD: 53.1, UC: 42.5 | Italy | [35] |
| 12 | 8 | 29 | Patients with idiopathic constipation | UBT | IBD: 60, C: 41 CD: NR, UC: NR | Italy | [36] |
| 45 | 66 | 77 | Patients with non-organic dyspepsia ¹ | histology | IBD: 66.7, C: 63.6 CD: 62.2, UC: 69.7 | Turkey | [37] |
| 0 | 90 | 120 | Healthy subjects | Histology, RUT | IBD: 30, C: 52.5 CD: NA, UC: 30 | Greece | [38] ² |
| 39 | 77 | 127 | Healthy subjects ¹ | <i>H. pylori</i> IgG (+) | IBD: 31.7, C: 55.1 CD: 28.6, UC: 33.1 | Greece | [39] ² |
| 19 | 21 | NA | NA | <i>H. pylori</i> IgG, IgA (+), histology | IBD: 0, C: NA CD: NA, UC: NA | Finland | [40] ³ |
| 94 | 185 | 70 | Healthy subjects ¹ | <i>H. pylori</i> IgG, IgA (+) | IBD: 24.4, C: 37.1 CD: 12.9, UC: 29.7 | Finland | [41] ² |
| 100 | 100 | 100 | Patients with acute bacterial diarrhoea ¹ | <i>H. pylori</i> IgG, IgA (+) | IBD: 15, C: 43 CD: 13, UC: 18 | Finland | [42] ² |
| 147 | 169 | 316 | Non-IBD patients ¹ | UBT | IBD: 25.3, C: 52.5 CD: 17.7, UC: 32 | Korea | [43] ² |
| 386 | 0 | 277 | Blood donors ¹ | <i>H. pylori</i> IgG, IgA (+) | IBD: 17.4, C: 35.4 CD: 17.4, UC: NA | Nederland | [44] ² |
| 90 | 0 | 525 | Non-IBD patients | Histology | IBD: 16.7, C: 40.2 CD: 16.7, UC: NA | Japan | [45] ² |
| 38 | 0 | 12 | Healthy subjects ¹ | UBT | IBD: 8, C: 42 CD: 8, UC: NA | Japan | [46] ² |
| 80 | 39 | 98 | Non-IBD patients ¹ | <i>H. pylori</i> IgG (+) | IBD: 27.5, C: 41.7 CD: 13.5, UC: 30.8 | Israel | [47] ² |
| 51 | 82 | 200 | Non-IBD patients ¹ | UBT | IBD: 12.8, C: 39 CD: 13.7, UC: 12.2 | Hungary | [48] ² |
| 36 | 0 | 36 | Healthy subjects ¹ | Histology | IBD: 8.3, C: 36.1 CD: 8.3, UC: NA | Germany | [49] ² |
| 75 | 0 | 200 | Non-CD patients | Histology | IBD: 30.5, C: 35.2 CD: 33, UC: NA | Germany | [50] |
| 56 | 0 | 382 | Non-CD patients | Histology | IBD: 32.1, C: 46.1 CD: 32.1, UC: NA | USA | [51] ³ |
| 371 | 560 | 64451 | Non-IBD patients | Histology | IBD: 4.5, C: 9 CD: 4, UC: 5 | USA | [52] ² |
| 0 | 42 | 74 | Non-IBD patients | <i>H. pylori</i> IgG (+), UBT | IBD: 52.4, C: 51.4 CD: NA, UC: 52.4 | Brazil | [53] |
| 43 | 0 | 74 | Non-IBD patients | UBT | IBD: 51.2, C: 70.3 CD: 51.2, UC: NA | Brazil | [54] |
| 50 | 44 | 194 | Non-IBD patients | Histology, RUT | IBD: 9.6, C: 38.5 CD: 14, UC: 4.5 | Poland | [55] ^{2,3} |
| 21 | 23 | 76 | Non-IBD patients | <i>H. pylori</i> IgG (+) | IBD: 54.5, C: 68 CD: 52.2, UC: 57.1 | Mexico | [56] |
| 104 | 104 | 416 | Healthy subjects ¹ | UBT | IBD: 19.7, C: 48.8 CD: 18.3, UC: 21.2 | Chinese | [57] ² |
| 229 | 0 | 248 | Non-CD patients | UBT, culture, histology | IBD: 27.1, C: 47.9 CD: 27.1, UC: NA | Chinese | [58] ² |
| 0 | 153 | 121 | Non-UC patients | UBT, culture, histology | IBD: 30.5, C: 57 CD: NA, UC: 30.5 | Chinese | [59] |
| 30 | 30 | 20 | Non-IBD patients ¹ | UBT | IBD: 43, C: 40 CD: 50, UC: 37 | Spain | [60] |

¹Age and sex matched; ²Statistically significant result (IBD vs control group); ³Paediatric population. CD: Crohn's disease; UC: Ulcerative colitis; C: Controls; IBD: Inflammatory bowel disease; GI: Gastrointestinal; *H. pylori*: *Helicobacter pylori*; Ref: References; NA: Not applicable; NR: Not reported; FAT: Serology fecal antigen test; RUT: Rapid urease test; UBT: Urea breath test.

Table 2 Prevalence of both *Helicobacter pylori* negative and positive gastritis in patients with inflammatory bowel disease in different populations

| CD | UC | C | Control group | Biopsies | <i>H. pylori</i> (+) gastritis (%) | <i>H. pylori</i> (-) gastritis (%) | Ref. |
|-----|-----|------|------------------|-----------------------|------------------------------------|------------------------------------|------|
| 37 | 43 | 41 | Non-IBD patients | Antrum, body | CD: 27, UC: 37.2 C: 53.7 | CD: 29.6, UC: 22.2 C: 10.5 | [61] |
| 141 | 79 | 141 | Non-IBD patients | Antrum, angulus, body | CD: 33, UC: 47 C: 60 | CD: 43, UC: 12 C: 19 | [34] |
| 75 | 0 | 200 | CD-free patients | Antrum, body | CD: 33.3, UC: NA C: 48 | CD: 39, UC: NA C: 0.8 | [50] |
| 208 | 280 | 4943 | Non-IBD patients | Antrum, body | CD: 4, UC: 6 C: 7 | CD: 5, UC: 0 C: 0 | [63] |
| 67 | 41 | 43 | Healthy subjects | Antrum, body | CD: 17.6, UC: 6.4 C: 20 | CD: 45.4, UC: 15.6 C: 30 | [33] |
| 62 | 0 | 0 | NA | Antrum, corpus | CD: 9.7, UC: NA C: NA | CD: 32, UC: NA C: NA | [64] |

CD: Crohn's disease; UC: Ulcerative colitis; C: Controls; IBD: Inflammatory bowel disease; *H. pylori*: *Helicobacter pylori*; NA: Not applicable.

microbiota may be strong confounders, further mechanistic studies in *H. pylori* infection using mouse models are necessary to further define the mechanism of this negative association. Furthermore, when looking for explanations for the lower prevalence of *H. pylori* infection in IBD, some authors have suggested that treatment with sulfasalazine and other aminosalicic compounds could be responsible for “spontaneous eradication” of *H. pylori* infection^[32,34,35,38], although their possible role has not been confirmed by other studies^[29-31,37,39,41-45,55,57,60,99]. Various studies have suggested that sulfasalazine, but not 5-aminosalicylic acid (5-ASA), could account for the lower prevalence of *H. pylori* infection^[32,34], whereas Piodi *et al*^[35] found the opposite. Ishikawa *et al*^[100] observed a lower prevalence of *H. pylori* infection in rheumatoid arthritis patients receiving sulfasalazine, whereas Taha *et al*^[101] did not find any statistically significant difference. The mechanisms of how these agents prevent *H. pylori* infection is still unknown, but prevention may be the result of a direct action against germ adhesion to the gastric mucosa or due to immuno-modulatory actions of the drugs^[30,102,103]. It has also been hypothesized that prolonged treatment with antibiotics used in IBD (especially metronidazole) could account for spontaneous eradication and lower prevalence of *H. pylori* infection. Indeed the prevalence of *H. pylori* infection was significantly lower in CD patients who had received antibiotics for ≥ 2 wk^[45] while in another study, antibiotic therapy was negatively associated with *H. pylori* infection (20.5% *vs* 55%, $P = 0.0001$)^[39]. Moreover, other studies have shown that prior treatment with ciprofloxacin and/or metronidazole had no influence on *H. pylori* status in IBD patients^[48,104,105].

Finally, the data on the prevalence of virulent *H. pylori* strains in IBD patients are limited. Wagtmans *et al*^[44] showed that the majority (66%) of *H. pylori* seropositive patients with CD were infected by *H. pylori* cagA (+) strains although a similar proportion of controls (69.4%) were also infected by these strains. These findings deserve further investigation as it is well known that the intense host responses, specifically to *H. pylori* cagA

(+) strains may further alter Th1- and Th2-type immune responses with subsequent induction of immune-regulatory lymphocytes^[106].

POTENTIAL PROTECTIVE MECHANISMS OF *H. PYLORI* AGAINST IBD

It is plausible to suggest that *H. pylori*, by attempting to promote its own survival, may benefit the host via a variety of mechanisms against other chronic inflammatory conditions such as IBD. Several mechanisms have been proposed to explain the inverse association between *H. pylori* and IBD. In CD, Th1 immune responses prevail, whereas in UC, Th2 or Th1/Th2 immune responses may be predominant^[5,107,108]. These altered immune responses to lumen antigens in IBD may influence the way the host responds to *H. pylori* infection. Conversely, a perpetual bacterial infection in the stomach may either alter the host immune responses in a way that may be protective or render the host susceptible to IBD. The levels of numerous cytokines, including IFN- γ , TNF, IL-1 β , IL-6, IL-7, IL-8, IL-10, and IL-18, are increased in the gastric epithelial cells of *H. pylori* infected humans compared to uninfected humans^[109-111]. After activation of Toll-like receptors by *H. pylori*, dendritic cells (DC) may activate T cells in different ways, being capable of inducing either a Th1 or Th2/regulatory T cell (Treg) response by generation of IL-12 or IL-10, respectively^[112,113]. This finding was reported by D'Elia *et al*^[114] who observed that most (64%) of *H. pylori* specific T cell clones derived from uncomplicated chronic gastritis displayed a Th2-like phenotype, producing interleukin IL-4 or IL-5 together with INF- α , whereas only one third of *H. pylori*-specific gastric T cells were polarized with Th1 effectors.

Thus, a protective role of *H. pylori* infection against IBD may be due to the ability of this microbe to down-regulate pro-inflammatory immune responses. Considering that adoptive transfer of Treg is able to prevent and/or treat colitis in various animal models, it is reasonable to suggest that these cells produced in response to *H.*

pylori infection may act in the prevention of IBD^[115-119]. *H. pylori* can induce a Treg response and down-regulate the pro-inflammatory Th1/Th17 pathway^[65,66,120-123]. The importance of Treg in the pathogenesis of IBD was illustrated by the development of spontaneous colitis in mice deficient of IL-10, a key regulatory cytokine for Treg function^[124].

Moreover, the systemic levels of type I IFN were found to be lower in *H. pylori* infection-colonized IBD patients compared to non-colonized controls^[125]. Luther *et al*^[125] showed that prior oral administration of 20-50 µg *H. pylori* DNA ameliorated the severity of dextran sulphate sodium (DSS) induced acute or chronic colitis in mice in terms of both pathology and symptoms such as bleeding and weight loss. Thus, the protective properties of *H. pylori* DNA were attributed *in vitro* to inhibition of cytokine production by DC, which upon addition of the DNA failed to produce type I interferon and IL-12 in response to *E. coli* DNA^[125]. A protective effect of *H. pylori* colonization in mice against experimental colitis was also demonstrated by Higgins *et al*^[126]. Mice that were colonized with *H. pylori* SS1 6 wk prior to the induction of *Salmonella typhimurium* experimental colitis, experienced markedly less severity inflammation compared to mice that were not colonized with *H. pylori*. This result could be attributed to an up-regulation of IL-10 in the mesenteric lymph nodes and suppression of the Th-17 response in the cecum of the infected mice^[126], illustrating an extra-gastric immune-modulatory effect of the bacterium, an immunological crosstalk between the upper and lower gastrointestinal tract and providing mechanistic support for the epidemiological observation of a negative association between *H. pylori* status and the risk of IBD.

Another protective mechanism may operate via the development of antibodies against *H. pylori*, which may confer an immunization-type protection against other pathogenic *Helicobacter* or even different types of microbes implicated in IBD. Although *H. pylori*-specific antibodies do not eradicate this bacterium, they seem to confer a degree of protective immunity from a subsequent *Campylobacter* infection, indicating an antigenic cross-reactivity between these two bacterial species^[127-129]. It could also be that *H. pylori* induced reduction in acid secretion indirectly affects a different type of infection that ultimately results in IBD. Indeed, variable disease phenotype during dual infection by different *Helicobacter* species has been described by Lemke *et al*^[130] who demonstrated that *H. bilis* and *H. pylori* co-infection in mice attenuates *H. pylori* gastritis compared to those infected only with *H. pylori*.

The protective effect of *H. pylori* may simply be due to other confounding variables such as the presence of inherent genetic or environmental factors that favour *H. pylori* acquisition in some and the development of IBD in others. This scenario would fit well with the observation that IBD is associated with better hygiene, which in itself may be detrimental to *H. pylori* acquisition^[131,132]. The low prevalence of *H. pylori* infection in patients with

IBD compared to non-IBD patients strengthens the importance of the “hygiene hypothesis” in the development of autoimmunity and IBD. It suggests that inadequate microbial stimulation of gut-associated lymphoid tissue is a critical point for maturation of mucosal immunity^[133,134]. Improved access to a cleaner environment and the resulting decreased incidence of common childhood infections, including *H. pylori*, may be contributing to autoimmunity by altering susceptibility to certain diseases with an autoimmune component, such as IBD^[126].

Finally, regarding genetic factors, the CD variant of the autophagy gene ATG16L1 alters susceptibility to *H. pylori* infection with an enteric microbe in human subjects at the population level, supporting a role for altered autophagy in regulating the host response to enteric microbes in CD pathogenesis. It is interesting to speculate that due to increased susceptibility to infection, early exposure and acquisition of *H. pylori* in individuals with the ATG16L risk allele may decrease their risk for the subsequent development of IBD^[135].

ERADICATION OF *H. PYLORI* AND DEVELOPMENT OF IBD

There seems to be a rapid onset of CD after eradication of *H. pylori* infection, as illustrated by two cases^[136]. A similar experience was recently described by Jovanovic *et al*^[137], who described the onset of gastric CD only 6 mo after *H. pylori* infection eradication. Moreover, a steady rise in the incidence of UC was reported in *H. pylori* endemic regions after successful eradication of *H. pylori* infection^[128].

It is unknown why these patients developed CD after eradication of *H. pylori* infection, but this may be due to the induction of immune responses that in turn contributed to the development of the disease. Long-term *H. pylori* infection may cause an unstable equilibrium between the Th1 and Th2 phenotype pattern; eradication of *H. pylori* infection may diminish Th2 cytokine, with sudden consequent Th1 pattern prevalence and rapid increase of pro-inflammatory cytokines^[106]. In genetically predisposed subjects, this Th1 predominant pattern may suddenly favour the onset of a typical Th1-related disease such as CD. Further studies investigating the effect of eradication of *H. pylori* on the development and natural history of IBD are warranted.

CAUSAL ASSOCIATION OF *H. PYLORI* WITH COLORECTAL CANCER?

A meta-analysis of 13 studies suggested an increased risk of colorectal cancer due to *H. pylori* infection^[138]. Kapetanakis *et al*^[139] demonstrated the presence of *H. pylori* in malignant colonic tissue in 34 of 41 (82.9%) patients with colorectal cancer. *H. pylori* colonizing colonic tumour tissue seems to be associated with increased cell proliferation and impaired apoptotic process in malignant tissue

compared with adjacent normal colonic mucosa, thereby further contributing to colon cancer progression^[140]. Furthermore, *H. pylori* induced gastrin release can act as promoter of cell proliferation and differentiation (mainly by inducing COX-2 overexpression and PI3-kinase-mediated tyrosine phosphorylation of E-cadherin and b-catenin) in different gastrointestinal tract sites, including the colon^[141]. *H. pylori* infection is also accompanied by bone-marrow-derived stem cell (CD34+) recruitment that ultimately facilitates colon cancer progression^[142]. Finally, compared to normal gastric mucosa, *H. pylori* gastritis occurred more frequently among patients with hyperplastic polyps (OR = 1.24, 95%CI: 1.18-1.30), adenomatous polyps (OR = 1.52, 95%CI: 1.46-1.57), advanced adenomas (OR = 1.80, 95%CI: 1.69-1.92), villous adenomas or adenomas with high-grade dysplasia (OR = 1.97, 95%CI: 1.82-2.14), and adenocarcinomas (OR = 2.35, 95%CI: 1.98-2.80)^[143]. It has therefore been proposed that *H. pylori* eradication might inhibit IBD-related or non-colon neoplasia^[144].

CONCLUSION

Since the discovery of *H. pylori*, several epidemiological studies, therapeutic trials, case reports and/or *in vitro* studies have been published concerning a hypothetical damaging or protective role of *H. pylori* in the development of IBD. Whether the link between *H. pylori* and IBD is coincidental, epiphenomenal or mechanistic remains uncertain. There are contradictory data regarding both the causative and the protective role of *H. pylori* infection against IBD.

The discordance between studies may be explained by a number of confounding factors, such as variability in the power of the studies and the time periods in which these studies were conducted, geographical factors and the differences in the methods used to detect *H. pylori* infection^[145]. To be more specific, the urease breath test is more sensitive in detecting *H. pylori* than histology. Histology involves the examination of tissue samples that may be insufficient for a correct diagnosis and is more timely than serology, which also detects previous infections. Furthermore, one limitation of the studies using serology for the presence of *H. pylori* is the fact that after successful eradication of *H. pylori* infection, positive titres of antibodies normalize very slowly within several months, or even years, leading to the possibility that negative findings from *H. pylori* serology do not reflect eradication of *H. pylori* infection^[146]. Finally, from a clinical point of view, we must always bear in mind that any type of protection that exerts its influence on a general population level may not necessarily materialize in the individual patient.

In conclusion, the association between *H. pylori* infection and IBD is still controversial; however, it is worthy of further investigation, as the potential association of *H. pylori* with extra-gastric manifestations and disorders is always a very interesting and challenging research area^[147].

It is unclear whether the apparent protective effect of *H. pylori* is simply confounding due to other variables, but the effect of the presence of the live bacterium remains to be elucidated. More studies investigating the effect of *H. pylori* infection eradication on the risk of development of IBD and the natural history of IBD are needed.

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori infection: New pathogenetic and clinical aspects

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Abstract

Helicobacter pylori (*H. pylori*) infects more than half of the world's human population, but only 1% to 3% of infected people consequently develop gastric adenocarcinomas. The clinical outcome of the infection is determined by host genetic predisposition, bacterial virulence factors, and environmental factors. The association between *H. pylori* infection and chronic active gastritis, peptic ulcer disease, gastric cell carcinoma, and B cell mucosa-associated lymphoid tissue lymphoma has been well established. With the exception of unexplained iron deficiency anemia and idiopathic thrombocytopenic purpura, *H. pylori* infection has no proven role in extraintestinal diseases. On the other hand, there is data showing that *H. pylori* infection could be beneficial for some human diseases. The unpredictability of the long-term consequences of *H. pylori* infection and the economic challenge in eradicating it is why identification of high-risk individuals is crucial.

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Key words: *Helicobacter pylori*; Virulence factor; Host factors; Gastroduodenal diseases; Extraintestinal disorders

Core tip: *Helicobacter pylori* (*H. pylori*) infects more

than half of the world's human population. The association between *H. pylori* infection and chronic active gastritis, peptic ulcer disease, gastric cell carcinoma, and B cell mucosa-associated lymphoid tissue lymphoma, unexplained iron deficiency anemia and idiopathic thrombocytopenic purpura has been well established. *H. pylori* screening and treatment is a recommended gastric cancer risk reduction strategy in high-risk populations. The unpredictability of the long-term consequences of *H. pylori* infection and the economic challenge in eradicating it is why identification of high-risk individuals is crucial.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a micro-aerophilic, Gram-negative, slow-growing, spiral-shaped, and flagellated organism which infects more than half of the world's human population^[1]. *H. pylori* colonization itself does not cause any symptoms, and fewer than 20% of all infected patients will develop symptoms from their infection^[2]. Approximately 10% of infected individuals develop peptic ulcer disease, 1% to 3% develop gastric adenocarcinoma, and less 0.1% [mucosa-associated lymphoid tissue (MALT)] develop lymphoma^[3].

The outcome of *H. pylori* infection may involve a combination of bacterial, host, and environmental factors. The association between *H. pylori* infection and chronic active gastritis, peptic ulcer disease, gastric cell carcinoma, and B cell MALT lymphoma has been well established. On the other hand *H. pylori* infection could

Table 1 Summary of the pathogenetic and preventive role of *Helicobacter pylori*

| Pathogenetic role | | Preventive role |
|---------------------------------|--|---------------------------------|
| Proven | Suspected | Suspected |
| Gastro-duodenal diseases | Gastro-intestinal diseases | Gastroesophageal diseases |
| Peptic ulcer | Pancreatic cancer | Gastroesophageal reflux disease |
| Gastric cancer | Colorectal adenoma/carcinoma | Esophageal adenocarcinoma |
| MALT lymphoma | Liver cirrhosis, hepatocellular carcinoma | |
| Extra-intestinal diseases | Extra-intestinal diseases | Extra-esophageal diseases |
| Immune thrombocytopenic purpura | Laryngeal cancer | Bronchial asthma |
| Iron deficiency anemia | Lung cancer | |
| | Metabolic syndrome/insulin resistance | |
| | Cardiovascular diseases/ischemic heart disease | |
| | Chronic urticaria | |
| | Henoch-Schönlein purpura | |

MALT: Mucosa-associated lymphoid tissue.

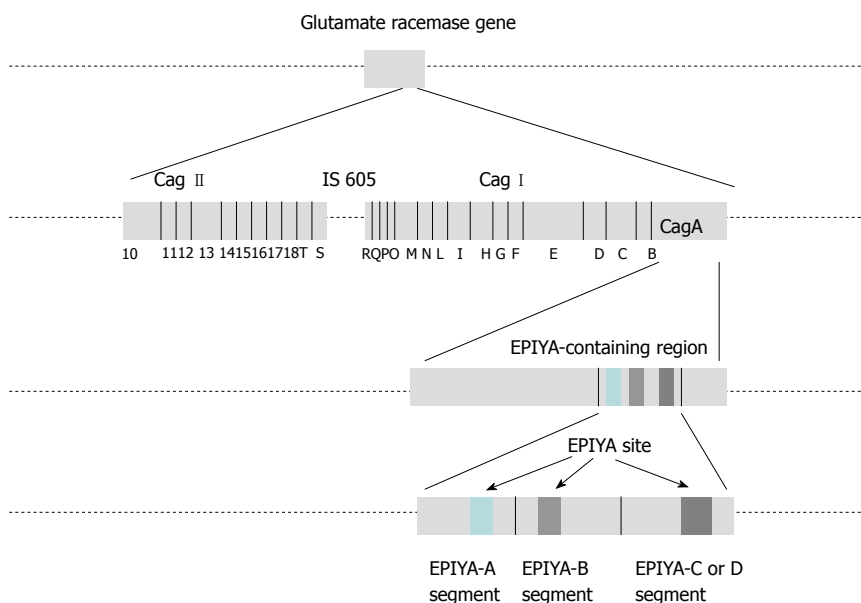


Figure 1 Cytotoxin-associated gene pathogenicity island. CagA: Cytotoxin-associated gene A product; EPIYA: Glutamate-proline-isoleucine-tyrosine-alanine.

be beneficial for humans^[2] (Table 1).

PATHOGENETIC ASPECTS

Virulence factors of *H. pylori*

Bacterial virulence factors play a significant role in the outcome and progression of *H. pylori* infection^[4]. The linkages of virulence factors may show how they interact with each other^[5].

The cag pathogenicity island (cag PAI) contains 27-31 genes flanked by a 31-p direct repeats. *H. pylori* exhibits a high degree of genetic heterogeneity due to genomic rearrangements, gene insertions, and/or deletion^[6].

At least 18 cag genes encode components of the bacterial type IV secretion system, which functions to export bacterial protein across the bacterial membrane and into host gastric epithelial cells. The presence of cag PAI (cag+) amplifies the risk for severe gastritis, atrophic gastritis, and distal gastric cancer in comparison with cag-deficient (cag-) bacteria^[6].

CagA: Cytotoxin-associated gene A product (CagA) is translocated into the host cell by the type IV secretion system. Phosphorylation of CagA at the glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs by the host Abl and Src kinases results in morphological changes to the cell (the so-called “hummingbird phenotype”). Four EPIYA motifs (-A, -B, -C, and -D) are distinguished with different degrees of phosphorylation and geographical distribution^[6]. EPIYA-A and EPIYA-B sites are less phosphorylated in comparison with EPIYA-C. EPIYA-C is typically found only in strains from Western countries (Europe, North America, and Australia), and is an indicator of gastric cancer risk. EPIYA-D is found in East Asian strains. EPIYA-D containing strains induce more relief of interleukin-8 (IL-8) from gastric epithelial cells^[6] (Figure 1).

Phospho-CagA interacts with numerous intracellular effectors, including eukaryotic tyrosine phosphatase with sustained activation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2), Crk adaptor, and C-terminal

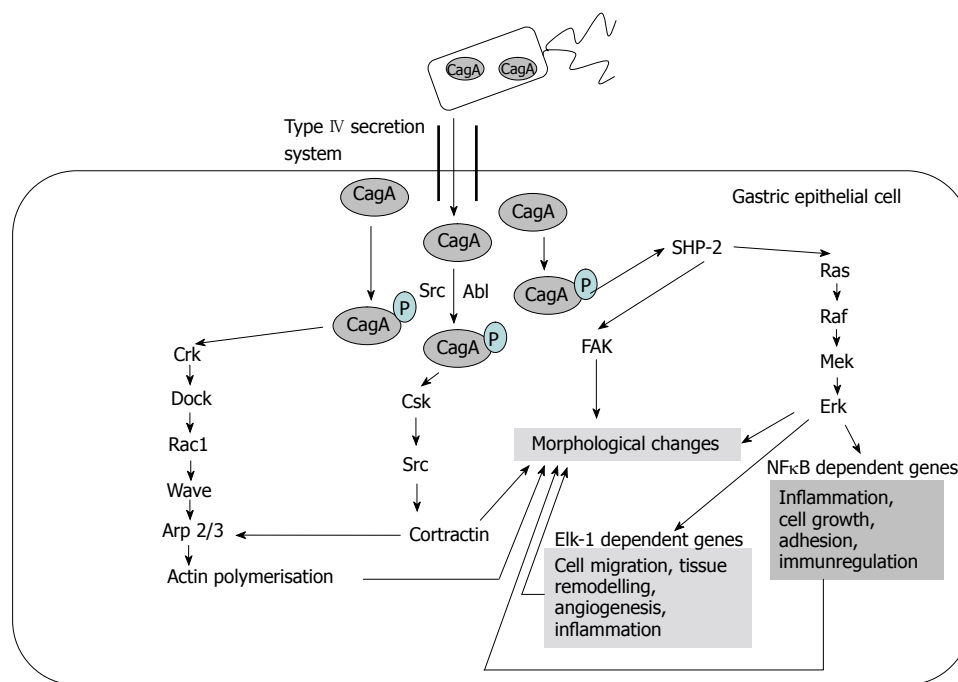


Figure 2 Targets of phosphorylated cytotoxin-associated gene A. Based on the article from Current Opinion in Microbiology, Hatakeyama M, SagA of CagA in *Helicobacter pylori* pathogenesis, 11, 30-37, Copyright (2008), with permission from Elsevier^[7]. CagA: Cytotoxin-associated gene A product; NFκB: Nuclear factor κB; FAK: Focal adhesion kinase; Csk: C-terminal Src kinase.

Src kinase^[6]. The activation of ERK and focal adhesion kinase with the tyrosine dephosphorylation of the actin binding proteins cortactin, ezrin, and vinculin leads to cell elongation^[1,6] (Figure 2).

The targets of non-phosphorylated CagA comprise E-cadherin, β-catenin, hepatocyte growth factor receptor c-Met, phospholipase C gamma, adaptor protein Grb2, kinase partitioning-defective 1b/microtubule affinity-regulating kinase 2, epithelial tight junction scaffolding protein zonula occludens 1, and the transmembrane protein junctional adhesion molecule A. The main effects are pro-inflammatory and mitogenic cell-cell junction disruption and loss of cell polarity that may be important in gastric carcinoma development^[1,6] (Figures 3 and 4).

Activity of CagA on tumor-suppressor pathways has also been investigated. CagA is able to modulate the *H. pylori* induced apoptotic signal, but the exact mechanism remains to be elucidated. The initial host response up-regulates p53 expression followed by the proteasomal degradation of p53^[8].

Almost all *cagA*+ strains are classified as *vacA* s1 genotypes (either m1 or m2), whereas almost all *cagA*- strains are classified as the *vacA* s2/m2 strain (see below)^[5]. Specific *vacA* genotypes of *H. pylori* strains are associated with a level of *in vitro* cytotoxin activity with clinical consequences^[9].

Peptidoglycans: Peptidoglycans translocated by the *cag* secretion system interact with the nucleotide-binding oligomerization domain 1 (Nod1) molecule which leads to the activation of nuclear factor κB (NF-κB), pro-inflammatory secretion of interleukin-8 (IL-8), and

β-defensin-2^[6,10]. *H. pylori* enhances the phosphoinositide 3-kinase Akt signaling pathway, leading to decreased apoptosis and increased cell migration. NOD1 ligand binding can activate the interferon (IFN)-stimulated gene factor 3 signaling cascade, resulting in type I IFN production usually associated with protection against viral infection and possibly other mucosal infections^[11].

VacA toxin: The cytotoxin gene *vacA* is present in all strains. The VacA cytotoxin induces the vacuolation, gastric epithelial barrier function disruption, disturbance of late endosomal compartments, and modulation of the inflammatory response. VacA reduces the mitochondrial transmembrane potential, releases cytochrome c from mitochondria, activates caspase 8 and 9, and induces apoptosis^[6,12].

Binding of VacA to receptor-type protein tyrosine phosphatase (RPTPβ) regulates cell proliferation, differentiation, and adhesion, which all play a role in ulcerogenesis^[13].

Variations in *vacA* gene structure (in the signal s: s1, s2, or in the middle regions m: m1, m2) make differences in vacuolating activity and specificity. The intermediate (i) region also plays role in the vacuolating activity of *H. pylori*. All s1, m1 strains were classified as i1 (vacuolating) type, and all s2, m2 strains were classified as i2 (non-vacuolating) type, while s1, m2 alleles could be i1 or i2. A novel intermediate variant (i3) has been identified. The fourth pathogenic region is d, a 69-81 bp-region between the m and i regions^[1,5].

The variants in s and m regions seem to be a good indicator of clinical outcomes. However the roles of i

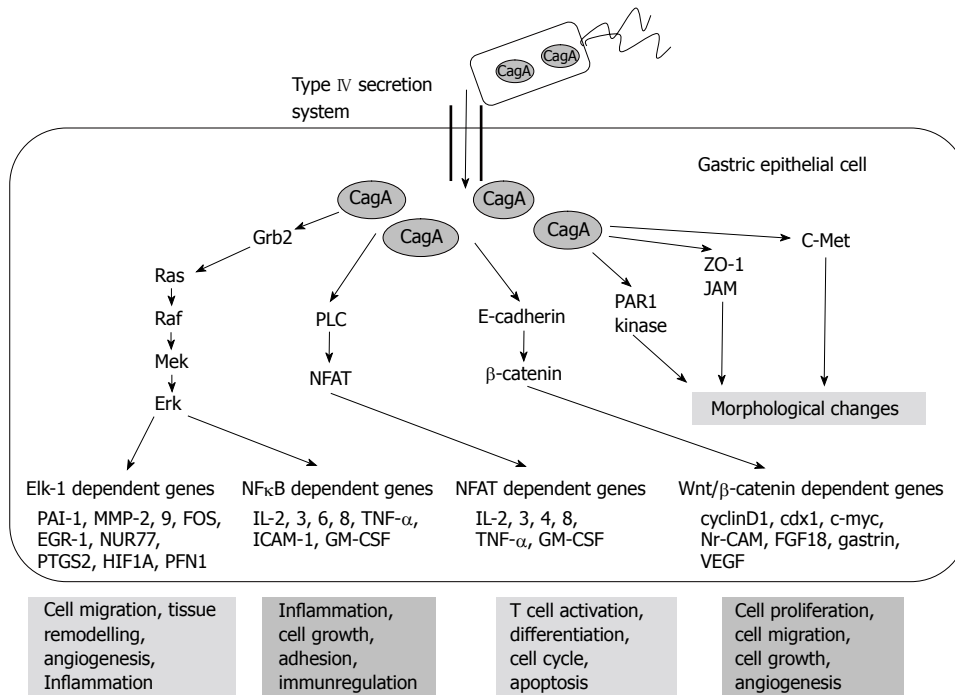


Figure 3 Targets of non-phosphorylated cytotoxin-associated gene a product. Based on the article from Current Opinion in Microbiology, Hatakeyama M, Saga of CagA in *Helicobacter pylori* pathogenesis, 11, 30-37, Copyright (2008), with permission from Elsevier^[7]. CagA: Cytotoxin-associated gene A product; PLC: Phospholipase C gamma; PAR1: Kinase partitioning-defective 1b; ZO-1: Zonula occludens 1; JAM: Junctional adhesion molecule A; NFκB: Nuclear factor κB; TNF-α: Tumor necrosis factor-α; IL: Interleukin.

and d regions should be further investigated^[5]. The s1, m1 strains can induce greater vacuolation, and are associated with peptic ulcer disease and gastric cancer in Western countries, but have no pathogenic role in East Asian countries^[16]. vacA i1 strains were associated with gastric cancer in Iranian patients^[14], but not in the East Asian or Southeast Asian populations^[14]. i1 genotype appeared to be a better predictor of carcinoma-associated *H. pylori* strains than the s or m genotype^[15]. In Western countries, d1 strains without the deletion of the d region are predictors of histological inflammation, atrophy, and an increased risk of peptic ulceration and gastric cancer, compared with the presence of the vacA s-, m-, and i-region strains^[16].

Adhesins and outer membrane proteins: 4% of the *H. pylori* genome encodes for outer membrane proteins (BabA, BabB, SabA, and OipA) which function as adhesins and porins, and are implicated in complement resistance and immune regulation^[17].

The blood group antigen binding adhesin BabA is thought to mediate host-bacterial interactions and maintain colonization of the *H. pylori* targeting human Lewis-b surface epitopes^[18,19]. The *babA2* gene is associated with duodenal ulcer and gastric cancer. When in conjunction with cagA and vacA s1 alleles ("triple-positive strains"), it is associated with a greater risk of the more severe duodenal ulcer and gastric adenocarcinoma in Western populations^[16,19].

Sialic acid-binding adhesin (SabA) binds to the car-

bohydrate structure sialyl-Lewis antigen expressed on the gastric epithelium. SabA can mediate the binding of *H. pylori* to neutrophils and erythrocytes, but the pathophysiological importance of these findings is uncertain^[1]. SabA positive status was associated with increased gastric cancer risk and a negative status associated with duodenal ulceration^[12].

The outer inflammatory protein (OipA) has a role in the increased expression of mucosal IL-1, -8, -17, tumor necrosis factor-α (TNF-α), and in gastric mucosal inflammation. Upregulation of matrix metalloproteinase 1, inhibition of glycogen synthase kinase 3β, and nuclear accumulation of β-catenin can influence carcinogenesis^[6]. OipA positive status was significantly associated with duodenal ulcer and gastric cancer^[12].

Others: Duodenal ulcer promoting gene (dupA) product induces the production of IL-8 and -12^[5]. DupA may enhance duodenal ulceration and /or decrease gastric cancer development in some populations^[1,5,6].

Variants of gene encoding flagellar proteins (flaA) of *H. pylori* may affect motility and colonization, and, therefore, the carcinogenic effect^[6].

Annexin family members (AnxA1 and AnxA4) are involved in epithelial cell membrane repair response induced by *H. pylori*-generated VacA and CagA-independent plasma membrane disruption. Plasma membrane disruption and AnxA4 can promote cell proliferation^[19].

TNF-α-inducing protein (Tipα) binds to cell-surface nucleolin and then enters the gastric cancer cells where

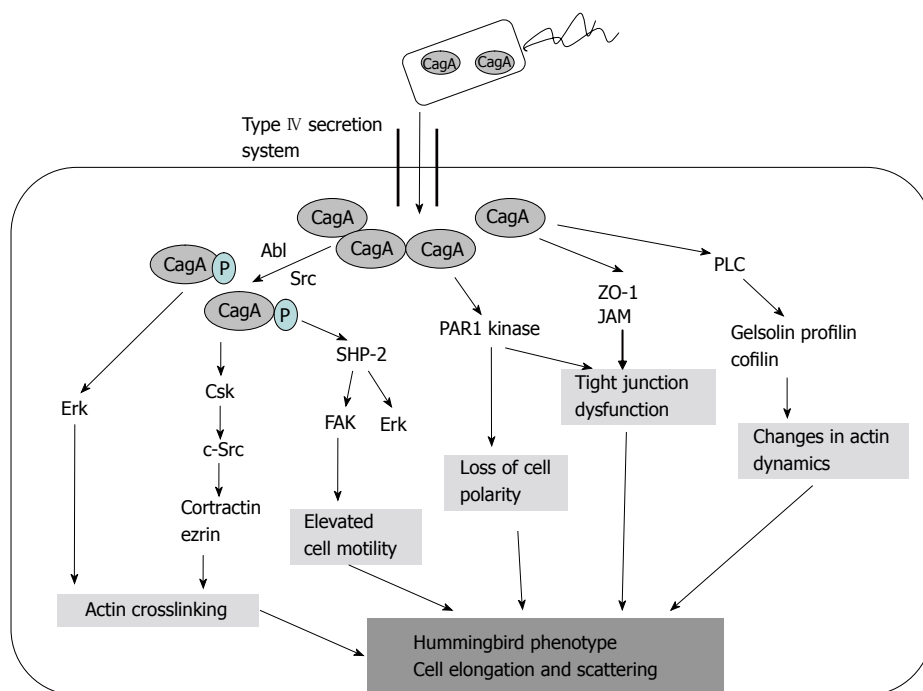


Figure 4 Development of “hummingbird phenotype”. CagA: Cytotoxin-associated gene A product; PLC: Phospholipase C gamma; PAR1: Kinase partitioning-defective 1b; ZO-1: Zonula occludens 1.

TNF- α and chemokine gene expressions are induced by NF- κ B activation in a cag PAI independent manner^[20].

Bacterial factors like urease, AmiE, AmiF, hydrogenase, and arginase are essential for *H. pylori* survival in the acidic gastric environment^[4].

Immune response to *H. pylori*: The host's innate and adaptive immune system plays a crucial role in the initiation and progression of *H. pylori* infection^[21].

Innate immunity effectors and a complex mixture of T helper (Th) 1, Th17, and regulatory T cells (Treg) adaptive immunity effectors are involved in *H. pylori* infection^[22].

H. pylori initially targets gastric epithelial cells which form part of the innate immune response via signaling through pattern recognition receptors, such as Toll-like receptors (mainly TLR2)^[21].

The neutrophil-activating protein of *H. pylori* polarizes Th1 cells, stimulating IL-12 and IL-23 secretion from neutrophils and macrophages. Th1 cytokines, such as gamma interferon (IFN- γ) and TNF- α , can increase the release of pro-inflammatory cytokines and augment apoptosis induced by *H. pylori*^[22,23].

IL-17 expressing Th17 cells are important in the pro-inflammatory immune response to *H. pylori*. Th17 cells produce IL-17, IL-21, and IL-22 cytokines^[6]. *H. pylori* infected macrophages produce IL-6, IL-23, and transforming growth factor (TGF)- β , which are required for Th17 cell development and maintenance^[6,21]. The literature on Th1 and Th17 *H. pylori*-associated gastric pathology is confusing and requires intensive investigation^[6].

Tregs (formerly suppressor T cells) are also implicated in the pathogenesis of *H. pylori* infection. TGF- β and IL-18 are responsible for Treg development^[21]. *H. pylori*-

specific Tregs suppress memory T cell responses that prolong the infection^[6]. Tregs suppress the inflammatory reaction driven by IL-17, thereby also favoring bacterial persistence^[24].

Antimicrobial defense of macrophages is nitric oxide (NO) dependent. *H. pylori*'s arginase enzyme can compete with macrophages for the inducible nitric oxide synthase (iNOS) substrate L-arginine so that host NO production is impaired; this leads to enhanced bacterial survival. *H. pylori* can evade macrophage phagocytosis. VacA protein prevents the fusion of phagosomes with lysosomes needed for phagocytosis. Fused phagosomes contain large numbers of live bacteria^[6].

The role of B cells in the host response to *H. pylori* has been suggested^[21]. Immunoglobulin (Ig) G and IgA antibody release from B cells in response to *H. pylori* may be involved in protective immunity, however it was suggested this antibody-mediated response may be counterproductive. B cells can also produce autoreactive antibodies that may be pathogenic^[6]. B cell activation and survival may have implications for MALT lymphoma development^[6].

CLINICAL ASPECTS

Gastrointestinal diseases

Peptic ulcer: Some *H. pylori* colonized individuals may develop corpus gastritis associated with gastric hypochlorhydria, gastric atrophy, gastric ulcer, and an increased risk of gastric cancer. Conversely, others may develop antral-predominant gastritis, which is associated with gastric hyperchlorhydria and an increased risk of duodenal ulcer^[8,25].

Since the discovery of *H. pylori* in the 1980s, the availability of effective eradication therapy has led to a decline in recurrent peptic ulcer disease and its complications. The pathogenetic role of *H. pylori* in 90% of duodenal ulcers and 80% of gastric ulcers is proven^[26,27]. Effective eradication decreased the yearly recurrence rate of duodenal and gastric ulcers from 80% and 60%, respectively, to less than 5%^[28].

Gastric cancer: *H. pylori* is a class I carcinogen in humans^[1]. It is considered to be the most common aetiological factor of infection-related cancers (followed by human papilloma, hepatitis B and C, Epstein-Barr, human immunodeficiency, and human herpesvirus-8)^[1,29]. *H. pylori* infection-related cancer represents 5.5% of the global cancer burden^[6].

Gastric cancer develops in 2.9% of *H. pylori* infected patients^[30]. *H. pylori* infection is responsible for about 75% of all non-cardia gastric cancers and 63.4% of all stomach cancers worldwide^[1]. *H. pylori* infection also plays a fundamental role in non-cardia gastric carcinogenesis, but its association with cardia cancer is still uncertain^[31].

The prevalence of infection is statistically significantly much higher in patients with intestinal-type gastric cancer (89.2%) compared to the diffuse-type (31.8%)^[32]. *H. pylori* infection is regarded as the trigger of intestinal-type gastric adenocarcinoma^[33]. According to Correa and Piazuelo, intestinal-type gastric carcinogenesis progresses as follows: normal gastric mucosa - no atrophic gastritis - multifocal atrophic gastritis without intestinal metaplasia - intestinal metaplasia of complete (small intestine) type - low-grade dysplasia - high-grade dysplasia - invasive adenocarcinoma^[34]. Altered cell proliferation, apoptosis, epigenetic modifications to the tumor suppressor genes, oncogene activation, and dysregulation of DNA repair may occur and eventually lead to inflammation-associated carcinogenesis^[35].

Eradication of *H. pylori* infection decreases the risk of premalignant lesions and gastric cancer in infected individuals^[36-38]. Follow-up endoscopy and histology is crucial, even in patients with apparently non-malignant gastric ulcers, in improving the malignancy detection rate in populations with a high prevalence of gastric cancer^[39].

H. pylori plays a role in the development and progression of gastric (MALT) lymphoma^[40]. The average prevalence of *H. pylori* infection in MALT lymphoma was 79%; it was higher in low-grade (79%) than in high-grade (60%) cases^[41]. Treatment for localized stage I gastric MALT lymphoma with *H. pylori* infection is eradication^[40]. Eradication of *H. pylori* resulted in a complete remission in 60%-80% of patients with MALT lymphoma^[42,43], and a 10-year sustained remission in up to 64% of cases^[44].

The carcinogenic effect of *H. pylori* can be modified by dietary and environmental factors. *H. pylori* infection is more frequent in less developed Asian countries (e.g., India, Bangladesh, Pakistan, and Thailand) in comparison with the more developed Asian countries (e.g., Japan and China). However, the frequency of gastric cancer is para-

doxically very low in these less developed regions than in Japan and China (the so-called "Asian enigma")^[33,45]. Several other large populations with high infection prevalence show a very low rate of gastric cancer. The so-called "African enigma" remains unexplained as well, but it does verify that not all *H. pylori* infected patients have an increased risk of gastric cancer^[32,33,46].

Host and environmental factors also affect the development of gastroduodenal diseases in *H. pylori* infected individuals^[6,47]. Individuals with a high-expression of IL-1 β polymorphisms (C-T or T-C transitions, at positions -511, -31, and +3954 base pairs from the transcriptional start site) have an increased risk for hypochlorhydria, gastric atrophy, and distal gastric adenocarcinoma in comparison with low-expression polymorphisms; they have no effect on cancers associated with high acid exposure such as esophageal adenocarcinomas and some cardia cancers^[47,48]. The combined effects of pro-inflammatory IL-1 genotypes and *H. pylori* bacterial virulence factors have been reported^[48].

Gene polymorphisms (-308 G > A) of the pro-inflammatory cytokine TNF- α that increase the expression of the cytokine and polymorphisms (promoter polymorphisms at positions -592, -819, and -1082) that reduce the production of the anti-inflammatory cytokine (IL-10) have been associated with an increased risk of distal gastric cancer^[48-50].

The effects of pro-inflammatory genotypes (IL-1 β , TNF- α and IL-10) are additive^[6,50].

High dietary salt intake increases the risk of gastric cancer by directly damaging gastric mucus and mucosa, improving temporary epithelial proliferation, increasing the incidence of endogenous mutations, upregulating cytokine production, and *H. pylori* gene expression modulation, especially that of virulence factors^[51-53].

Co-infection with helminths (*Ascaris lumbricoides*) and *Toxoplasma gondii* reduces the severity of *H. pylori*-induced gastritis via a reduced Th1 response with higher levels of Th2 cytokines^[54].

Fruit and vegetables are rich sources of carotenoids, vitamin C, folate, and phytochemicals, which may modulate xenobiotic-metabolizing enzymes and have antioxidant activity, thereby playing a preventive role in carcinogenesis^[6,55-57].

Smoking is an established risk factor for gastric cancer. Swallowed carcinogenic substances (nitrosamine and other nitroso compounds), greater concentrations of smoking-related DNA adducts in the gastric mucosa, lower levels of free radical scavengers (ascorbic acid and β -carotene), and increased mRNA expression of chemokines in the gastric mucosa are in the background^[58].

Pancreatic cancer

Epidemiological studies have suggested that *H. pylori* might be involved in the pathogenesis of pancreatic cancer (OR = 1.87, 2.1), however results are inconsistent^[59,60]. A meta-analysis showed significant association between *H. pylori* seropositivity and development of pancreatic

Table 2 Putative pathomechanisms of *Helicobacter pylori*

| Disease | Putative pathomechanisms |
|---------------------------------------|---|
| Pathogenetic role | |
| Pancreatic cancer | Inflammatory cytokine ↑ ^[61] Angiogenic factors ↑ ^[61] Reactive oxygen species ↑ ^[61] Somatostatin synthesis ↓ ^[64,65] Secretin release ↑ ^[64,65] Basal pancreatic bicarbonate output ↑ ^[64,65] Bacterial overgrowth, production of N-nitroso compounds ↑ ^[66] Absorption of antioxidants ↓ ^[67] |
| Colorectal adenoma/carcinoma | Direct damage ^[69] Inflammation ↑ ^[69] Bacterial overgrowth, bacterial fermentation (ammonia) ↑ ^[69,71,74,75] NO release ↑ ^[76] Hypergastrinemia ^[68,69] |
| Hepatobiliary disease | Ammonia ↑ ^[90] Endotoxemia ^[90] Inflammation ↑ ^[90] Hepatic fibrosis ↑ ^[87] Hepatoma cell adhesion and invasion ↑ ^[91] |
| Laryngeal cancer | Sensitivity to smoke and dust ↑ ^[92] Cell proliferation ↑ ^[92] Apoptosis ↓ ^[92] |
| Lung cancer | Direct damage ^[97] Sensitivity to smoke and dust ↑ ^[98] Inhalation of gastrin and urea ^[95] Hypergastrinemia ^[94] Activation of docking protein p130cas ^[95] |
| Insulin resistance/metabolic syndrome | Inflammation ↑ ^[94] Inflammation ↑ ^[103,105] Vasoconstrictor factors ↑ ^[103,105] Adiponectin ↓ ^[104] |
| Atherogenesis | Inflammation ↑ ^[108] Autoimmunity ^[108] Fibrinogen ↑ ^[112] Platelet aggregation ↑ ^[114] |
| Chronic urticaria | Vascular permeability ↑ ^[83] Complement consumption ↑ ^[83] Pathogenetic antibodies ↑ ^[83] |
| Henoch-Schönlein purpura | IgA ↑ ^[82] Cryoglobulins ↑ ^[82] C3 ↓ ^[82] |
| Possible preventive role | |
| Gastroesophageal reflux disease | Sympathetic tone ↑ ^[128] Cholinergic stimulation ^[128] |
| Esophageal adenocarcinoma | Sympathetic tone ↑ ^[128] Cholinergic stimulation ^[128] Acid production ↓ ^[129] |
| Bronchial asthma | Polarization of Th-1 ↓ ^[131] Allergic Th-2 response ↓ ^[131] Tregs ↓ ^[132,133] Interleukin-1 receptor associated kinase M (IRAK-M) ↑ ^[133] |

↑: Increase; ↓: Decrease.

cancer (pooled adjusted OR = 1.38), but further research is needed to confirm this result^[61,62].

Despite good scientific reasoning for the involvement of *H. pylori* in pancreatic diseases, direct pancreatic infection seems unlikely^[63] (Table 2).

Colorectal adenoma/carcinoma

On the basis of the epidemiological results showing

high mortality rates from gastric and colorectal cancer in similar areas, it can be speculated that gastric cancer and colorectal cancer have common risk factors like *H. pylori* infection^[68]. Although the role of *H. pylori* in colorectal carcinogenesis has been widely examined, the association has remained inconclusive^[69]. Several studies demonstrated conflicting positive and negative associations^[68,69]. A meta-analysis showed that *H. pylori* infection was associated with an increased risk of colorectal adenoma (OR = 1.66) and colorectal cancer (OR = 1.39), however there was significant heterogeneity among the studies^[70]. The inconsistent results might be due to sample bias, small sample size, varying frequencies of cagA+ strains in the study population, incomplete colonoscopies, and evaluation of *H. pylori* infection with the IgG serum test^[69].

H. pylori was detected in colorectal carcinoma tissue in a pilot study^[71]. Higher prevalence was proven in adenoma and colorectal cancer compared with control^[72,73]. *H. pylori* was more prevalent in moderate/severe dysplastic adenomas compared with mild dysplasia, and in tubular and tubulovillous adenoma compared with villous type^[72].

The pathogenetic mechanisms of *H. pylori* induced colorectal carcinogenesis are not fully understood^[69] (Table 2). However, not every study confirms the correlation between atrophic gastritis, hypergastrinemia, and colorectal cancer^[68]. Conversely, atrophic gastritis and hypergastrinemia demonstrated a significant elevation in the odds ratio (3.15) for rectal cancer^[68]. Overall, chronic atrophic gastritis did not seem to contribute to an increase in colorectal adenoma risk. Chronic atrophic gastritis and its progression appear to further increase the risk for proximal colorectal adenoma formation^[77]. The inconsistent results correlating hypergastrinemia and colorectal carcinogenesis may be explained by the fact that gastrin precursors (progastrin and glycine-extended gastrin) act as important promoters of colorectal carcinogenesis, but cannot be measured by most commercially available assays^[77,78].

Concomitant *H. pylori* infection with metabolic syndrome further increases the possibility of colorectal adenoma formation; however the pathomechanism for this possible association is still unclear^[69]. Insulin might exert proliferative effects on colonic tumor cells directly or indirectly via the insulin-like growth factor pathway^[79]. Chronic inflammation, increased pro-inflammatory cytokine production, and decreased anti-inflammatory adiponectin production might be associated with carcinogenesis^[69,80]. Triglycerides are energy sources for cancer cell growth and are linked with increased synthesis of bile acids, which have a carcinogenesis promoting effect^[81].

Extra-intestinal diseases

It has been shown that *H. pylori* may play a potential pathogenic role in extra-intestinal diseases via multiple mechanisms^[82]. Atrophic gastritis caused by infection, an increase in gastric vascular permeability and therefore increased exposure to alimentary antigens, release of inflammatory mediators, and systemic immune responses (auto-immunity, pro-inflammatory substances, and im-

mune complex formation induced by molecular mimicry and cross-reactive antibodies) have been suspected in the background^[82,83].

With the exception of unexplained iron deficiency anemia (evidence level 1a) and idiopathic thrombocytopenic purpura (evidence level 1b), *H. pylori* infection has no proven role in other extra-intestinal diseases^[82,84,85].

Hepatobiliary diseases

Helicobacter DNA has been detected in hepatic tissues from patients with various hepatobiliary diseases, hepatitis C virus-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)^[86,87]. The association between *H. pylori* and Child-Pugh classification is inconsistent^[87]. It can be proposed that *H. pylori* infection may play a role in hepatic carcinogenesis as well^[88]. The odds ratio for the association between *H. pylori* infection and the risk of HCC was 13.63^[89] (Table 2).

Respiratory tract disorders

Laryngeal cancer: Colonization of bacteria in the upper aerodigestive tract was confirmed, however the relationship between *H. pylori* infection and laryngeal cancer risk have produced conflicting results. Meta-analysis showed a 2.03-fold increased risk^[92].

H. pylori was detected in larynx cancerous tissue. The presence of the *cagA* gene in larynx cancer tissues significantly decreased survival rate and increased the possibility of disease recurrence^[93] (Table 2).

Lung cancer: The results of previous studies of *H. pylori* seropositivity and lung cancer are inconclusive^[94], with an odds ratio between 1.24 and 17.78 on the basis of the epidemiological studies^[95]. The NHANES study observed an inverse association between *H. pylori* and lung cancer in older participants, with a significant inverse association for *cagA*+ strains; this was without histological examination^[96]. A case-control study found no evidence of an association between *H. pylori* and lung cancer in Finish male smokers. Neither overall *H. pylori* seropositivity nor *CagA*-specific *H. pylori* seropositivity were associated with lung cancer^[94]. Causal relationships must be confirmed with exact determination of smoking status^[95] (Table 2).

Insulin resistance and metabolic syndrome

Epidemiological studies showed significant associations with metabolic syndrome (OR = 1.39)^[99,100]. Furthermore, multiple linear regression analysis showed that *H. pylori* seropositivity was significantly associated with higher systolic blood pressure, lower high-density lipoprotein (HDL)-cholesterol level, and higher low-density lipoprotein (LDL)-cholesterol level^[99]. It has been suggested that *H. pylori* eradication could lead to an improvement of atherogenic blood lipid profile, insulin resistance, and low-grade inflammation, which were deduced from a decreased C-reactive protein level^[101]. Other studies did not find an association between *H. pylori* infection and insulin resistance^[102,103].

The relationship between *H. pylori* infection and metabolic syndrome is both poorly understood^[104] (Table 2) and controversial^[106-108].

Cardiovascular diseases

Studies investigating the pathogenetic role of *H. pylori* in cardiovascular diseases have produced conflicting results^[108-111]. A meta-analysis of 18 epidemiological studies involving 10,000 patients did not find any positive association between *H. pylori* and cardiovascular risk factors and coronary heart diseases^[112]. A higher prevalence of more virulent *cagA*+ *H. pylori* was reported in patients with ischemic heart disease, unstable angina, acute myocardial infarction, and restenosis after percutaneous transluminal coronary angioplasty and essential hypertension^[108,111,113].

Evidence on the relationship between *H. pylori* infection and ischemic heart disease is weak, with some inconclusive, albeit plausible, mechanisms (Table 2). There are also no adequate interventional studies done to demonstrate that *H. pylori* eradication is associated with a lower incidence of ischemic heart disease^[108].

Dermatological disorders

Chronic urticaria: A correlation between *H. pylori* infection and chronic urticaria has been suggested (Table 2). *H. pylori* eradication in patients with chronic urticaria leads to symptomatic improvement in some patients, while others showed no improvement^[83].

Hematological disorders

Immune thrombocytopenic purpura: The prevalence of *H. pylori* infection in patients with immune thrombocytopenic purpura (ITP) is significantly higher than that in age- and gender-matched controls^[108,115,116]. The most plausible mechanism is cross-mimicry involving *H. pylori*, platelet antigens, and infected host factors (antibody production cross-reacts with platelet glycoprotein antigens)^[108,117].

Eradication of *H. pylori* results in an increasing platelet count in nearly half of infected ITP patients, although geographical differences in the efficacy of eradication were also presumed^[83,115,116]. The European *Helicobacter* Study Group consensus in 2012 and the Second Asia-Pacific Consensus Guidelines have recommended *H. pylori* infection eradication in patients with chronic idiopathic thrombocytopenic purpura^[84,85]. However, larger randomized controlled trials with long-term follow-up are still required before a firm conclusion can be drawn^[108].

Henoch-Schönlein purpura: A study in China found increasing evidence suggesting that Henoch-Schönlein purpura (HSP), especially abdominal HSP, might be associated with *H. pylori* infection (OR = 4.62); this underlines the necessity of screening *H. pylori* infection in children with HSP with gastrointestinal manifestations^[82].

It was found that eradication of *H. pylori* infection resulted in prompt resolution of the HSP, or at least prevented its recurrence^[118].

More investigations are needed to confirm the pathogenetic role of *H. pylori* in HSP (Table 2). HSP children with serious gastrointestinal symptoms must be screened and treated for *H. pylori* infection^[82].

Iron deficiency anemia: Several epidemiological studies have shown lower ferritin levels among patients with *H. pylori* infection, although there were studies that produced a negative association^[108]. Meta-analyses showed an association between *H. pylori* infection and iron deficiency anemia (IDA)^[119,120]. *H. pylori* eradication improves iron absorption^[121].

Possible pathomechanisms are: increased iron loss due to active hemorrhage secondary to gastritis, peptic ulcer, gastric cancer, reduced iron absorption caused by achlorhydria induced by chronic pancreatitis, reduced secretion of ascorbic acid to the gastric mucosa, and iron utilization for protein synthesis by the bacterium for colonization in the host environment^[122]. Elevated serum prohepcidin might also indicate the role of inflammation in its aetiology^[123].

Testing and eradication of *H. pylori* for unexplained IDA are supported by the current evidence and approved by the Maastricht IV Consensus and the Second Asia-Pacific Consensus Guidelines^[84,85]. However, larger sample randomized controlled trials are necessary to clarify the reason why only a small proportion of *H. pylori*-positive patients develop IDA^[108].

Possible beneficial clinical consequences of *H. pylori* infection

Gastroesophageal reflux disease/esophageal adenocarcinoma: A meta-analysis showed that *H. pylori* infection displays a negative association with the development of endoscopic gastroesophageal reflux disease (GORD). Eradication of the infection may be a risk factor for development of *de novo* GORD^[124].

H. pylori infection protects against gastroesophageal reflux^[2]. *H. pylori*-induced corpus gastritis and profound suppression of gastric acid secretion have also been shown to prevent patients from developing GORD^[125]. *cagA*+ *H. pylori* strains have a more protective effect against GORD^[126], and it was found that *H. pylori* infection was inversely associated with Barrett's esophagus^[127].

The Maastricht consensus IV confirmed a negative association between the prevalence of *H. pylori* and the severity of GORD. The consensus stated that *H. pylori* status exerts no effect on symptom severity, recurrence, or treatment efficacy in GORD. *H. pylori* eradication does not exacerbate pre-existing GORD or affect treatment efficacy^[85].

Esophageal adenocarcinoma risk due to *H. pylori* infection was 0.58-fold, and squamous cell carcinoma risk was 0.80-fold compared with that of controls. Compared with *cagA*- *H. pylori*, *cagA*+ *H. pylori* markedly decreased esophageal cancer risk^[128].

The underlying mechanism in the background of the protective effect of *H. pylori* against GORD is not fully

understood (Table 2).

H. pylori infection acts as neither a preventive factor nor a risk factor for squamous cell carcinoma. This discrepancy might be due to the relatively small number and heterogeneity of the included studies^[129].

There is further need to assess the benefits of *H. pylori* in connection with GORD and its complications.

Bronchial asthma: An infection in the early phase of life is essential for the normal maturation of the immune system, achieving a balance between T-helper type 1 (protective immunity) and T-helper type 2 (allergic diseases) cytokine responses, which can reduce the risk of atopy later^[129].

H. pylori infection might play a role in the development of chronic bronchitis, bronchiectasis, tuberculosis, and lung cancer^[130]. Moreover, *H. pylori* might have an influence on the developing immune system, which might reduce the risk of asthma in later life^[131].

The associations between *H. pylori* and asthma were contradictory. Inverse associations were reported, but other studies demonstrated different results^[131]. A meta-analysis found weak evidence (OR = 0.81, 0.84) for an inverse association between *H. pylori* infection and asthma in children and adults, respectively^[131,132]. Another meta-analysis failed to prove a significant association between *H. pylori* infection and asthma risk^[130].

The mechanism of the preventive effect of *H. pylori* on asthma has been unambiguous (Table 2).

It seemed that *H. pylori* infection (especially *cagA*+ strains) may prevent children from developing asthma, but must be studied in the future^[131] due to the inconsistent result^[134].

CONCLUSION

The clinical outcome of *H. pylori* infection is determined by host genetic predisposition, bacterial strain factors, and environmental factors^[1]. Bacterial virulence factors (*VacA*, *CagA*) can modulate the immune response involved in the initiation of the carcinogenesis in the stomach. Host genetic factors including IL-1 β , IL-10, and TNF- α influence the inflammatory response and the exasperation of mucosal damage. Environmental factors, including salt intake and smoking tobacco, are well-known harmful aetiological factors. The ingestion of fruit and vegetables has some protective effect^[135].

The mechanisms of *H. pylori*-associated gastric carcinogenesis are still poorly defined; further recognition may provide possibilities to develop effective strategies for gastric cancer prevention and treatment^[1].

Indications for *H. pylori* therapy have been extended and now include idiopathic thrombocytopenic purpura, iron deficiency anemia, and vitamin B12 deficiency. New data are presented on the role of *H. pylori* in neurodegenerative disorders and in metabolic syndrome. *H. pylori* is associated with a small increase in the risk for colorectal adenoma and colon cancer^[80] (Table 3).

Table 3 Other possible pathogenetic roles of *Helicobacter pylori*^[83,94,110,117,136,137]

| |
|--|
| Renal diseases |
| Renal resistive index, proteinuria |
| Hepatobiliary diseases |
| Alcoholic damages of the liver, cholestatic autoimmune liver diseases (primary biliary diseases, primary sclerosing cholangitis), cholelithiasis, cholangiocellular carcinoma |
| Pancreatic disorders |
| Autoimmune pancreatitis |
| Intestinal diseases |
| Enteric diseases, inflammatory bowel diseases |
| Neurological diseases |
| Alzheimer-disease, idiopathic parkinsonism |
| Dermatological diseases |
| Alopecia areata, atopic dermatitis, lichen planus, chronic prurigo multiformis, nodular prurigo, pruritus, psoriasis, recurrent aphthous stomatitis, rosacea, Sweet's syndrome |
| Ophthalmological diseases |
| Glaucoma, central serous chorioretinopathy, uveitis, blepharitis |
| Autoimmune disorders |
| Autoimmune thyroiditis, Behçet's disease, Sjögren's syndrome, progressive systemic sclerosis |
| Others |
| Impaired bioavailability of medication such as thyroxine and l-dopa, pre-eclampsia, chronic prostatitis, growth retardation |

H. pylori screening and treatment is a recommended gastric cancer risk reduction strategy in high-risk populations. In low-risk populations for gastric cancer, *H. pylori* screening is not recommended^[84]. The removal of *H. pylori* from a large section of the population may be economically difficult, and the long-term consequences are still unpredictable. Identification of high-risk individuals is thus very important^[40].

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Efficacy of tailored *Helicobacter pylori* eradication therapy based on antibiotic susceptibility and *CYP2C19* genotype

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Abstract

The cure rates of *Helicobacter pylori* (*H. pylori*) eradication therapy using a proton pump inhibitor (PPI) and antimicrobial agents such as amoxicillin, clarithromycin, and metronidazole are mainly influenced by bacterial susceptibility to antimicrobial agents and the magnitude of the inhibition of acid secretion. Annual cure rates have gradually decreased because of the increased prevalence of *H. pylori* strains resistant to antimicrobial agents, especially to clarithromycin. Alternative regimens have therefore been developed incorporating different antimicrobial agents. Further, standard PPI therapy (twice-daily dosing) often fails to induce a long-term increase in intragastric pH > 4.0. Increasing the eradication rate requires more frequent and higher doses of PPIs. Therapeutic efficacy related to acid secretion is influenced by genetic factors such as variants of the genes encoding drug-metabolizing enzymes (*e.g.*, cytochrome P450 2C19, *CYP2C19*), drug transporters (*e.g.*, multidrug resistance protein-1; ABCB1),

and inflammatory cytokines (*e.g.*, interleukin-1 β). For example, quadruple daily administration of PPI therapy potentially inhibits acid secretion within 24 h, irrespective of *CYP2C19* genotype. Therefore, tailored *H. pylori* eradication regimens that address acid secretion and employ optimal antimicrobial agents based on results of antimicrobial agent-susceptibility testing may prove effective in attaining higher eradication rates.

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Key words: *Helicobacter pylori*; Tailored eradication therapy; Proton pump inhibitor; Cytochrome P450 2C19; Clarithromycin

Core tip: The eradication for *Helicobacter pylori* infection is mainly influenced by antibiotic susceptibility and insufficient acid inhibition [*e.g.*, cytochrome P450 2C19 (*CYP2C19*) genotype, proton pump inhibitor (PPI) dose, and PPI treatment schedule]. When a PPI is administered to *CYP2C19* rapid metabolizers and intermediate metabolizers, plasma levels of PPIs cannot be maintained between once-daily doses. The intragastric pH attained with four-times-daily-dosing of PPI is significantly higher than those observed when PPI is administered as once-daily-dosing of four-fold doses or twice-daily-dosing of two-fold doses. We describe a tailored treatment that was designed according to pharmacogenomics and antimicrobial susceptibility to achieve an eradication rate exceeding 95%, irrespective of different *CYP2C19* genotypes.

Sugimoto M, Furuta T. Efficacy of tailored *Helicobacter pylori* eradication therapy based on antibiotic susceptibility and *CYP2C19* genotype. *World J Gastroenterol* 2014; 20(21): 6400-6411 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6400.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6400>

INTRODUCTION

The Maastricht IV/Florence Consensus Report issued by the European *Helicobacter* Study Group in 2012^[1] recommends *Helicobacter pylori* (*H. pylori*) eradication therapy as first-line treatment for patients with upper gastrointestinal disorders such as peptic ulcer disease, gastric mucosa associated-lymphoid tissue lymphoma {Evidence level in support of the recommendations formulated in the Maastricht IV/Florence Consensus Report: 1a [Systematic review of randomized controlled trial (RCT)], Grade of recommendation: A}, atrophic gastritis [2a (Systematic review of cohort studies) and B], intestinal metaplasia (2a and B), and functional dyspepsia (1a and A) as well as for patients with extra-gastrointestinal disorders such as idiopathic thrombocytopenic purpura [1b (Individual RCT with narrow CI) and A], vitamin B12 deficiency [3b (Individual case-control study) and B], and iron-deficiency anemia (1a and A)^[2-11].

“Test and treat” is a strategy involving a noninvasive test (e.g., serum anti-*H. pylori* IgG or urea breath test) given to patients with dyspepsia to diagnose *H. pylori* infection of the gastric mucosa, with treatment initiated upon detection^[1]. In Japan, first-line *H. pylori* eradication therapy is limited to a regimen that employs a proton pump inhibitor (PPI) administered twice-daily (*bid*) using a standard dose (e.g., omeprazole, rabeprazole, lansoprazole, and esomeprazole), amoxicillin (AMPC) 750 mg *bid*, and clarithromycin (CAM) 200 mg or 400 mg *bid* for 1 wk. Unfortunately, the prevalence of CAM-resistant *H. pylori* strains in Japan is increasing (> 30%)^[12,13]. Therefore, alternative regimens are designed to consist of different antimicrobial agents with susceptibility to *H. pylori* strain, increased dosing dosages of antimicrobial agents and PPIs, increased dosing times of drugs and prolonged treatment periods^[14-17].

In this review article, we consider first the factors that influence the cure rate of *H. pylori* eradication therapy and the importance of inhibiting acid secretion. We then propose optimal treatment strategies based on the most effective antimicrobial agents and the patient's genotype.

FACTORS CONTRIBUTING TO THE SUCCESS OF *H. PYLORI* ERADICATION THERAPY

The cure rates of *H. pylori* infection are influenced by several factors such as antibiotic susceptibility^[12,13,18], insufficient inhibition of acid secretion [e.g., cytochrome P450 2C19 (*CYP2C19*) genotype, PPI dose, and PPI treatment schedule]^[19], bacterial genotypes that reduce virulence (e.g., *cagA*-negative strains and the *vacA* s2 genotype), the environment (e.g., smoking), and compliance (Table 1).

Antibiotics targeted to *H. pylori* strains with known drug sensitivities will likely increase the eradication rate^[20,21]. When patients are treated with antibiotics that are targeted specifically to the infecting *H. pylori* strain, increased eradication rates are achieved. Therefore,

Table 1 Major factors preventing eradication of *Helicobacter pylori* infection

| Factor | | |
|------------------------------|------------------------------------|---|
| Antibiotics | Resistance to antibiotics | Clarithromycin Metronidazole Levofloxacin Amoxicillin Rapid metabolizer |
| Inhibition of acid secretion | <i>CYP2C19</i> | |
| | <i>CYP2C19</i> *17 | *17 carrier |
| | <i>ABCB1</i> 3435 | C/C genotype (Caucasian) |
| | <i>IL-1B</i> -511 | C/C genotype |
| | <i>IL-1B</i> -31 | T/T genotype |
| | Acid inhibitory drug dosing time | Low frequency (<i>oid</i>) |
| <i>H. pylori</i> phenotype | Acid inhibitory drug dosing dose | Insufficient dose |
| | <i>H. pylori</i> virulence factors | <i>cagA</i> -negative |
| | | <i>vacA</i> s2 genotype |
| Environment | Volume Smoking Compliance | Much Many Poor |

CYP2C19: Cytochrome P450 2C19; *IL*: Interleukin; *ABCB1*: Multidrug resistance protein-1; *H. pylori*: *Helicobacter pylori*.

determining the antibiotic susceptibility of *H. pylori* using either or both culture or genetic testing is of great import, particularly in a population with a high rate of infection with drug-resistant strains. These tests should be conducted before treatment, particularly with second- and third-line treatment^[1]. The Maastricht IV Consensus Report^[1] recommends that, after a second failure, if culture and susceptibility testing is not possible, molecular genetic tests should be conducted to detect *H. pylori*. Recently, a novel fully-automated rapid genetic analyzer was developed which was capable of determining CAM resistance (e.g., 23S rRNA gene point mutations of A2143G and A2144G) within 60-120 min^[20], whereas culture tests required 7-10 d. Note that the treatment strategy should take into account the drug resistance of *H. pylori* in different patient populations in different localities.

The optimal intragastric pH condition with potent acid inhibition in the stomach makes selected antibacterial agents more stable and bioavailable^[21,22]. Controlling intragastric pH with PPIs depends on dosing schedules, dose, and combination of acid inhibitors^[23-27], and polymorphisms in the genes encoding drug-metabolizing enzymes and drug transporter genes such as *CYP2C19* and multidrug resistance protein-1 (*ABCB1*) influence pH during treatment^[23-29]. In the gastrointestinal tract, *ABCB1* is expressed on the apical surfaces of superficial columnar epithelial cells of distal small bowel and the pharmacokinetics of PPIs are influenced by the activity of *ABCB1*. Polymorphism of *ABCB1* is one of the determinants of successful eradication of *H. pylori* by the triple therapy with lansoprazole, amoxicillin and clarithromycin, and eradication rates for *H. pylori* infection are 82%, 81% and 67% in patients with the *ABCB1* 3435

C/C, C/T and T/T genotype, respectively ($P = 0.001$)^[30]. In particular, patients' pharmacogenetic characteristics [*e.g.*, CYP2C19 rapid metabolizer (RM), interleukin (IL)-1 β -511 C/C, and *ABCB1* 3435 T/T genotype] require a modified treatment regimen to effectively inhibit acid secretion for 24 h^[23-29].

The increased levels of IL-1 β induced by *H. pylori* infection potently inhibit acid production, and IL-1 β is one hundred times more potent an inhibitor than PPIs on a molar basis^[31]. Polymorphisms in the gene encoding IL-1 β are associated with individual differences in IL-1 β levels^[32], and presence of the *IL-1B*-511 polymorphism has been shown to be related to eradication rate^[33-35]. Indeed, the eradication rate achieved for patients with the low IL-1 β -producer genotype *IL-1B*-511 C/C (77.4%) is lower than that of the C/T and T/T genotypes (87.2%; odds ratio for failure: 1.98, 95%CI: 1.38-2.84, $P = 0.0002$)^[35]. Further, *H. pylori* virulence factors (*e.g.*, *cagA* and *vacA*), which play critical roles in gastric mucosal injury and inflammation, determine cure rates^[19]. In a meta-analysis, individuals infected with strains with the *cagA*-negative 69.9% (95%CI: 65.7%-73.9%) *vs* 83.1% (80.7%-85.3%) for *cagA*-positive genotypes and *vacA* s2 genotype [72.1% (64.8%-78.7%) *vs* *vacA* s1 genotype 79.2% (75.1%-83.0%)] are at increased risk of failure of eradication therapy^[19]. The presence of *dupA*, which is associated with the development of duodenal ulcers, influences the cure rate of eradication therapy^[36,37].

CAM RESISTANCE AND ERADICATION TREATMENT

CAM is a key component of *H. pylori* eradication therapy, exerting its antimicrobial effects by binding to the sub-unit 23S of the bacterial ribosome, which inhibits protein synthesis. In general, although the cut-off concentration used to define resistance to CAM is higher than 1.0 mg/mL^[38], bacterial susceptibility to CAM in most strains is conferred by a single nucleotide polymorphism at either position 2142 or 2143 of the *H. pylori* 23S rRNA gene (A2142G or A2143G). The most frequent mutation is A2143G (69.8%), followed by A2142G (11.7%) and A2142C (2.6%)^[39,40].

From 1990 to 2000, the eradication rates achieved in Japan using CAM-based triple therapy ranged from approximately 85%-91%^[12]; in contrast, eradication rates for patients infected with CAM-resistant strains were markedly low (10%-30%)^[41,42]. The frequencies of resistance of *H. pylori* strains to CAM in Japan and Europe exceed 35% and 20%, respectively, and account for the relatively low eradication rates with the CAM-based regimen^[12,13,42,43].

The Maastricht IV consensus report recommends first-line eradication treatment using a CAM-based regimen [PPI-CAM-AMPC or -metronidazole (MNZ)] and an alternative eradication using a bismuth-containing quadruple treatment in areas where prevalence of CAM-resistant strains is low, and a bismuth-containing qua-

druple treatment in areas of high resistance^[1]. Extending the duration of CAM-based triple treatment from 7 to 10-14 d improves the eradication success rate by approximately 5%^[44,45]. When CAM-based treatment fails, either bismuth-containing quadruple therapy or levofloxacin-based therapy is recommended. In areas of high CAM resistance, levofloxacin-based treatment is recommended after quadruple therapy fails.

IMPORTANCE OF INHIBITING GASTRIC ACID SECRETION DURING ERADICATION THERAPY

Rapid and potent neutralization of intragastric pH after treatment with drugs that inhibit acid secretion is required to cure acid-related diseases. Thus, intragastric pH during treatment is associated with the cure rates of peptic ulcers^[46], gastroesophageal reflux disease^[47], and aspirin-induced gastroduodenal mucosal injury^[48]. Further, eradication treatment fails if acid secretion is not sufficiently inhibited^[49-51].

Whereas *H. pylori* survives with a periplasmic pH of 4.0-8.0, it only grows between pH 6.0-8.0^[52]. When bacterial urease activity elevates the pH to 4.0-6.0, *H. pylori* survives but does not divide^[52]. The consistent and potent action of drugs that inhibit acid secretion increases the stability and bioavailability of acid-sensitive antimicrobial agents such as CAM and AMPC by preventing their degradation in the stomach. Further, these inhibitors increase the concentration of antimicrobial agents in the gastric mucosa^[21,52,53]. Raising pH from 3.5 to 5.5 increases the *in vitro* effectiveness of AMPC more than 10-fold^[21]. Ampicillin is bactericidal at pH 4.5-7.4 but not at pH 3.0, as this pH inhibits the expression of genes encoding cell envelope components and proteins required for cell division^[54]. The activity of CAM is higher at pH 7.4 than at pH 5.0, and activity is intermediate at pH 6.8^[55]. Inhibiting acid secretion also allows *H. pylori* to grow and become more sensitive to antimicrobial agents^[52].

In a previous study, the 24-h intragastric pH level in patients treated successfully with eradication therapy (omeprazole 20 mg *bid* and AMPC 1 g *bid*) was found to be higher than in patients that failed 7-d treatment^[51]. When 24-h pH exceeds 5.5, *H. pylori* can be eradicated using dual PPI/AMPC therapy without a second antimicrobial agent such as CAM, MNZ, or both^[51]. Eradication using PPI/AMPC therapy is preferred for patients with pH > 4.0 for 75% of total treatment duration and above 5.5 in 4-h pH^[49]. Univariate analysis shows that pH values depend on the dose of omeprazole ($P = 0.003$), CYP2C19 genotype ($P = 0.001$), and *H. pylori* density ($P = 0.044$)^[49]. We demonstrate further that the median 24-h pH was 6.4 (range, 5.0-7.6) for successful eradication therapy compared with that for failed therapy [pH 5.2 (2.2-6.2), $P = 0.0131$] and that median percentage time at pH < 4.0 for successful cures [0.5% (0.0%-31.6%)] is significantly shorter compared with failures [26.7%

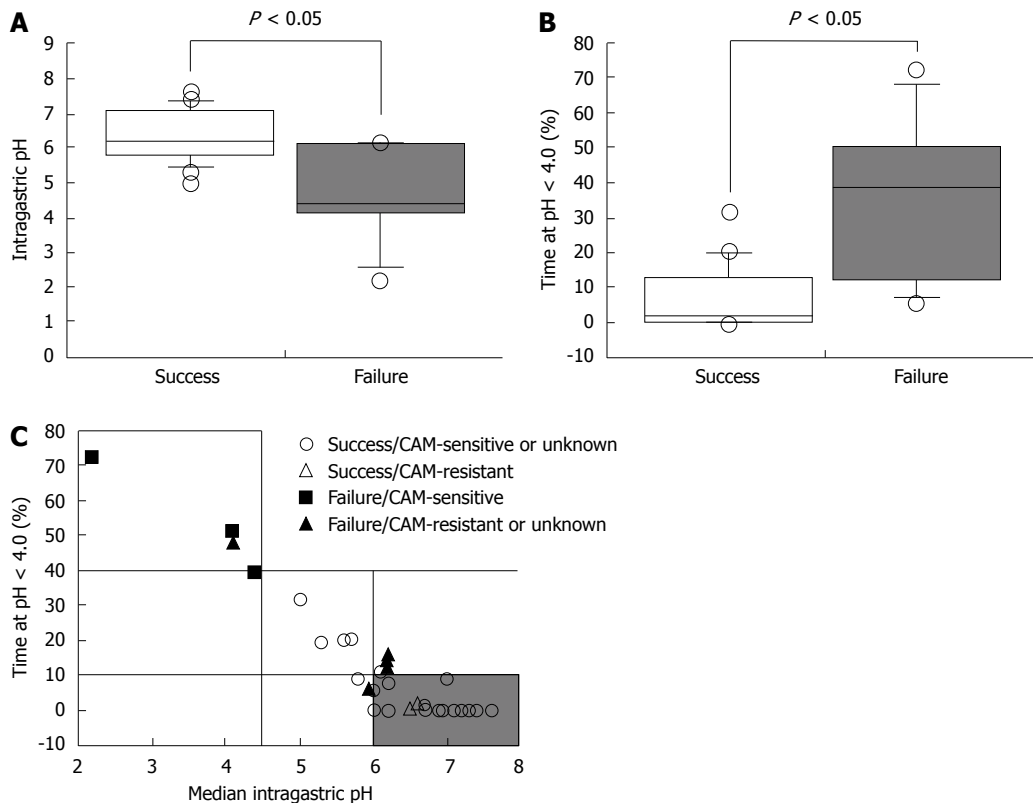


Figure 1 Success of *Helicobacter pylori* eradication treatment as a function of pH. A, B: Median 24-h pH values (A) and the percentage of the times when pH < 4.0 during eradication therapy according to successful and failed treatment (B); C: Variation of pH and the percentage of time at pH < 4.0^[51]. The median pH of successfully treated patients was significantly higher than compared with patients that failed treatment (A). The median percentage of the time when pH < 4.0 in successfully treated patients was significantly shorter compared with unsuccessfully treated patients (B). The majority of patients were cured using triple therapy when the percentage of time at pH < 4.0 during the 24-h post-dose period was < 10% and the 24-h pH was > 6.0 (shaded area) (C). CAM: Clarithromycin.

(6.0%-72.2%), $P = 0.0017$; Figure 1A and B^[50]. Therefore, the degree and duration of acid suppression are related to cure rate, and we may conclude that pH > 4 should be maintained for 24 h and 24-h pH higher than 6.0 (Figure 1C). Unfortunately, the PPI standard-dose *bid* treatment does not maintain pH values > 4.0 long enough to satisfy the above criteria in all of patients^[23].

CYP2C19 POLYMORPHISMS AND *H. PYLORI* ERADICATION THERAPY

Pharmacokinetics and pharmacodynamics of PPIs according to CYP2C19 genotype

PPIs delivered through the circulatory system are absorbed in the small intestine and reach gastric parietal cells where they bind irreversibly to and alter the function of $H^+/K^+-ATPase$, which potently inhibits acid secretion^[56,57]. Therefore, PPIs are currently used as the first-line treatment of acid-related diseases^[33,50,58,59].

PPIs undergo extensive hepatic metabolism by the CYP system, which includes CYP2C19 and CYP3A4 (Figure 2)^[60]. CYP2C19 polymorphisms therefore influence both pharmacokinetics [*i.e.*, peak plasma concentration (C_{max}) and area under the curve (AUC) of the plasma concentration] and pharmacodynamics [*i.e.*, intragastric pH] of PPIs (Figure 3A and B). At least 20 CYP2C19 variants (Figure 4) have been identified, with the major-

ity classified into three genotypes: RMs, intermediate metabolizers (IMs), and poor metabolizers (PMs)^[60-63]. The inhibition of acid secretion achieved using PPIs (*e.g.*, omeprazole and lansoprazole) to treat PMs is enhanced compared with IMs or RMs because of the different pharmacokinetics among the three CYP2C19 genotypes^[26,27,29,64]. In contrast, rabeprazole reduces acid secretion mainly *via* a non-enzymatic reaction, with minor involvement of CYP2C19 (Figure 2)^[60,65], and its acid inhibitory effect is less influenced by CYP2C19 genotypes than that of omeprazole or lansoprazole. Esomeprazole, which is the S-isomer of omeprazole, more effectively inhibits acid secretion than omeprazole^[66,67], and this relatively high activity can be attributed to its less extensive metabolism during first-pass hepatic metabolism compared with omeprazole. The increased systemic bioavailability of esomeprazole offers the prospect of improved clinical efficacy and more effective management of acid-related diseases^[68]. A 2006 study shows that the CYP2C19*17 variant is an ultrarapid metabolizer genotype^[69]. Interethnic differences have been reported in the frequencies of genotypes^[63,70,71], with frequency of the *17 allele approximately 20% among Caucasian, African-American, Swedish, and Ethiopian populations but only 4% among Asians.

Several studies have compared the level of acid inhibition attained among CYP2C19 genotypes on PPI *bid*

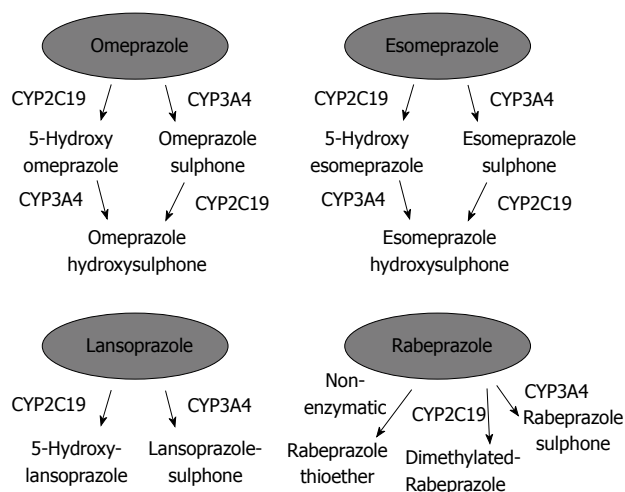


Figure 2 Metabolism of omeprazole, lansoprazole, rabeprazole, and esomeprazole by cytochrome P450 isoenzymes. Reproduced from Chang *et al.*^[61].

administration^[23,33,68,72,73]. Effects of lansoprazole (30 mg, *bid*) have been found to vary markedly with *CYP2C19* genotype^[33,68], whereas little or no differences in efficacy were observed in patients treated with rabeprazole (10 mg) or esomeprazole (20 mg) *bid*, regardless of *CYP2C19* genotype^[68,72,73]. Further, *CYP2C19* genotype-dependent differences in intragastric pH attained using esomeprazole and rabeprazole are smaller than those noted with lansoprazole and omeprazole^[68]. As such, twice-daily dosing with a second-generation PPI may attenuate the influence of *CYP2C19* genotype, but not completely remove it.

We reported that the median 24-h pH [5.4 (3.5–6.8)] of *H. pylori*-negative *CYP2C19* RMs administered esomeprazole 20 mg *bid* was significantly higher than that achieved with omeprazole [5.0 (2.4–5.9), $P = 0.018$], rabeprazole [4.8 (2.5–6.4), $P = 0.002$], or (check) lansoprazole [4.7 (3.7–5.5), $P = 0.017$]^[68]. However, these findings suggest that although esomeprazole inhibits acid secretion in *CYP2C19* RMs, twice-daily dosing of omeprazole, lansoprazole, and rabeprazole as well as with esomeprazole does not sustain high pH for 24 h for all patients.

Influence of *CYP2C19* polymorphisms on PPI-based eradication of *H. pylori*

The rates for eradicating *H. pylori* infection using triple therapy (omeprazole 20 mg *bid* or lansoprazole 30 mg *bid*, AMPC 250 mg *tid*, and CAM 200 mg *tid* for 1 wk) vary with *CYP2C19* genotype as follows: 72.7%, RMs; 92.1% IMs; and 97.8%, PMs^[18]. The frequency of *CYP2C19* RMs is relatively high among patients that experience eradication failure (58.6%, *vs* 2.9% for PMs). Schwab *et al.*^[74] also noted significant differences in eradication rates among RMs (80.2%), IMs (97.8%), and PMs (100%) ($P < 0.01$), which were associated with corresponding changes in median serum lansoprazole levels (753 ng/mL, PMs; 59 ng/mL, IMs; and 21 ng/mL, RMs; $P < 0.001$). A meta-analysis performed by those authors^[74] further revealed that failure to eradicate *H. pylori* infection using PPI-based regimens is strongly influenced by the *CYP2C19*

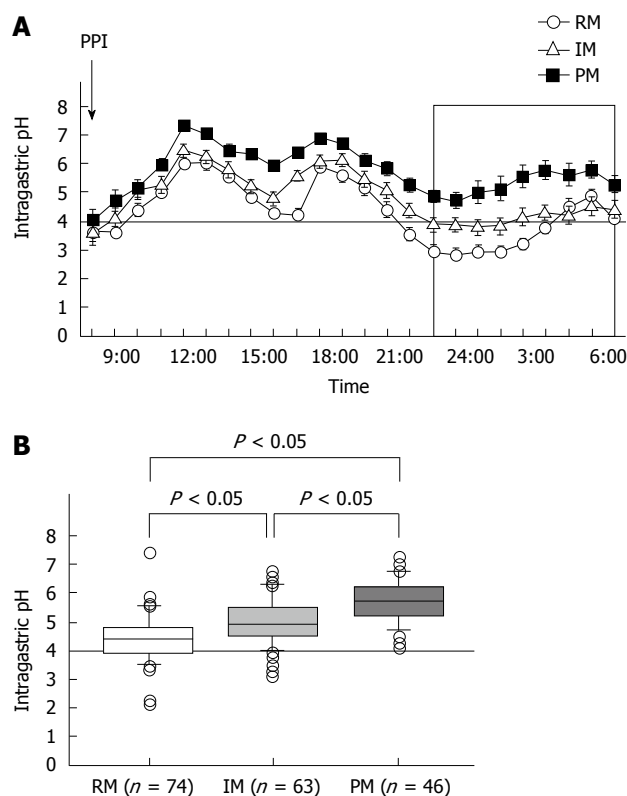


Figure 3 Median 24-h intragastric pH profiles (A) and median 24-h pH values after administering a standard dose of a proton pump inhibitor to patients with the three *CYP2C19* genotypes (B). Proton pump inhibitor (PPI) treatment of poor metabolizers (PMs) inhibited gastric acid secretion more effectively than that of rapid metabolizers (RMs) and intermediate metabolizers (IMs).

genotype.

A more recent meta-analysis performed by Tang *et al.*^[75] on the effects of *CYP2C19* loss-of-function variants revealed significant differences in rates between RMs and IMs (OR = 0.72; 95%CI: 0.59–0.88), between RMs and PMs (0.51; 0.38–0.68), and between IMs and PMs (0.69; 0.52–0.92) regardless of the PPI administered. Although other studies have reported significant differences in eradication rates using other PPI-based eradication therapies (*e.g.*, rabeprazole, esomeprazole, or pantoprazole) among the different *CYP2C19* genotypes^[76,77], Tang *et al.*^[75] found no significant differences in efficacy between rabeprazole or esomeprazole for loss-of-function *CYP2C19* variants. Therefore, *CYP2C19* loss-of-function variants such as *CYP2C19* PMs are associated with increased *H. pylori* eradication rates in patients taking PPI-based triple therapies with either omeprazole or lansoprazole^[75].

EFFICACY OF DIVIDED DOSING WITH PPIS

When PPIs are administered to *CYP2C19* RMs and IMs, plasma levels of PPIs cannot be maintained between once-daily doses^[23,26,29,78]. RMs rapidly eliminate PPIs from the systemic circulation, and plasma levels of PPIs before dosing and 3 h later are often below detectable

CYP2C19: chromosome 10
(10q24.1-10q24.3)

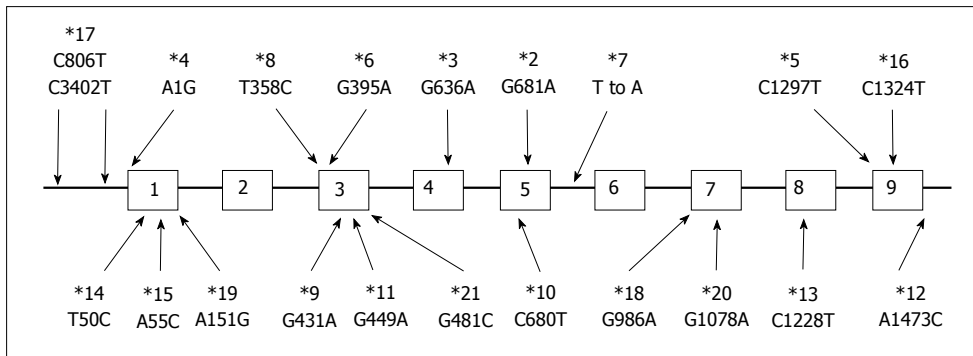


Figure 4 Genetic polymorphisms of *CYP2C19*. More than 20 variants have been discovered.

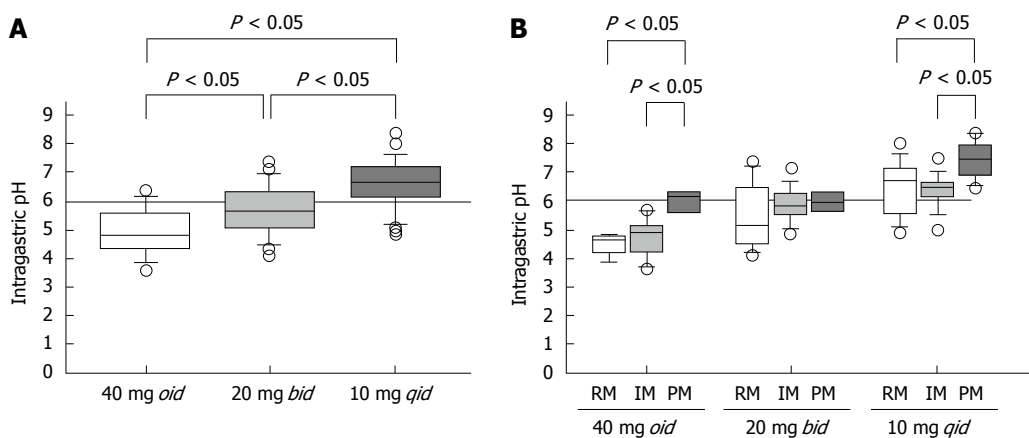


Figure 5 Median 24-h pH values as a function of dosing frequency using 40 mg of rabeprazole (A), and the pH attained using three different dosing regimens as a function of *CYP2C19* genotype (B). Reproduced from Sugimoto *et al.*^[25]. PMs: Poor metabolizers; RMs: Rapid metabolizers; IMs: Intermediate metabolizers.

levels (plasma half-life, 2-3 h). Following rapid elimination of PPIs, activated or newly synthesized H^+ , K^+ -ATPase expressed in gastric parietal cells secretes gastric acid^[23,26,29,78]. Although the C_{max} for multiple-dosing PPI regimens (e.g., *bid* or *qid*) does not increase compared with *oid* administration, plasma levels of PPIs reached using multiple doses can be sustained throughout the 24-h period and continue to inactivate H^+ , K^+ -ATPase consistently for 24 h, achieving sufficient inhibition of acid secretion.

We reported that when rabeprazole (40 mg *oid* or 20 mg *bid*) is administered to *CYP2C19* RMs, the levels of plasma rabeprazole are often below the limit of detection. However, when rabeprazole (20 mg *bid* or 10 mg *qid*) is administered to IMs and RMs, respectively, plasma levels are sustained above 10 ng/mL throughout the 24-h period, and sufficient suppression of acid secretion is achieved^[23]. When rabeprazole is administered once, twice, or four times to achieve a daily dose of 40 mg, the median pH values are 4.8 (3.6-6.4), 5.7 (4.1-7.4), and 6.6 (4.9-8.4), respectively (Figure 5A)^[25]. Administering 10 mg rabeprazole, 30 mg lansoprazole, and 10 mg esomeprazole *qid* to RMs achieves sufficient inhibition of acid secretion^[23,64,73].

However, when patients with the same *CYP2C19*

genotype were treated with different doses of rabeprazole on different dosing schedules, the median pH attained with 10 mg *qid* was significantly higher than those observed when the drug was administered as 40 mg *oid* or 20 mg *bid* (Figure 5A). Further, multiple doses of a PPI decreased the influence of *CYP2C19* genotype on pH (Figure 5B)^[25]. Fischbach *et al.*^[41] reported that inhibition of acid secretion attained using esomeprazole 20 mg *bid* or 10 mg *qid* was similar among *CYP2C19* genotypes but differed markedly from that achieved with 40 mg *oid*. We may therefore reasonably assume that, in order to maintain plasma PPI levels above a certain threshold level throughout the 24 h period, a multiple-dosing regimen would be more effective in inhibiting acid secretion by increasing the dose than by increasing either or both the C_{max} or the AUC value.

Previously, sufficient eradication rates were achieved for RM patients treated with either lansoprazole 30 mg *qid* or rabeprazole 10 mg *qid* plus AMPC 500 mg *qid* for 2 wk. These findings suggest that potent inhibition of acid secretion using more frequent dosing intervals than at present *bid* may help to improve the eradication rate^[18,43,79-81]. In addition, interestingly, rabeprazole 20 mg *bid* plus AMPC 1000 mg *bid* attains a 59.6% cure rate

irrespective of administration of high daily doses of a PPI and AMPC^[82]. This observation may be explained by findings that inhibition of acid secretion is insufficient when RMs are treated with rabeprazole 20 mg *bid*.

EFFICACY OF DIVIDED DOSING WITH AMPC

Similarly, AMPC should have been administered at 500 mg *qid*, not 1000 mg *bid*. We therefore believe that administering AMPC using a regimen of 750-1000 mg *bid* is theoretically inappropriate according to pharmacological considerations, as antibiotics with a beta-lactam ring, such as AMPC, exert little post-antibiotic effects on gram-negative rods^[83]. Their antibacterial effect depends largely on the duration for which their concentration is maintained at levels above the MIC and not the AUC or C_{max}. The regimens reported to achieve high re-eradication rates (96.8%-100%) using dual therapy use a PPI *qid* plus AMPC *qid*^[18,43,79,84].

EFFICACY OF TAILORED ERADICATION TREATMENT FOR *H. PYLORI* INFECTIONS BASED ON SUSCEPTIBILITY TO ANTIBIOTICS AND *CYP2C19* GENOTYPE

Tailored eradication therapy shows promise for delivering significantly more successful outcomes than the standard therapies described above. For example, in their preliminary trial, Kawai *et al.*^[85] determined the efficacy of a regimen based on bacterial drug susceptibility to CAM that included 70 *H. pylori*-positive patients administered the following drugs: PPI/AMPC/CAM for patients with CAM-sensitive strains and PPI/AMPC/MNZ for CAM-resistant strains. The tailored treatment regimen achieved a 94.3% eradication rate, which is significantly higher than that achieved with standard treatment (71.4%, PPI/AMPC/CAM)^[85], suggesting that treatment based on susceptibility to CAM will be effective, particularly in areas such as Japan with a high prevalence of CAM-resistant strains.

A second example of the increased efficacy of a tailored therapy comes from our own studies in which we administered PPIs according to *CYP2C19* genotype and sequence analysis of the gene encoding *H. pylori* 23S rRNA^[86]. Patients infected with a CAM-sensitive strain were treated with CAM 200 mg *tid*, AMPC 500 mg *tid*, and personalized doses of lansoprazole (*e.g.*, RMs, 30 mg *tid*; IMs, 15 mg *tid*; and PMs, 15 mg *bid*) for 1 wk. Patients infected with a resistant strain were treated with AMPC 500 mg *qid* and a personalized dose of lansoprazole (*e.g.*, RMs, 30 mg *qid*; IMs, 15 mg *qid*; and PMs, 15 mg *bid* for 2 wk)^[86]. The ITT analyses of eradication rates for standard triple regimen (PPI/AMPC/CAM) are 70.0% compared with 96.0% ($P < 0.0001$) for tailored treatment (graded A, excellent)^[86].

Although this tailored treatment may be optimal with high eradication rate, a setting of drug selection and dosing doses of PPI and antimicrobial agents may be complicated. We therefore propose that a regimen based on administering rabeprazole *qid* for all patients, irrespective of their *CYP2C19* genotype, may achieve higher eradication rates than using a regimen employing a PPI *bid*, in particular in RMs. We assessed the efficacy of the tailored eradication regimens that control acid secretion using a PPI *qid* and selected antimicrobial agents based on the CAM-susceptibility of the patient's *H. pylori* strain (Figure 6A). Patients infected with CAM-sensitive strains were treated with a tailored regimen of rabeprazole (RPZ) *qid*, AMPC 500 mg *qid*, and CAM 200 mg *bid* for 1 wk, while those infected with resistant strains were given RPZ *qid*, AMPC 500 mg *qid*, and MNZ 250 mg *bid* for 1 wk, irrespective of *CYP2C19* genotype. The overall eradication rate achieved using the standard regimen was 77.8% (95%CI: 72.0%-85.5%) according to ITT analysis and that of the tailored regimen was 98.0% (94.3%-99.6%) (Figure 6B). The eradication rates using CAM-based and MNZ-based treatment were similar (96.5% and 98.4%, respectively, $P = 0.469$), and the eradication rates using the tailored regimen were similar among different *CYP2C19* genotypes (RM, 94.3%; IM, 98.3%; and PM, 100%). In contrast, the outcomes achieved using the standard regimen were as follows: RM, 75.7%; IM, 81.7%; and PM, 87.0%) (Figure 6C). A tailored *H. pylori* eradication regimen based on CAM susceptibility that inhibits acid secretion for 24 h using RPZ 10 mg *qid* is more effective than the standard therapy used in Japan, as its eradication rate exceeds 95% irrespective of *CYP2C19* genotype. Benefits of this tailored treatment are to save a cost of genotyping test and to prevent increased CAM-resistant *H. pylori* strain. We added limitation of this treatment in revised version. However, because not all patients are *CYP2C19* RM and more frequent dosing with the PPI for IMs and PMs is more costly, this tailored treatment may be applicable third-line treatment. To identify efficacy of this tailored treatment for first- and second-line treatment (*i.e.*, eradication rate and cost benefit), further study will be required.

CONCLUSION

This review focuses on *H. pylori* eradication therapy in relation to pharmacogenomics and susceptibility to antimicrobial agents. We highlight the many genetic factors that influence therapeutic outcomes of *H. pylori* eradication therapy using a PPI and antimicrobial agents. We describe a tailored treatment that was designed according to pharmacogenomics and antimicrobial susceptibility to achieve an eradication rate exceeding 95%, irrespective of eradication history, that overcomes differences among *CYP2C19* genotypes. Although a tailored regimen based on an individual's *CYP2C19* genotype is a valid therapeutic consideration, our strategy saves the cost of *CYP2C19* genotyping. However, using increased doses of PPIs may

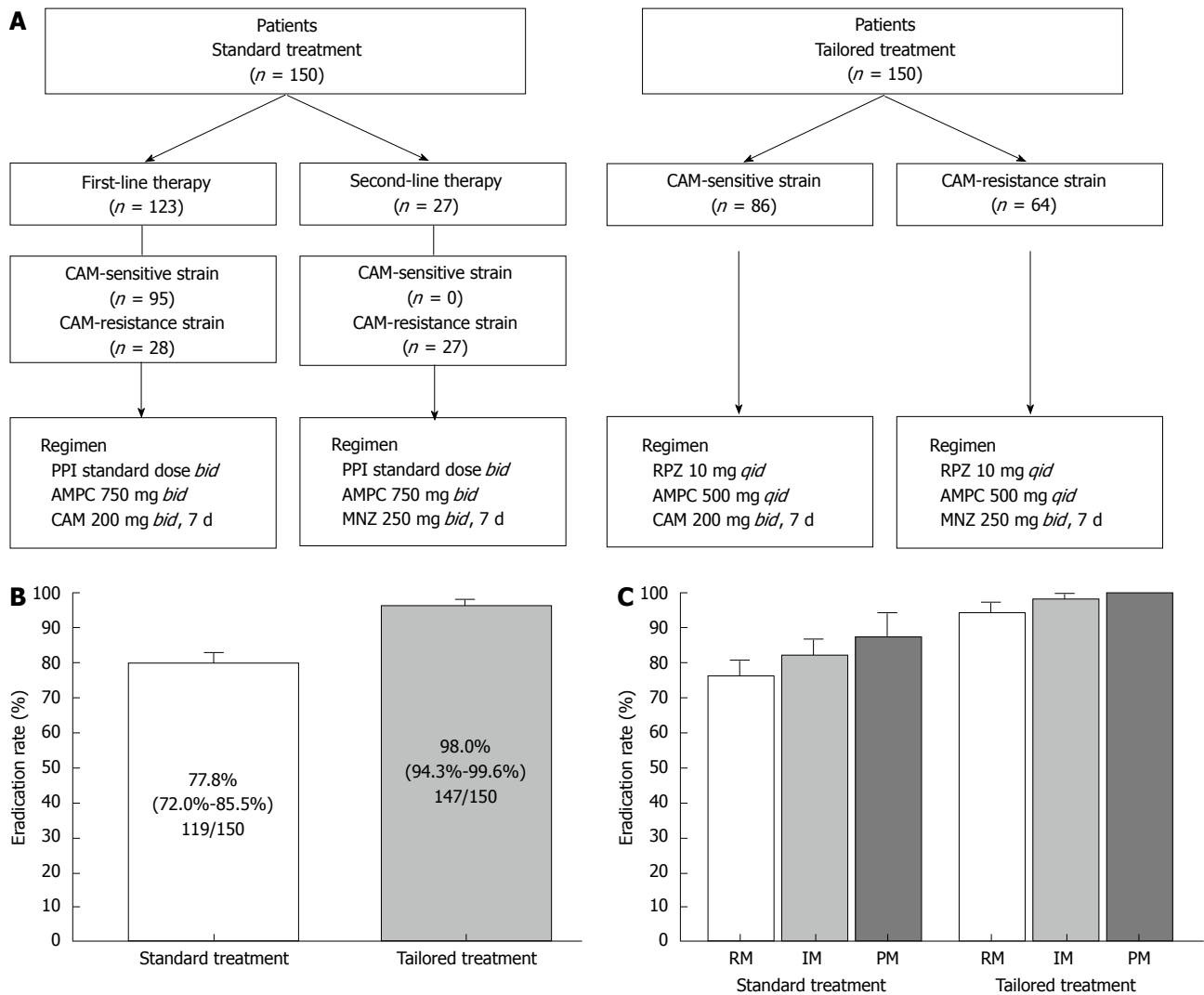


Figure 6 Study design and outcomes. A: Patients were classified into two treatment regimens: standard treatment group (first- or second-line standard Japanese regimen) and tailored treatment group (based on clarithromycin-susceptibility); B: Eradication rates for the standard and tailored regimens for eradication of *Helicobacter pylori*; C: Eradication rates for the standard and tailored regimens among different *CYP2C19* genotypes. CAM: Clarithromycin; PPI: Proton pump inhibitor; AMPC: Amoxicillin; MNZ: Metronidazole; RPZ: Rabeprazole.

not be universally welcomed and may not be tolerated by some patients. In addition, because there are other genetic factors to influence in eradication rate as listed in Table 1, it may be better to consider effects of genetic factors for optimal tailored treatment.

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Critical pathogenic steps to high risk *Helicobacter pylori* gastritis and gastric carcinogenesis

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Abstract

Helicobacter pylori (*H. pylori*) gastritis may progress to high risk gastropathy and cancer. However, the pathological progression has not been characterized in detail. *H. pylori* induce persistent inflammatory infiltration. Neutrophils are unique in that they directly infiltrate into foveolar epithelium aiming the proliferative zone specifically. Neutrophilic proliferative zone foveolitis is a critical pathogenic step in *H. pylori* gastritis inducing intensive epithelial damage. Epithelial cells carrying accumulated genomic damage and mutations show the Malgun (clear) cell change, characterized by large clear nucleus and prominent nucleolus. Malgun cells further undergo atypical changes, showing nuclear folding, coarse chromatin, and multiple nucleoli. The atypical Malgun cell (AMC) change is a novel premalignant condition in high risk gastropathy, which may progress and undergo malignant transformation directly. The pathological significance of AMC in gastric carcinogenesis is reviewed. A new diagnosis system of gastritis is proposed based on the critical pathologic steps classifying low and high risk gastritis for separate treatment modality. It is suggested that the regulation of *H. pylori*-

induced neutrophilic foveolitis might be a future therapeutic goal replacing bactericidal antibiotics approach.

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Key words: *Helicobacter pylori*; Atypical Malgun cell; Neutrophilic foveolitis; Stomach; Cancer; Gastritis; Pathology; Premalignant

Core tip: Two critical pathogenic steps of high risk *Helicobacter pylori* (*H. pylori*) gastritis and gastric carcinogenesis are reviewed. Neutrophilic proliferative zone foveolitis is a critical pathogenic step in *H. pylori* gastritis inducing intensive epithelial damage. It is suggested to provide a new therapeutic goal replacing traditional bactericidal antibiotics approach. Atypical Malgun cell change is a novel premalignant condition in high risk gastropathy, which may progress and undergo malignant transformation directly. A new diagnosis system of gastritis is proposed based on the critical pathologic steps classifying low and high risk gastritis for separate treatment modality.

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INTRODUCTION

It has been 3 decades since *Helicobacter pylori* (*H. pylori*) was first identified^[1]. *H. pylori* is now accepted as the major cause for gastritis and gastric cancer. Numerous investigations for the pathogenesis of *H. pylori* have been done^[2,3], heightening expectations for curative remedies of most gastric diseases. However, the clinical transla-

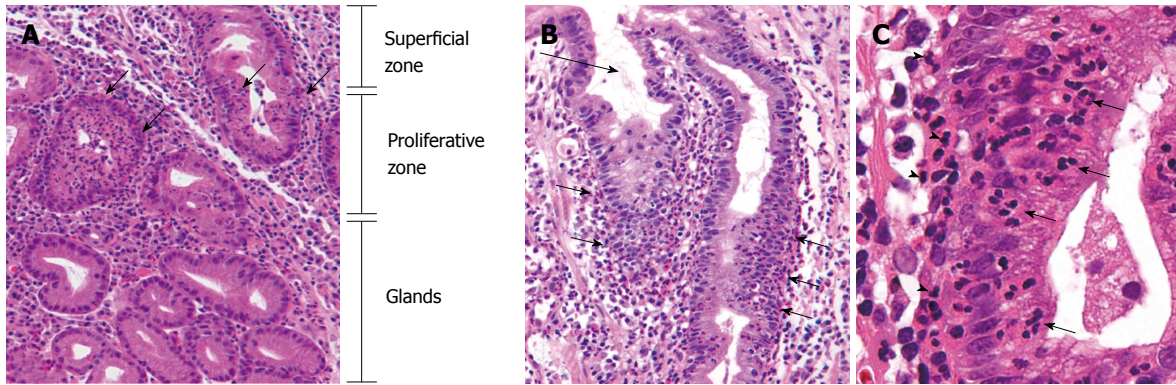


Figure 1 Neutrophilic proliferative zone foveolitis. A: Intense infiltration specifically focused on the proliferative zone of gastric pits (arrows). (HE, $\times 40$); B: Neutrophils targeting the proliferative zone foveolar epithelium (arrows), while the surface is free of significant infiltration (long arrow) (HE, $\times 30$); C: Infiltrate consisting of pure neutrophils in single cells or small aggregates (arrows). Neutrophils in lamina propria close to the foveolitis (arrowheads), suggesting an active movement into the epithelium (HE, $\times 150$).

tion of the progress has been so sluggish that current management for gastric diseases is not so much different from the pre-*H. pylori* era fundamentally. The major therapeutic goal remains to be early detection and curative resection of gastric cancer, and cancer prevention and clinical management at the premalignant stage have a long way to be established.

Antibiotics treatment for *H. pylori* has been such a controversial issue that it is still not well established as to who should be treated. The high 'recurrence rate', potential long term complications, and socioeconomic usefulness of the bactericidal treatment have been disputed. Even the benefits of *H. pylori* have been suggested such as reducing excessive gastric secretion^[4]. However, such potential benefits may not be taken into account in countries like South Korea where the *H. pylori* infection and gastric cancers are so rampant^[5]. To detect gastric cancers at the early stage, endoscopic examinations are done massively nation-wide in Korea. More than 31000 gastric biopsies are done annually in Asan Medical Center alone for all stages of gastritis and advanced lesions. More than 10% of the patients eventually undergo mucosal/surgical resections for cancers. The huge pathologic samples provide an exceptional opportunity to investigate the pathogenesis and progression of *H. pylori* gastritis to high risk gastropathies.

Most cancers develop after a multi-step carcinogenic process for a long period. However, the pathological progression to high risk *H. pylori* gastritis and cancer is poorly understood. Gastric cancers are so heterogeneous that it is not easy to define major carcinogenic routes. It probably reflects that most gastric cancers develop after accumulated random genomic damage in persistent inflammation without strong genetic predispositions. Common premalignant lesions, such as tubular adenomas in colon, have been elusive to identify in stomach. Colon-type tubular adenomas are not so frequent in *H. pylori* gastritis, far less than cancers. Taken together, it is strongly suspected that we have missed or overlooked critical pathogenic steps of *H. pylori* gastritis and premalignant changes developing under persistent inflammatory pres-

sure. They are likely to be rather common and subtle pathological lesions.

Here, major pathological routes of gastric carcinogenesis including critical pathologic steps and clinical implications are reviewed. Neutrophilic proliferative zone foveolitis is the pathogenic core of *H. pylori* gastritis the biological significance of which has largely been overlooked. Surface neutrophilic foveolitis appears to be associated with predisposition for erosion. The atypical Malgun cell (AMC) change is a newly recognized premalignant lesion present in advanced high risk *H. pylori* gastritis. A new pathologic diagnosis system of gastritis is proposed for practical use.

NEUTROPHILIC PROLIFERATIVE ZONE FOVEOLITIS: THE CORE OF *H. PYLORI* GASTRITIS PATHOGENESIS

H. pylori gastritis shows various inflammatory infiltrates including neutrophils, lymphocytes, plasma cells, eosinophils, macrophages, and mast cells. Neutrophils are of particular pathobiological significance, because they directly infiltrate into foveolar epithelium while other infiltrates are mostly in lamina propria. Neutrophilic foveolitis is present in *H. pylori* gastritis worldwide^[5], and correlates directly with *H. pylori* infection^[5-7].

Neutrophilic foveolitis consists of two specific forms, *i.e.* neutrophilic proliferative zone foveolitis and surface neutrophilic foveolitis. Neutrophilic proliferative zone foveolitis (PNF) is the most characteristic feature of *H. pylori* gastritis, which is shown rarely in other gastritis of alcoholic, chemical, and viral causes. PNF is present in most *H. Pylori* gastritis regardless of the stage^[4-7]. The neutrophilic infiltration is specifically localized to the pit proliferative zone (Figure 1A and B). Lamina propria infiltrates are mostly present close to the foveolar infiltrates (Figure 1C), supporting active movement of neutrophils to the proliferative zone epithelium.

Surface neutrophilic foveolitis (SNF) consists of neutrophilic infiltrates into the surface epithelium (Figure 2).

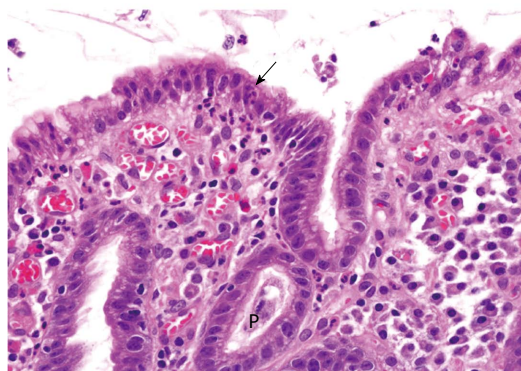


Figure 2 Surface neutrophilic foveolitis. A biopsy from a male subject with recurrent episodes of erosive gastritis, showing a small focus of neutrophilic infiltration in the surface epithelium (arrow). P: Proliferative zone (HE, $\times 50$).

SNF is not as common as the proliferative zone foveolitis, and may be present with or without PNF. SNF is usually associated with a subgroup of patients with intractable recurrent erosion and/or ulcer. In those patients, uninvolved “normal” mucosa often shows surface neutrophilic infiltration, suggesting a genetic predisposition to the epithelial erosion. “Neutrophilic infiltration” has been described in the Sydney system as one of the inflammatory infiltrates in *H. pylori* gastritis^[8]. However, specific details and pathobiological significance have largely been overlooked. Regrettably, it is often described indiscriminately as “chronic active gastritis”. Such a description should be discarded, because it does not provide useful clinical information but rather gives a misconception that any gastritis without prominent neutrophils is “inactive”. *H. pylori* gastritis is a progressive disease, and should be taken as “active” at any stage. Neutrophilic foveolitis may not be readily found in small biopsies including intestinal metaplasia or advanced high risk gastritis where *H. pylori* are rare locally.

It is of interest how neutrophils specifically aim the proliferative zone epithelium. Given that *H. pylori* penetrate into the borders between epithelial cells^[2,3], it is tempting to postulate that neutrophils infiltrate into the proliferative zone where the epithelial junctions are relatively weak compared to surface or deep glandular zones so that *H. pylori* would easily disrupt the epithelial integrity. The precise mechanism(s) of specific targeting may provide us a future therapeutic target replacing controversial bactericidal antibiotics therapy.

MALGUN (CLEAR) CELL CHANGE IN FOVEOLAR EPITHELIUM

Intense neutrophilic infiltration targeting the proliferative zone has a strong pathobiological impact. Activated neutrophils secrete abundant inflammatory agents damaging the pits, which enhances epithelial proliferation in turn. Such agents as reactive oxygen and nitrogen species are mutagenic, inducing accumulated genomic damage in vulnerable epithelial cells in the close vicinity.

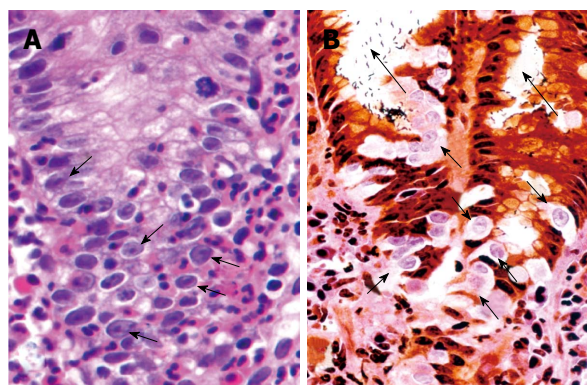


Figure 3 Malgun cell change. A: Malgun cell change of the epithelial cells at the site of neutrophilic infiltration (arrows), showing large, pale nucleus, prominent nucleolus, and smooth nuclear envelope. They often show characteristic “perinuclear halo” reflecting artificial retraction of cytoplasm (HE, $\times 150$). B: Malgun cells are not stained by silver impregnation in triple silver staining (arrows), demarcating them clearly from other cells. They develop as single cells at the proliferative zone in association with neutrophilic foveolitis, and make small clusters as they move upward to the surface suggesting a clonogenic potential. Note numerous *Helicobacter pylori* attached to the epithelium and in the lumen (long arrows). (Triple silver staining, $\times 150$).

In the proliferative zone with intense neutrophilic infiltrates, epithelial cells begin to show the Malgun (clear) cell change characterized by clear, enlarged nuclei and prominent nucleolus (Figure 3A). The word “Malgun” represents “clear” or “transparent” in Korean^[7,9]. The cytoplasm is so tender that a characteristic artifact of “perinuclear halo” is seen frequently due to shrinking in the fixation and embedding process. Malgun cells are demarcated clearly by negative silver staining in contrast to other cells and *H. pylori* (Figure 3B). Malgun cells have considerable genomic damage, supporting that activated neutrophils indeed induces significant mutagenesis in proliferating epithelial cells^[9]. Despite the genomic damage, they manage to keep the proliferative activity and may expand in groups (Figure 3B). Typical Malgun cells may be regarded to reflect the genomic damage inside. The pale face of Malgun cells reminds me of “the Scream” by Edvard Munch, trying to warn the upcoming tragedy loudly to someone. We may have overlooked them, because there are so many all over.

CHRONIC METAPLASTIC GASTRITIS

Intestinal metaplasia is very frequent in progressive *H. pylori* gastritis. By definition, the word “metaplasia” represents a switch from a differentiated cell type to another. What we actually refer to, however, represents a low grade dysplastic condition “similar to other cell types”. Strictly speaking, it is a misnomer, but I follow it just to avoid additional unwanted confusion. Nonetheless, it should be reminded that the intestinal metaplasia does not represent “normal” intestinal mucosa but a progressive dysplastic condition at relatively early stage.

Gastritis is a highly variegated process. *H. pylori* organisms are difficult to find in the metaplastic area. They move to adjacent non-metaplastic mucosa to induce neu-

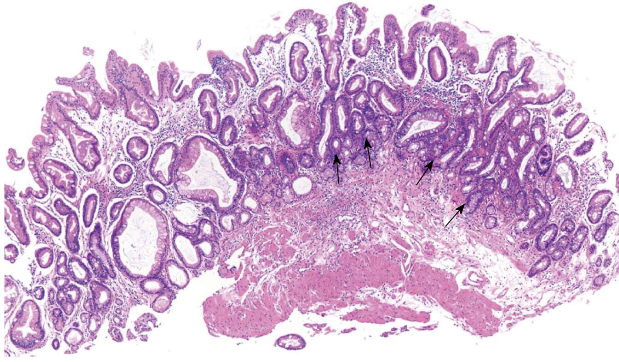


Figure 4 Chronic metaplastic gastritis with atypical Malgun cell change. Panoramic view showing irregular arrangement of pits with multifocal crowding of small atypical glands (arrows) (HE, $\times 10$).

trophilic foveolitis in the new colony. Intestinal metaplasia may be regarded as the second best measure for gastric mucosa to avoid fierce neutrophilic foveolitis and to earn some time to take a breath from the relentless attack. However, it represents just another way of mucosal instability with slow progression to high-risk gastritis. In “advanced” or “complete” metaplasia, the mucosa is entirely replaced by metaplastic pits showing paneth cells. It would then undergo further atypical changes progressively.

ATROPHIC AND FOLLICULAR GASTRITIS

Upon persistent neutrophilic foveolitis, mucosal damage and repeated erosions give rise to atrophic gastritis eventually. Mucosal atrophy itself would represent not only a poor physiological function but also a limited capacity for “intrinsic protection” against progressive epithelial atypia.

It has been proposed to take the degree and extent of mucosal atrophy for “staging” of gastritis^[10]. Mucosal atrophy may indeed be associated with the progression to high risk gastropathy. However, the reproducibility of measurement for atrophy is very doubtful, because it is difficult to examine endoscopic gastric biopsies in standard orientations. Furthermore, the degree of atrophy varies considerably throughout the mucosa. For a practical approach, mucosal atypia rather than atrophy should be the focus of reproducible pathologic examination.

Lymphoid follicles are not present in gastric mucosa normally but often appear in atrophic gastritis, suggesting that atrophy somehow induces lymphoid follicle formation. Individual predisposition may play a role, because not all atrophic gastritis shows lymphoid follicles. Numerous plasma cells in lamina propria would represent a follicular gastritis even if no follicle is present in the given biopsy.

The control of ad hoc follicles in aberrant location seems to be precarious. In advanced follicular gastritis, the mantle and marginal zones often shows irregular expansion which may precede MALT lymphoma. However, such a progression is rare and usually very slow. The fact that “MALT lymphomas” may often be treated with an-

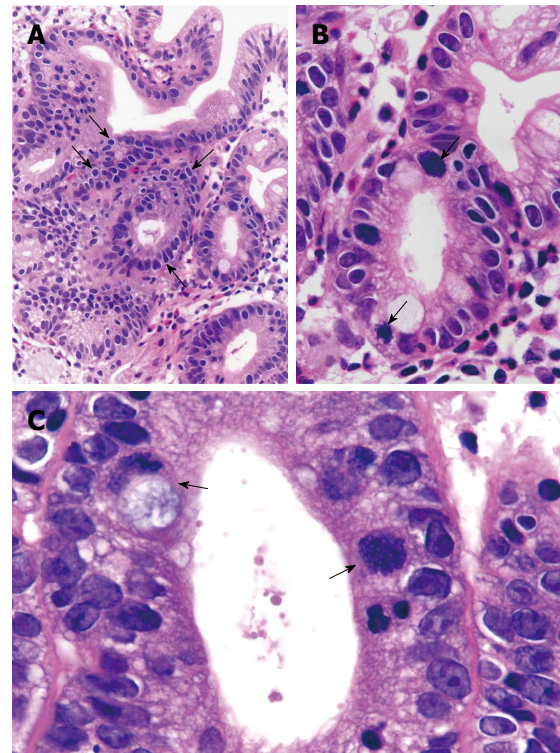


Figure 5 Atypical Malgun cells. A: Atypical malgun cells (AMC) develops at the proliferative zone, showing characteristic “perinuclear halo” but hyperchromatic nuclei (arrows) (HE, $\times 45$). B: Magnified view of A, showing 2 mulberry cells (arrows) in AMC (HE, $\times 100$). C: High power view of AMC and Mulberry cells. Atypical Malgun cells show irregular nuclear folding, hyperchromatism, coarse chromatin, and multiple nucleoli nuclear folding. Two Mulberry cells with extreme nuclear folding are indicated by arrows. The left one shows cytoplasmic mucin (HE, $\times 400$).

tibiotics strongly suggests a practical over-diagnosis of gastric lymphomas. Nonetheless, excessive expansion of lymphoid follicles and/or evident epithelial infiltration should be included in the pathology report.

AMC CHANGE

As *H. pylori* gastritis progresses, the mucosa shows irregular configuration and arrangement with frequent crowding of pits (Figure 4), where Malgun cells gradually show evident atypical change. The AMC change first develops at the proliferative zone and spreads out (Figure 5A). In metaplastic pits, AMC tends to appear at the mucosal bottom (Figure 4), which represents the proliferative zone for the intestinal crypts. AMC have large nuclei and “perinuclear halo” as in classical Malgun cells. However, nuclei are not clear and round anymore; they show irregular folding, hyperchromatism, coarse chromatin, and multiple nucleoli (Figure 5B and C). AMC often show extreme nuclear folding like a mulberry (Figure 5B and C), which may be confused with mitotic figures. Mitoses are also frequent with occasional atypical figures (Figure 6). “Mulberry cells” may be regarded as one of the diagnostic hallmarks for AMC. Histopathological and cytological characteristics of atypical *vs* classical Malgun cell changes are summarized in Table 1.

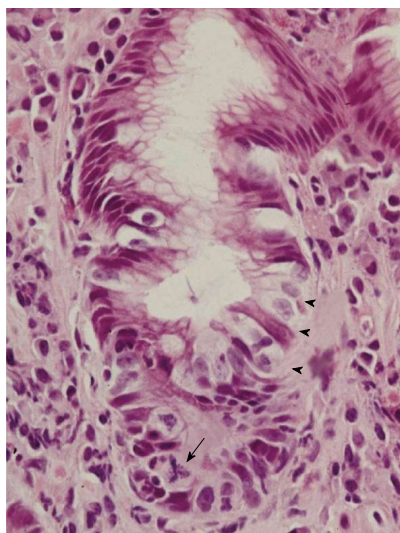


Figure 6 Atypical Malgun cells at the proliferative zone of pit (arrowheads). Note an atypical mitotic figure (arrow) (HE, × 150).

AMC IS A PROGRESSIVE PREMALIGNANT LESION

AMC represents a progressive high risk gastropathy. The cellular and structural atypia increases as it progresses, and atypia of variable degrees may be present simultaneously in a patient (Figure 7). AMC may progress to overt “epithelial dysplasia” consisting of crowded glands of high grade atypia. Colon type tubular adenomas may develop in metaplastic gastritis, but are relatively infrequent.

Like AMC, gastric carcinomas first develop at the proliferative zone and spreads out laterally in the mucosa. Cancers frequently develop in AMC without any other evident premalignant lesion (Figure 8A). Cytologic figures of early cancers are often very similar to adjacent AMC (Figure 8B and C), supporting that cancers, particularly diffuse type carcinomas, develop directly from AMC. As cancers grow, the histological features may change depending on which cell clones emerge to dominate. Other evidences also support the pathobiological role of AMC in carcinogenesis. On long-term follow up, gastric cancers seem to develop much more frequently in AMC compared to gastritis without AMC, supporting the notion that AMC is a premalignant lesion. A prospective epidemiological study is under progress.

MAJOR ROUTES OF GASTRIC CARCINOGENESIS

Major carcinogenic routes from chronic *H. pylori* gastritis are summarized in Figure 9. *H. pylori* infection induces neutrophilic proliferative zone foveolitis that is by far the most critical initial pathogenic step causing intense and persistent pit damage and mucosal erosion. Epithelial cells then show the Malgun cell change and/or intestinal metaplasia. As genomic damage is accumulated, typical Malgun cells and metaplastic cells progress

Table 1 Atypical vs typical malgun cell change

| | "Typical" malgun cell | Atypical malgun cell |
|--------------------------|------------------------------|-------------------------|
| Location | Proliferative/surface zone | Proliferative zone |
| Pits | Elongated proliferative zone | Irregular with crowding |
| Cell size | Enlarged | Enlarged |
| Perinuclear halo | + | + |
| Nuclei | Clear | Hyperchromatic |
| Chromatin | Euchromatin | Heterochromatin |
| Nucleoli | 1 (or 2) | 2 (or multiple) |
| Nuclear envelope | Smooth | Folded |
| Mulberry cells | - | + |
| In metaplasia | - | +/- |
| Malignant transformation | - | + |

to AMC, a critical pathological feature representing high risk gastritis. Cancers, particularly diffuse type carcinomas, may develop directly from AMC. Intestinal type cancers mostly develop from AMC in metaplastic gastritis with or without overt epithelial dysplasia. Mucosal atrophy, persistent inflammatory pressure, and other carcinogenic factors would promote the malignant transformation together. Lymphomas may develop in association with chronic follicular gastritis arising in atrophic mucosa. The proposal is to depict major carcinogenic routes for the majority of gastric cancers but not to cover all pathways.

PATHOLOGIC DIAGNOSIS SYSTEM FOR GASTRITIS AND CLINICAL IMPLICATIONS

A practical system for pathologic diagnoses for gastritis is proposed in Table 2. Superficial congestive gastritis (SCG) represents an acute process with mucosal edema, capillary congestion, and mild inflammatory infiltration. Regenerative atypia may be found in elderly subjects (SCG-Ad), which may reflect healed epithelial “scarring”.

Erosive gastritis (EG) shows inflammatory infiltration with epithelial damage. EG-PNF represents EG with prominent neutrophilic proliferative zone foveolitis. Antibiotics treatment may be recommended to prevent rapidly progressive mucosal damage. EG with superficial neutrophilic foveolitis represents an evident superficial epithelial neutrophilic gastritis with or without EG-PNF. It consists of a minor population of EG prone to intractable erosive disease for whom anti-acid and/or -secretory regulators would be helpful. Advanced EG (EG-Ad) represents longstanding EG with elongated proliferative zone, epithelial damage, and frequent typical Malgun cells. Cellular and glandular atypia should not be significant. EG with AMC (EG-AMC) should be regarded as a high risk gastritis so that patients are followed-up regularly. EG-Ad progresses to EG-AMC gradually. The points for the differential diagnosis are summarized in Table 1.

Chronic atrophic gastritis (CAG) is confirmed when the tissue sample is oriented well so that the entire length

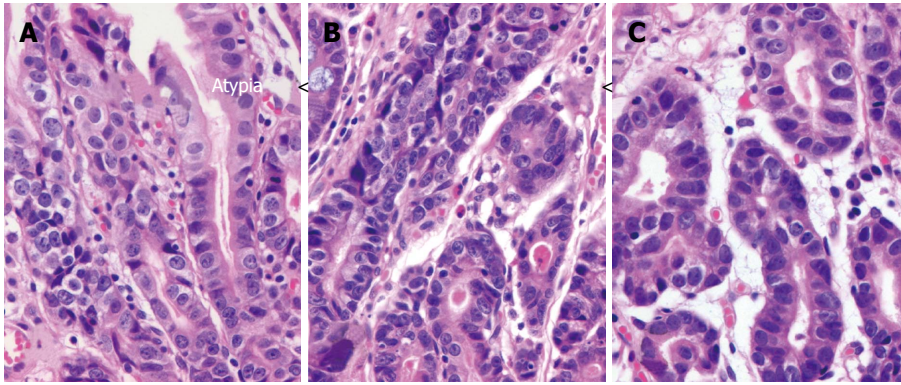


Figure 7 Atypical Malpighian cells with progressive atypia. A-C: Three nearby loci of Atypical malpighian cells (AMC) at antrum of a male patient showing progressive glandular and cytological atypia from A to C (HE, $\times 75$).

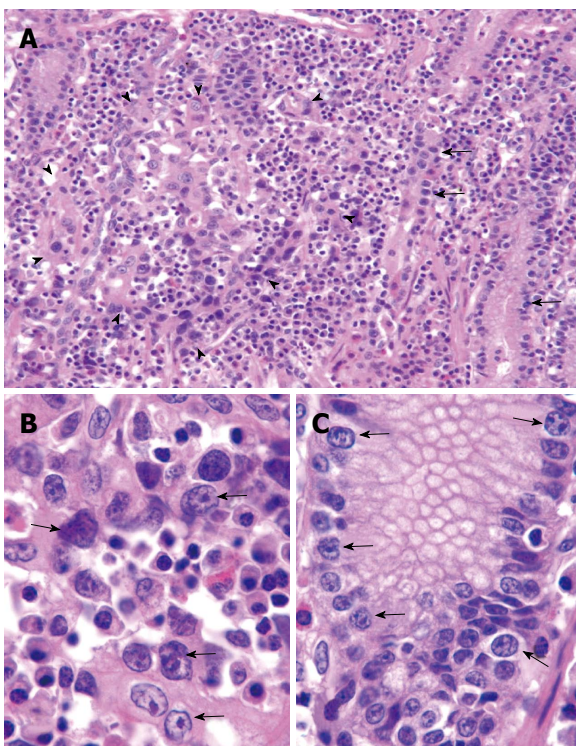


Figure 8 Gastric cancer developing in atypical Malpighian cells. A: Small focus of early mucosal gastric cancer (arrowheads) developing next to Atypical malpighian cells (AMC) (arrows) at the proliferative zone (HE, $\times 30$); B, C: High power views of carcinoma (B) and AMC (C) in A, respectively. Although cancer cells (B) are larger than AMC (C) at the same magnification, they keep cytological characteristics similar to AMC. No other “pre-malignant lesion” is present, supporting direct malignant transformation from AMC (HE, $\times 120$).

of pits may be assessed. CAG should not be confused with “chronic active gastritis”, which should be discarded at once for many reasons as described. Advanced CAG (CAG-Ad) represents severe mucosal atrophy with or without significant epithelial atypia. Both CAG-Ad and CAG-AMC are regarded as high-risk groups. Chronic follicular gastritis (CFG) is mostly associated with mucosal atrophy. Advanced CFG represents gastritis with enlarged lymphoid follicles and irregularly expanded mantle zone into of adjacent pits. No lymphoid cellular atypia or overt

epithelial infiltration is to be included.

Chronic metaplastic gastritis (CMG) consists of intestinal metaplasia and mild inflammatory infiltration. Local *H. pylori* and neutrophilic foveolitis are absent or rare in CMG. Advanced CMG represents an extensive metaplasia replacing the whole mucosal layers with frequent paneth cells. The cellular atypia should be minimal. CMG with AMC (CMG-AMC) is quite common form of high risk gastritis. AMC shows considerable cellular atypia, increased mitoses, and occasional Mulberry cells. Gastric cancers may develop directly from CMG-AMC.

Epithelial dysplasia is regarded to be a neoplastic change, showing crowded atypical glands. Colon-like tubular adenomas developing in CMG are included in this category. High grade dysplasia shows severe cellular and structural atypia of pits, and represents an imminent malignant change. Local mucosal resection should be considered whenever applicable.

Multiple diagnoses in the proposal may be made for a given case. In such a case, the diagnosis of grave clinical implication should be placed first. The histological presence of *H. pylori* may also be described. We do not mention it anymore in our pathology report unless it is in high risk gastritis or an antibiotics therapy is warranted such as EG-PNF.

CONCLUSION AND FUTURE DIRECTIONS

Gastric cancers represent a typical inflammation-induced malignancy arising after accumulated mutations and genomic damage mostly induced by *H. pylori* infection. Neutrophilic proliferative zone foveolitis is the core of pathogenesis of *H. pylori* gastritis and cancers. Detailed descriptions of neutrophilic foveolitis should be included in the pathological diagnosis of gastritis. Surface neutrophilic foveolitis may be treated as a separate subgroup prone to erosion and/or ulcer.

Neutrophilic foveolitis may be implicated in the future development of therapeutics for *H. pylori* gastritis. The “eradication” of *H. pylori* using traditional bactericidal antibiotic therapy is controversial. After all, the bacteria have been together with us through the evolu-

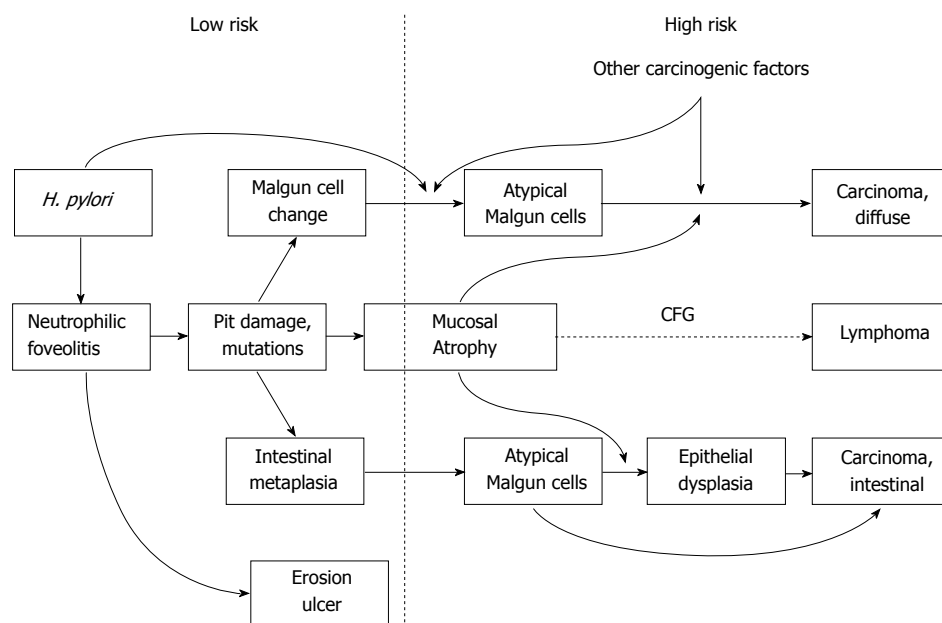


Figure 9 Major routes for gastric carcinogenesis from *Helicobacter pylori* gastritis. Low and high risk gastritis are delineated. CFG: Chronic follicular gastritis; *H. pylori*: *Helicobacter pylori*.

Table 2 Pathologic diagnosis system of gastritis/gastropathy

| |
|---|
| Superficial congestive gastritis (SCG) |
| SCG with epithelial regenerative change (SCG-Ad) |
| Erosive gastritis (EG) |
| EG with prominent neutrophilic proliferative zone foveolitis (EG-PNF) |
| EG with superficial neutrophilic foveolitis (EG-SNF) |
| EG, advanced (EG-Ad) |
| EG with atypical Malgun cell change (EG-AMC) |
| Chronic atrophic gastritis (CAG) |
| CAG, advanced (CAG-Ad) |
| CAG with AMC (CAG-AMC) |
| Chronic follicular gastritis (CFG) |
| CFG, advanced (CFG-Ad) |
| Chronic metaplastic gastritis (CMG) |
| CMG-Ad |
| CMG-AMC |
| Epithelial dysplasia (DYS) |
| DYS-high grade (DYS-H) |
| General addendum, if necessary |
| <i>H. pylori</i> +/- |
| dominant infiltrate, etc. |

H. pylori: *Helicobacter pylori*.

tion as a member of human microbiota. We may now consider changing the direction of therapeutic approach. Control of neutrophilic foveolitis induced by *H. pylori* may be a good alternative goal for future therapy. The mechanism(s) of inducing neutrophilic foveolitis should be investigated thoroughly. I suggest to screen widely for safe remedies, synthetic or natural, to control the motility of *H. pylori* for immediate clinical trials. Active intercellular penetration of *H. pylori* is likely to be critical for inducing neutrophilic infiltration particularly at the proliferative zone of vulnerable epithelial cells.

AMC represents a premalignant lesion from which gastric cancers may develop directly. It has been largely

overlooked, because, ironically, it is so common in advanced *H. pylori* gastritis. The molecular mechanisms of carcinogenesis from AMC need to be investigated in detail. The proposed diagnostic system would be useful for defining high risk group gastritis objectively. Separate clinical management of high risk gastritis would be important for appropriate patient care as well as efficient use of the medical resources socioeconomically. High risk group gastritis/gastropathy consists of about 5%-10% of all gastric biopsies in our population. Considering that biopsies are taken from a minor group of endoscopic examinations, high risk group gastritis of the whole population should be within manageable range for follow-up care even in such a high prevalence society of *H. pylori* gastritis as Korea.

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WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*Simple animal model of *Helicobacter pylori* infection

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Core tip: *Helicobacter pylori* (*H. pylori*) causes significant gastroduodenal diseases. Experimental animal models play an important role in helping us understand the pathogenesis and discovering new therapeutic strategies. Previous *H. pylori*-associated gastritis candidate animal models have included gnotobiotic piglets, non-human primates, pigs, dogs, cats, gerbils, and mice. Rat models of *H. pylori* infection use a difficult technique and take a long time to establish. In this study, we developed a simple model of *H. pylori* infection in rats for further research.

Abstract

Helicobacter pylori (*H. pylori*) has become accepted as a human pathogen for the development of gastritis and gastroduodenal ulcer. To develop a simple rat model of chronic *H. pylori* infection, male Sprague-Dawley rats were pretreated with streptomycin suspended in tap water (5 mg/mL) for 3 d. The rats were inoculated by gavage at 1 mL/rat with *H. pylori* suspension (5×10^8 - 5×10^{10} CFU/mL) twice daily at an interval of 4 h for three consecutive days. Two weeks after inoculation, rats were sacrificed and the stomachs were removed. Antral biopsies were performed for urease test and the stomachs were taken for histopathology. Successful *H. pylori* inoculation was defined as a positive urease test and histopathology. We reported a 69.8%-83.0% success rate for *H. pylori* infection using the urease test, and hematoxylin and eosin staining confirmed the results. Histopathological analysis detected bacteria along the mucous lining of the surface epithelium and crypt lumen and demonstrated mild to moderate gastric inflammation in successfully inoculated rats. We developed a simple rat model of chronic *H. pylori* infection for research into gastric microcirculatory changes and therapy with plant products.

Werawatganon D. Simple animal model of *Helicobacter pylori* infection. *World J Gastroenterol* 2014; 20(21): 6420-6424 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6420.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6420>

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral bacterium that causes infection with many different clinical outcomes. It has been established as a major etiological agent of chronic gastritis and peptic ulcer disease, which includes duodenal and gastric ulcer^[1]. The role of *H. pylori* infection in gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma has also been recognized^[2].

ANIMAL MODELS OF *H. PYLORI* INFECTION

Increasing evidence reveals that *H. pylori* is a significant gastroduodenal pathogen. Experiment animal models are needed to help us understand better its pathogenic mechanisms, and to verify the pathogenesis as well as the rela-

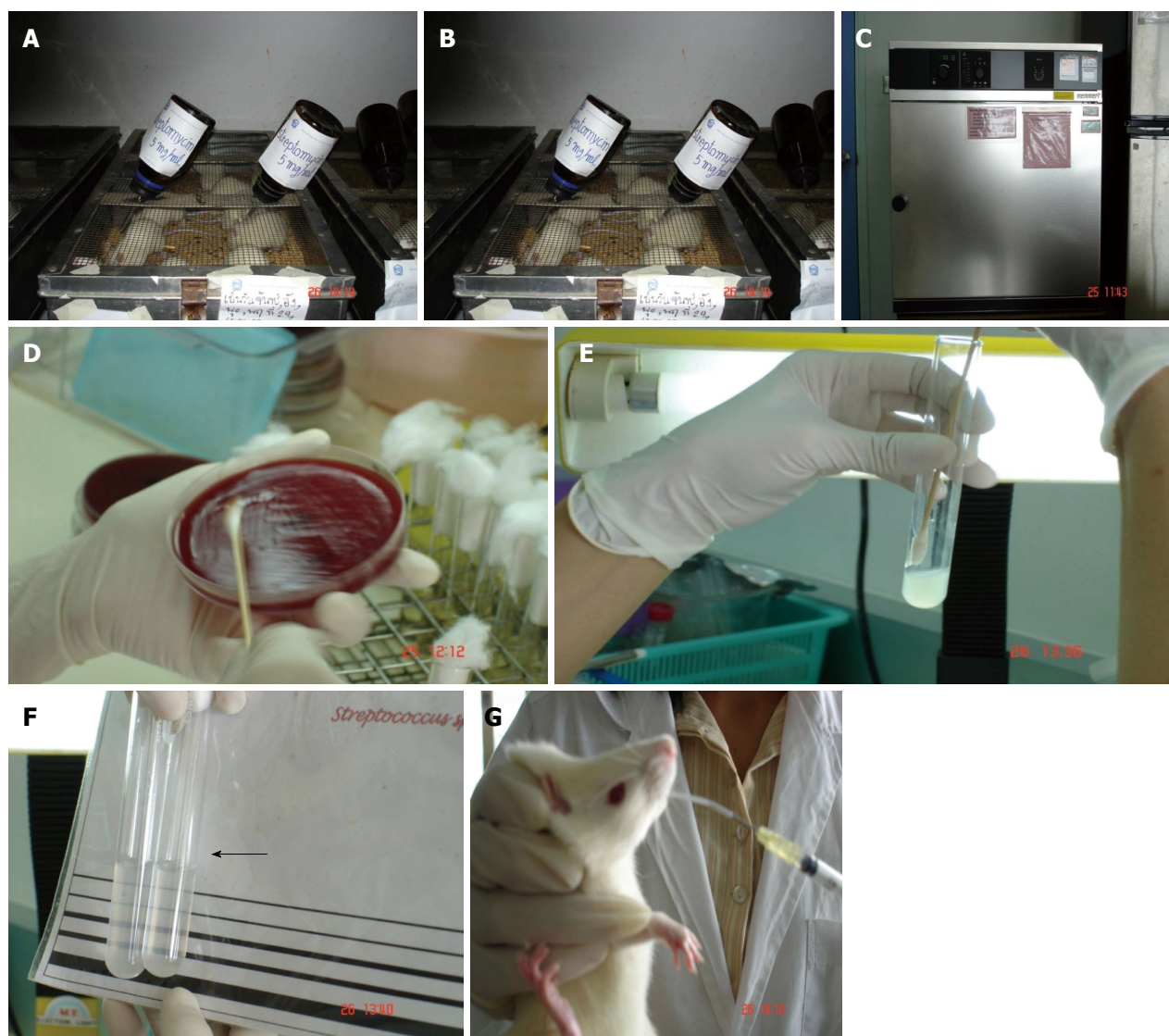


Figure 1 Illustration of *Helicobacter pylori* rat inoculation. A: Male Sprague-Dawley rats 120-150 g; B: Pretreatment with streptomycin (5 mg/kg) for three consecutive days; C and D: *H. pylori* in microaerophilic condition: 5% O₂, 50% CO₂, 37 °C; E and F: *H. pylori* 10⁸-10¹⁰ CFU/mL, suspended in saline; G: Gavage, 1 mL/rat twice daily at an interval of 4 h, for three consecutive days. *H. pylori*: *Helicobacter pylori*.

tionship of this bacterium to gastric injury. Experimental animal models also play an important role in discovering new therapeutic strategies including application of plant products for efficient treatment against *H. pylori* infection^[3]. Previous *H. pylori*-associated gastritis candidate animal models have included gnotobiotic piglets, non-human primates, pigs, dogs, cats, gerbils, and mice^[4-7].

Some studies have been successful for development of models of *H. pylori* infection in rats, pretreated with oral omeprazole to reduce acidic conditions in the stomach, and intragastric administration of *H. pylori* to colonize the stomach^[7].

These animal models were designed and used to establish gastritis that closely resembled the disease commonly found in humans. Animal models offer many benefits and have proved useful in conducting studies to understand better human gastritis in animal counterparts. Rats are one of the most commonly used laboratory animals in gastrointestinal research, and their gastric physiolo-

gy has been thoroughly investigated. Even though other *Helicobacter*-infected animal models have yielded important information, an *H. pylori*-infected rat model would be useful for studying pathophysiological events in the gastrointestinal tract during chronic *H. pylori* infection^[8].

In the past, *H. pylori* bacteria or bacterium-free *H. pylori* filtrates have been used to inoculate rats with normal mucosa and surgically produced gastric ulcers^[3]. Recently, rat models to study reactions from rat gastric mucosa during long-term *H. pylori* infection have been established^[9]. Another model of *H. pylori* infection in rats was also reported by Zeng *et al*^[10], who developed mouse and rat models of *H. pylori* infection by using the Sydney strain 1 *H. pylori* (SS1 Hp) to colonize the stomach. They used a difficult technique over a long period of time and found that *H. pylori* could lead to chronic active gastritis after 8, 12 and 24 wk.

Our model was a simple rat model of chronic *H. pylori* infection developed to research gastric microcirculatory

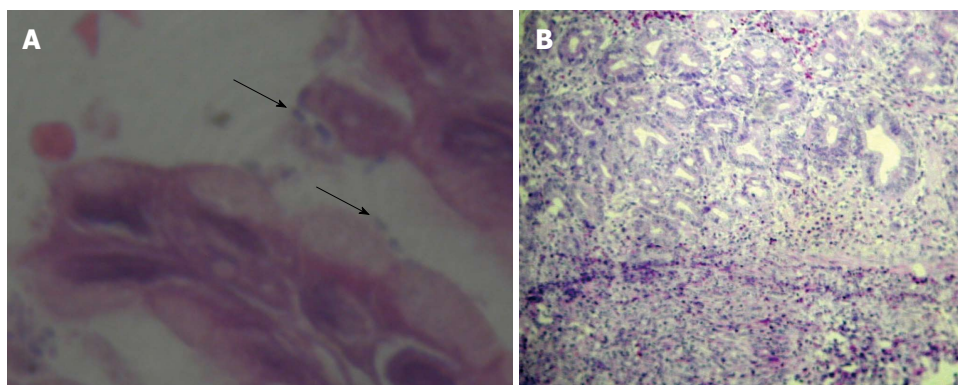


Figure 2 Antral mucosa from *Helicobacter pylori*-infected rats with hematoxylin and eosin staining. A: *Helicobacter pylori* organism in the gastric mucosa (arrow) (600 ×); B: Gastric mucosa with erosion and scattered infiltration of inflammatory cells (250 ×).

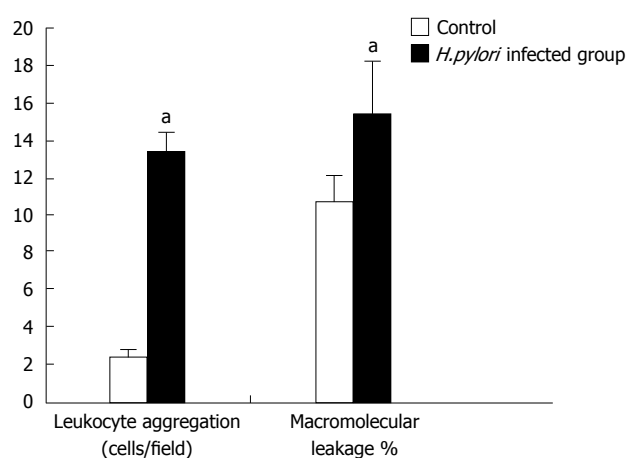


Figure 3 Bar graph of the mean \pm SE of adherent leukocytes and macromolecular leakage of control group compared with *Helicobacter pylori*-infected group. The leukocyte adhesion and macromolecular leakage in the *H. pylori*-infected group were significantly increased compared with the control group ($^aP < 0.01$ vs control group). *H. pylori*: *Helicobacter pylori*.

changes and treatment with plant products. Sprague-Dawley rats (120–150 g) were pretreated with streptomycin suspended in tap water (5 mg/mL) for 3 d before the first *H. pylori* inoculation. The rats were then inoculated by gavage at 1 mL/rat with *H. pylori* suspension (5×10^8 – 5×10^{10} CFU/mL) twice daily at an interval of 4 h for three consecutive days (Figure 1). We reported a 69.8–83.0% success rate of *H. pylori* infection^[11–13] using the urease test. Hematoxylin and eosin staining confirmed these results. The level of bacterial colonization was evaluated by using a grading system and gastric inflammation levels were scored following the updated Sydney System (Figure 2).

GASTRIC MICROCIRCULATORY CHANGES IN A RAT MODEL OF *H. PYLORI* INFECTION

The effects of topical administration of *H. pylori* on the mesenteric microcirculation were detected by using intravital microscopy^[14]. The exposed mesentery that was subjected to *H. pylori* extracts showed an increase in leu-

kocyte adhesion and emigration in the venules. *H. pylori* extracts exhibited changes in the rat mesenteric microcirculation. However, *H. pylori* infection was localized in the stomach, and the leukocyte involvement demonstrated within the mesentery may not be mirrored in the gastric mucosa. Kalia *et al*^[14–16] studied gastric mucosal microcirculation changes caused by *H. pylori* extracts using intravital fluorescent *in vivo* microscopy. *H. pylori* water extracts were applied to rat gastric mucosa and macromolecular leakage, leukocyte adherence, leukocyte rolling, and platelet activity were observed for 90 min. *H. pylori* induced increases in macromolecular leakage after 5 min, and induced adherent platelet thrombi and circulating platelet emboli after 5 and 15 min, respectively.

In our study, we explored the effects of *in vivo* chronic *H. pylori* infection on changes in rat gastric microcirculation using intravital fluorescent microscopy to understand better the pathogenic mechanism of inflammation by monitoring macromolecular leakage and leukocyte-endothelium interaction^[12,13]. Twenty-four male Sprague-Dawley rats were divided into two groups (12 control and 12 *H. pylori* infected). In the *H. pylori*-infected group, rats were inoculated by gavage with bacterial suspension (5×10^8 – 5×10^{10} CFU/mL) twice daily at an interval of 4 h for three consecutive days. Two weeks after inoculation, intravital fluorescence microscopy was performed to examine leukocyte adhesion to post-capillary venules. Macromolecular leakage was examined at 0 and 30 min after fluorescein isothiocyanate-dextran average molecular weight 250K (FITC-dx-250) injection on the posterior surface of the stomach. In the *H. pylori*-infected group, leukocyte adhesion per 100 μ m vessel length was 13.40 ± 1.0 cells, which increased significantly ($P < 0.01$) compared with the control group (2.47 ± 0.62 cells). The average macromolecular leakage was $15.41\% \pm 2.83\%$ and $10.69\% \pm 1.43\%$ in *H. pylori*-infected and control groups, respectively ($P < 0.01$) (Figures 3–5).

The strain of *H. pylori* plays an important role in the pathogenesis of gastroduodenal diseases. *H. pylori* obtained from peptic ulcer patients or other pathogenic strains can increase infection rates and develop pathogenesis in the animal stomach. In contrast, intragastric admin-

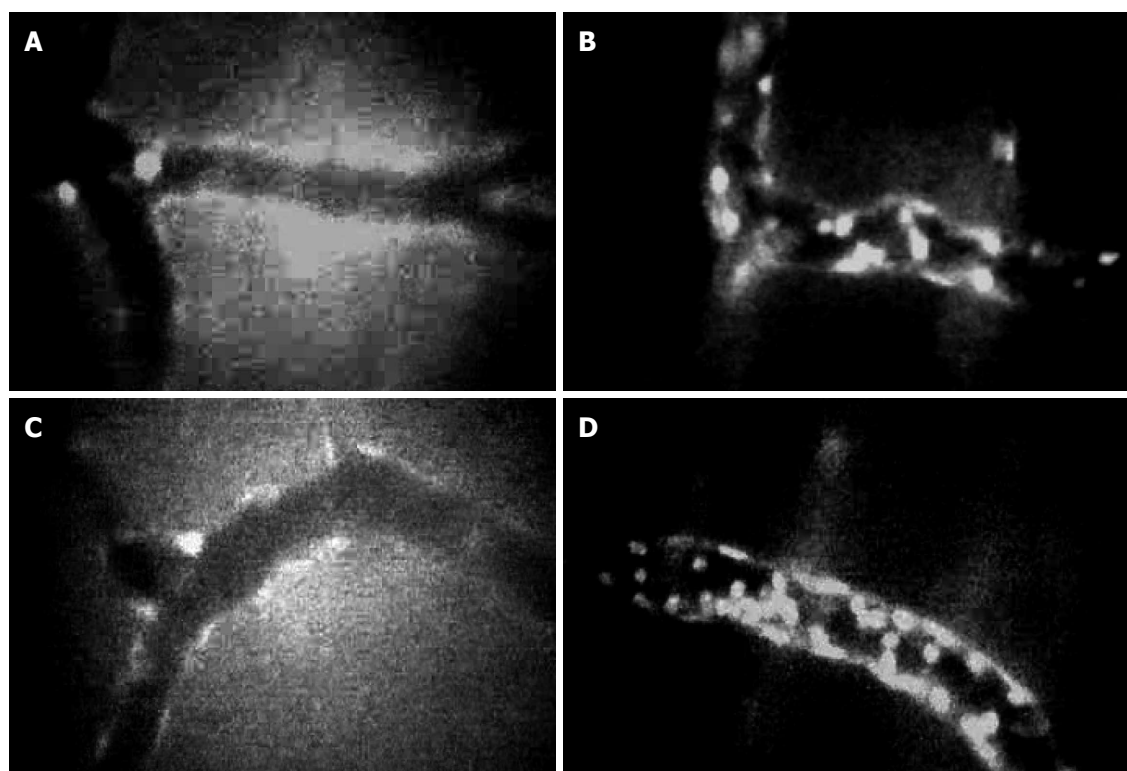


Figure 4 Intravital microscopy demonstrated leukocyte adhesion in control group (A and C) and *Helicobacter pylori*-infected group (B and D) (40 ×).

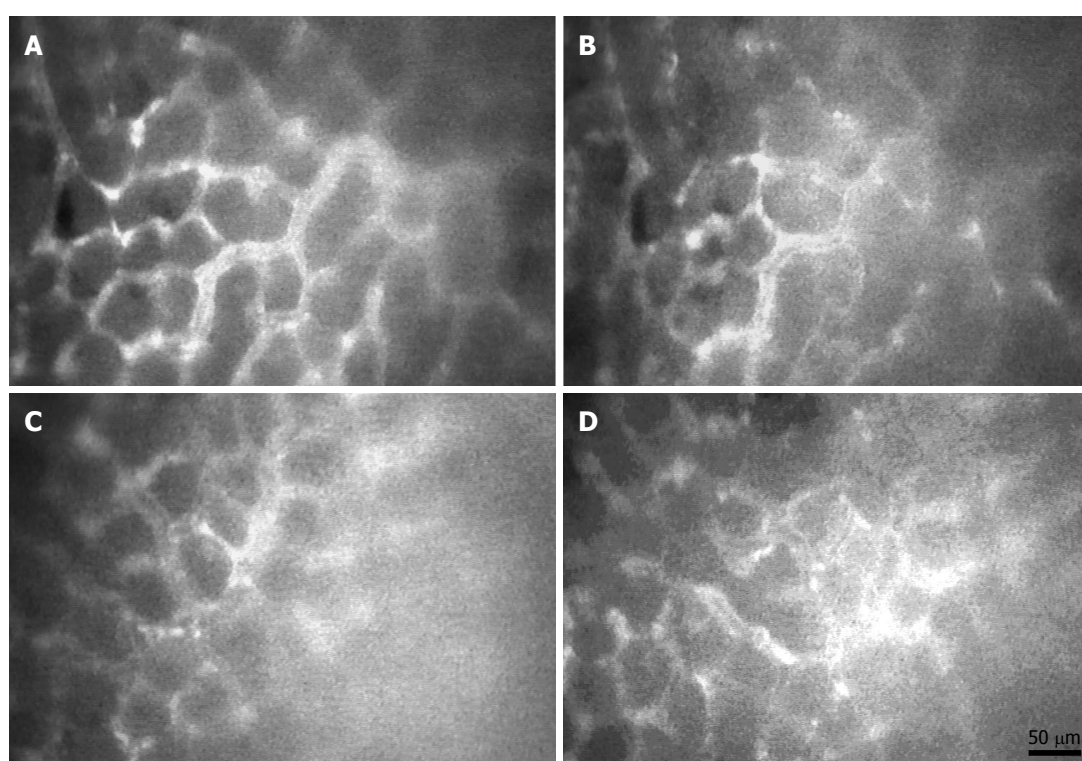


Figure 5 Intravital fluorescent microscopic images (20 ×) demonstrate macromolecular leakage from vessels to the interstitial fluid at 0 and 30 min after injection of control group (A and B) and *Helicobacter pylori*-infected group (C and D). FITC-dx-250 injection (0 min) (A and C); same area at 30 min after FITC-dx-250 injection (B and D).

istration of a nontoxigenic strain to normal rat stomach was unsuccessful at inducing chronic inflammation, and

only resulted in low-level colonization^[3]. Gastric infection with *H. pylori* expressing *cagA*- and *vacA*-encoded cyto-

toxins delayed healing of ischemia/reperfusion-induced acute gastric lesions due to impairment of gastric micro-circulation^[17]. To conclude, after 2 wk inoculation, *H. pylori* successfully colonized Sprague-Dawley rats, with development of mild to moderate gastric inflammation^[18].

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Endoscopic therapy for early gastric cancer: Standard techniques and recent advances in ESD

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to prevent procedural complications. To overcome this disadvantage of ESD, there have been various advances in the knives and other accessories used for this procedure.

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Abstract

The technique of endoscopic submucosal dissection (ESD) is now a well-known endoscopic therapy for early gastric cancer. ESD was introduced to resect large specimens of early gastric cancer in a single piece. ESD can provide precision of histologic diagnosis and can also reduce the recurrence rate. However, the drawback of ESD is its technical difficulty, and, consequently, it is associated with a high rate of complications, the need for advanced endoscopic techniques, and a lengthy procedure time. Various advances in the devices and techniques used for ESD have contributed to overcoming these drawbacks.

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Key words: Endoscopic submucosal dissection; Early gastric cancer; Standard techniques; Recent advances; Complications; Indications

Core tip: Endoscopic submucosal dissection (ESD) enables en bloc resection of early gastric cancer, facilitating detailed histopathological evaluation. ESD needs to be performed by highly skilled endoscopists in order

INTRODUCTION

Endoscopic submucosal dissection (ESD) enables en bloc resection of early gastric cancer, facilitating detailed histopathological evaluation^[1]. ESD needs to be performed by highly skilled endoscopists in order to prevent procedural complications^[2,3]. To overcome this disadvantage of ESD, there have been various advances in the knives and other accessories used for this procedure. I previously reported a review of endoscopic therapy for early gastric cancer in 2009^[4]. The current review of ESD will provide an overview of standard techniques, devices and recent advances in the procedure since 2010.

STANDARD ESD

Standard ESD requires special cutting knives, such as a needle knife (KD-1L-1, Olympus, Tokyo, Japan)^[5], an insulation-tipped electrosurgical (IT) knife (KD-610L/KD-611L, Olympus)^[1,2,6-9], a hook knife (KD-620LR/KD-620QR, Olympus)^[10,11], a flex knife (KD-630L, Olympus)^[12], a dual knife (KD-650L/KD-650Q, Olympus), a flush knife (DK2618JB/DK2618JN, Fujifilm, Saitama, Japan)^[13], a triangle-tip (IT) knife (KD-640L, Olympus)^[14], a mucosectome (DP-D2518/DP-D2622, Pentax, Tokyo,

Japan)^[15], a grasping type scissor forceps (DP2618DT, Fujifilm)^[16,17], SB knife (MD-47706/MD-47704 and MD-47703, Sumitomo Bakelite, Akita, Japan)^[18], a Fork knife (Kachu Technology, Seoul, South Korea)^[19] and a Cap knife (Create Medic, Yokohama, Japan)^[20].

Standard ESD is performed with a standard, single accessory-channel endoscope. Typical procedural steps include marking, incision and submucosal dissection with simultaneous hemostasis. After making several marking dots outside the lesion, various submucosal solutions, including a normal saline and epinephrine mixture, glycerol mixture and hyaluronic acid, are injected. A circumferential incision into the mucosa is made using one of the special cutting knives. Direct dissection of the submucosal layer is carried out with one of the specified knives until complete removal is achieved. During ESD, we perform endoscopic hemostasis either with the knife itself or with a hemostatic forceps whenever active bleeding is noticed. After the ESD, we perform preventive endoscopic hemostasis for any oozing or exposed vessel. High-frequency generators (Erbotom ICC 200 or VIO 300D; ERBE, Tuebingen, Germany) are used for marking, incision of the gastric mucosa, gastric submucosal dissection and endoscopic hemostasis.

INDICATIONS FOR ESD

Absence of lymph node metastasis in the stomach is considered a prerequisite for ESD for early gastric cancer. A large series of patients with well-differentiated intestinal mucosal cancer without ulcers and no size limit, with ulcers less than 30 mm in size, patients with submucosal cancer limited to sm1 infiltration (< 500 µm deep in the submucosa, starting from the muscularis mucosae) that was less than 30 mm in diameter, and patients with undifferentiated cancer without ulcers or with ulcer size less than 20 mm were found to satisfy this criterion^[21]. Therefore, ESD is indicated in such patients.

COMMERCIAL CUTTING KNIVES

IT knife

The IT knife consists of a small ceramic ball attached to the tip of a high-frequency needle knife. The ceramic ball functions as an insulator for the tip of the needle knife, so that incision and dissection of the mucosa and submucosa can be performed safely. The insulator helps to prevent perforation due to accidental cutting of the muscularis propria. A specialized feature of the IT knife is that the portion between the insulator tip and the sheath is used for incision, sweeping off the tissue with the blade portion of the knife instead of the tip. This feature makes a pull-cut, which limits the direction of the incision; however, a straightforward incision is difficult while looking directly at the incision line or submucosa (Figure 1A).

The IT knife 2 is an improved version of the IT knife, with a small metallic plate mounted inside the ceramic tip, facilitating procedures in the traverse direction.

Hook knife

The top of the hook-type knife is right-angled and is 1 mm in size. Compared to the use of a needle knife, use of this knife is associated with greater safety because the submucosal tissue is hooked and pulled before the incision. This knife has a rotating function so that the operator can select the optimal direction of the hook (Figure 1B).

Flex knife

The point of the flex knife is rounded and has a twisted wire that serves as a snare. The sheath is soft and flexible. This knife is less likely to cause perforation when it reaches the muscular layer, as its tip is rounded, and the entire knife is soft and flexible. As the tip of the sheath is thick and functions as a stopper, operators can control the depth of the incision very easily (Figure 1C).

Dual knife

This knife has a small ball-like process on the top, which prevents it from slipping (unlike the Needle Knife). When the tip is retracted, only 0.3 mm of the ball-like process protrudes, increasing the ease of creating markings on the lesion. The thicker end of the sheath prevents inadvertent tissue perforation (Figure 1D).

Flush knife (Water jet short needle knife)

The Flush knife is a characteristic knife with a needle 0.4 mm in diameter, and five projecting parts 1, 1.5, 2, 2.5 and 3 mm in length. The knife clamp at the tip of the sheath is made of ceramic for heat protection. The outer sheath is 2.6 mm in diameter, and water emission is possible through the lumen of the sheath by connecting a water pump to it. The water jet is swiftly activated by pressing the foot pedal of the conduction pump. The conductor of the sheath lumen is insulated in order to prevent electric current dispersion (Figure 1E).

TT knife

The TT knife has evolved along with the process of ESD, which began with the IT knife. The triangular tip of the knife can be used for either cutting or coagulating, and has been designed to operate in any direction (Figure 1F).

Mucosectome

The mucosectome is composed of a flexible plastic shaft and cutting wire (5 mm long in the standard device and 2.5 mm long in the Mucosectome 2). By operation of the handle, the top of this device turns freely, which enables the cutting wire to face in the proper direction. The plastic shaft moves the muscular layer aside, and the cutting wire moves the mucosal layer away from the submucosa during ESD, so that the procedure itself can be performed safely (Figure 1G).

Grasping type scissor forceps

Each step of ESD (circumferential incision, submucosal excision, hemostatic treatment) can be achieved by the following three operations: (1) grasping the target

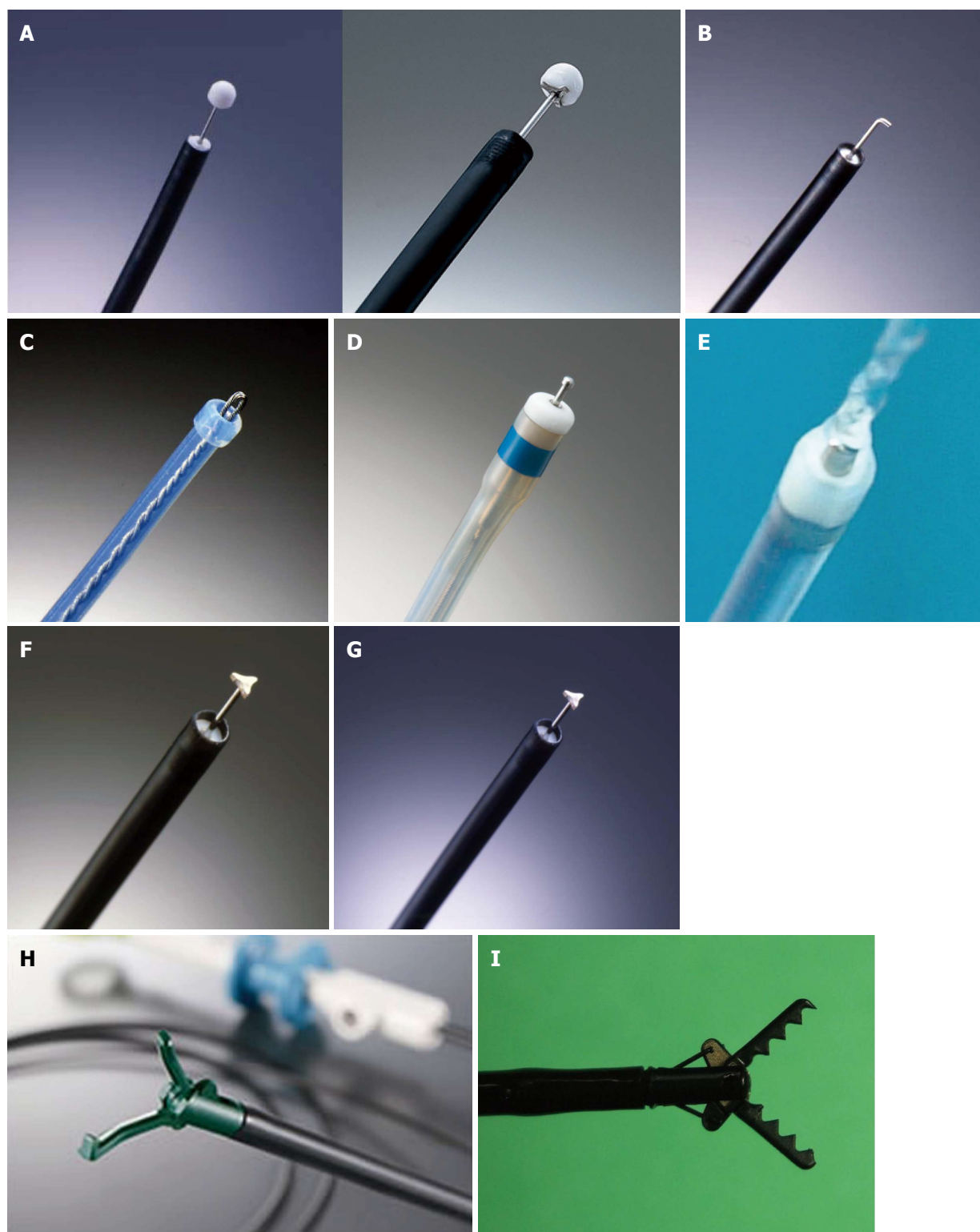


Figure 1 Insulation-tipped knife 1 and 2 (A), Hook knife (B), Flex knife (C), Dual knife (D), Flush knife (E), TT knife (F), Mucosectome (G), Grasping type scissor forceps (H), SB knife (I) and Cap knife (J).

tissue (fixation); (2) lifting up the grasped tissue (separation of the grasped tissue from the underlying muscle layer); and (3) cutting the grasped tissue (or coagulating the blood vessel) using an electrosurgical current. These operations are simple and as easy as the bite biopsy technique (Figure 1H).

SB knife and SB knife Jr.

These forceps have a claw and curved scissors to prevent unnecessary injury to the normal muscle layer. The SB knife line includes the following types of knives: standard type (7 mm knife), short type (6 mm knife) and thin type (SB knife Jr.; 3.5 mm knife). The use of specially

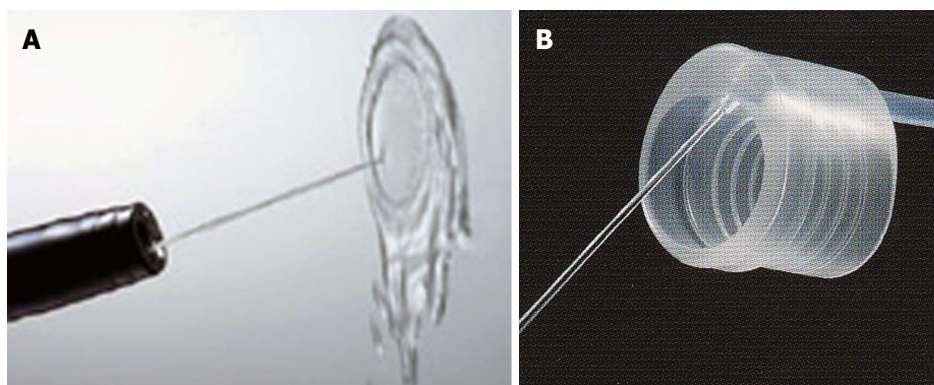


Figure 2 Water jet endoscope (A) and Irrigation hood (B).



Figure 3 Small-caliber-tip transparent hood.

designed transparent hoods (SB hoods) is recommended when these knives are used^[18] (Figure 1I).

Cap knife

The author developed a novel partial (one-third) transparent hood that facilitates endoscopic hemostatic procedures while simultaneously allowing irrigation at the site of bleeding^[22]. The one-third partial hood is easily placed on the tip of the endoscope, although the hood has to be fitted to the right side of the endoscope. The hood-knife was fabricated by drilling an extra side hole, in addition to the hole of the irrigation tube at the cap portion of the transparent end hood^[23]. A snare forceps was glued to the exterior surface over the hole and was attached using short tubes at the inside of the cap. Based on this prototype, the irrigation cap-knife [the cap-knife attachment (Type KUME) with a fixed snare] was developed^[20] (Figure 1J).

ESD procedures using the hood-knife are performed as follows. After the tumor is separated from the surrounding normal mucosa by complete incision around the lesion using the IT knife, the endoscope is removed and the hood-knife is placed on the tip and fixed with tape. A grasping forceps is passed through the accessory channel and the lesion is pushed away from the muscle layer. In submucosal exfoliation, the hood-knife is only slid by the coagulation current on the muscle layer.

Fork knife

The Fork knife has two interchangeable knives, a fixed flexible snare and a forked knife, which form a single working unit, and an inlet for material injection or saline irrigation during the procedure. The knives can be changed during the procedure by using two switches, the fork knob and core knob, located on the center of the body.

WATER JET

Water jet endoscope

By washing the surgical field with a water jet, the source of bleeding can be immediately identified and coagulated, although it can be difficult to identify the bleeding source in a small number of cases with erupting venous bleeding (Figure 2A).

Irrigation hood

The author developed an end hood that facilitates endoscopic hemostatic procedures while simultaneously allowing irrigation of the hemorrhage site. The end hood piece was fabricated by drilling a side hole in the cap portion of a conventional transparent hood, and the irrigation tube was glued to the exterior surface of the hole^[22,24]. The fabricated transparent hood was placed at the tip of the endoscope. Based on this prototype, the irrigation hood [the irrigation cap (Type KUME)] was developed (Create Medic) (Figure 2B).

Transparent hood

A transparent hood facilitates better visualization of the operating field. In particular, good visualization of the submucosal tissue with the aid of a small-caliber-tip transparent hood makes the cutting procedures easy and safe^[25] (Figures 2C and 3).

THERAPEUTIC ENDOSCOPE

Multibending scope

There are certain tumor locations where it is difficult to perform EMR using the conventional scope, such as the



Figure 4 EndoLifter.

lesser curvature or posterior wall of the gastric body, and the cardia. To facilitate EMR of tumors at these locations, a two-channel scope with two independently curving segments, that is, a multibending scope (the “M-scope”), was developed^[26]. The M-scope consists of a distal flexible segment that can bend in any of the four major directions, and a proximal flexible segment that can bend in two directions. Combined operation of the segments allows the operator to obtain a variety of visual fields, to randomly approach or recede from the lesions, and to obtain an en face view.

Multibending double-channel therapeutic endoscope

The multibending double-channel therapeutic endoscope (the “R-scope”) was designed for lifting lesions and for improved dissection by the incorporation of two movable channels^[27,28]. The R-scope has two movable instrument channels: one moves vertically and the other swings horizontally. During the operation, the two instruments can be manipulated with a knob and lever that surround the angulation control knobs of the R-scope.

TRACTION METHODS

Magnetic anchor system

The magnetic anchor (Pentax) consists of three parts: a hand-made magnetic weight made of magnetic stainless steel, microforceps, and a connecting thread. The weight is designed to facilitate gastric ESD by use of an extracorporeal hands-free electromagnet, whereby magnetic forces allow suitable countertraction for submucosal dissection^[29].

Percutaneous traction

A small snare is introduced into the gastric lumen through a percutaneous gastric port (2-mm diameter), to grasp and pull the lesion away from the muscularis propria, thus facilitating resection^[30].

External grasping forceps

In ESD using an external grasping forceps, oral traction applied with the external forceps can elevate the lesion and make the submucosal layer on the aboral side wider and more visible, thereby facilitating submucosal dissection under direct vision^[31].

EndoLifter

In ESD using an external grasping forceps through the EndoLifter (LA-201, 202, Olympus), traction applied with the external forceps can elevate the lesion and make the submucosal layer wider and more visible, thereby facilitating submucosal dissection under direct vision (Figure 4).

INJECTION SOLUTIONS

There are two types of solutions for submucosal injection: an isotonic solution (normal saline, hyaluronic acid) and a hypertonic solution (hypertonic saline, glucose, Glyceol[®])^[25,32-35]. The advantage of hypertonic solutions is better mucosal elevation and hemostatic effect than normal saline. However, a hypertonic solution is more likely to damage tissue in the resected sample, post-resection ulcer or the surrounding mucosa, compared with an isotonic solution.

Hyaluronic acid solution (MucoUp, Johnson and Johnson, Tokyo, Japan) makes a better, long-lasting submucosal cushion without tissue damage, as compared to other available solutions^[25,32].

RECENT ADVANCES AFTER 2010

New devices and methods

Water jet-assisted knife: The water jet-assisted knife (ERBEJET, Erbe Elektromedizin GmbH, Tuebingen, Germany) with an outer diameter of 2.1 mm allows injection or hydrodissection without a needle, with a preselected effect setting through a standard working channel of the endoscope. Without switching instruments, the operator can alternately use the tool for marking the targeted lesion, circumferential cutting, dissection, and coagulation by radiofrequency application^[36].

Thulium laser: The thulium 2- μ m wavelength laser system (RevoLix; LISA Laser Products OHG, Katlenburg-Lindau, Germany) is used during ESD procedures for marking, mucosal incision and submucosal dissection. The original device, which consists of a 550- μ m flexible silica with metallic attachment, is inserted through the working channel of the endoscope instead of endoscopy knives^[37].

Master and Slave Transluminal Endoscopic Robot:

The Master and Slave Transluminal Endoscopic Robot is deployed through a two-channel therapeutic endoscope. By remote operation of the two-armed master interface, the endoscopist can intuitively control the slave endoscopic tools at the distal end of the endoscope^[38].

Double-endoscope: The use of two endoscopes for ESD provides a good field of vision and allows countertraction to be applied to the lesion, clearly facilitating submucosal dissection. Submucosal dissection is performed with the main scope. Countertraction is applied to the lesion with the use of the grasping forceps, introduced

through the forceps channel of the sub-scope^[39].

Yo-yo technique: The yo-yo technique is a traction method. The endoscopic snare is mobilized through the patient's nose independently of the endoscope. The partially resected specimen can be pushed or pulled according to the snare movements, exposing the dissection plane and the distal luminal area, respectively, and facilitating submucosal dissection under direct vision^[40].

Carbon dioxide insufflation: Compared with air insufflation, CO₂ insufflation during ESD reduces the volume of residual gas in the digestive tract (air *vs* CO₂ = 1047 mL *vs* 643 mL, *P* < 0.001), but not the 100-mm visual analog scale score for abdominal pain and distension after the procedure^[41].

Local steroid injection: Local steroid injection (triamcinolone acetonide 50 mg/5 mL) into the floor of a post-ESD artificial ulcer promotes the formation of granulation tissue at an early stage of the healing process, leading to regeneration of gastric mucosa without mucosal convergence or gastric deformity^[42].

Complications and risk factors

Antithrombotic drugs: Continuous aspirin use increases the risk of bleeding after ESD. It is reported that post-ESD bleeding occurred in 4.1% (21/514) of patients, and was more frequent in continuous aspirin users (4/19, 21.1%) than in those who never used aspirin (15/439, 3.4%, *P* = 0.006) and those with interrupted aspirin use (2/56, 3.6%, *P* = 0.033)^[43]. Thus, aspirin use should be discontinued in patients with a low risk for thromboembolic disease who undergo ESD, to minimize bleeding complications.

Interruption of antithrombotic drug therapy may be adequate for preventing early post-ESD bleeding; however, reinitiating antithrombotic drug therapy is a significant independent risk factor for delayed post-ESD bleeding^[44].

Deep vein thrombosis: ESD procedures carry a moderate risk for venous thromboembolism. D-dimer measurements were higher in patients with DVT than in patients without deep vein thrombosis (DVT). Kusunoki *et al*^[45] reported that according to receiver operating characteristic curve analysis, the resulting cut-off value of the D-dimer level on the day after ESD was 1.9-μg/mL for ESD patients, with superior association to pre-ESD or immediate post-ESD levels as compared to. High D-dimer levels the day after ESD and the presence of comorbidities are associated with DVT development.

Second-look endoscopy after ESD: A second-look endoscopy after ESD may contribute little to the prevention of delayed bleeding^[46].

and highlighted the progresses in ESD. It should be emphasized that the performance of ESD, which involves invasive endoscopic procedures, requires a high level of technical skill and sufficient knowledge.

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CONCLUSION

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Epigenetics: An emerging player in gastric cancer

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Abstract

Cancers, like other diseases, arise from gene mutations and/or altered gene expression, which eventually cause dysregulation of numerous proteins and noncoding RNAs. Changes in gene expression, *i.e.*, upregulation of oncogenes and/or downregulation of tumor suppressor genes, can be generated not only by genetic and environmental factors but also by epigenetic factors, which are inheritable but nongenetic modifications of cellular chromosome components. Identification of the factors that contribute to individual cancers is a prerequisite to a full understanding of cancer mechanisms and the development of customized cancer therapies. The search for genetic and environmental factors has a long history in cancer research, but epigenetic factors only recently began to be associated with cancer formation, progression, and metastasis. Epigenetic alterations of chromatin include DNA methylation and histone modifications, which can affect gene-expression profiles. Recent studies have revealed diverse mechanisms by which chromatin modifiers, including writers, erasers and readers of the aforementioned modifications, contribute to the formation and progression of cancer. Furthermore,

functional RNAs, such as microRNAs and long noncoding RNAs, have also been identified as key players in these processes. This review highlights recent findings concerning the epigenetic alterations associated with cancers, especially gastric cancer.

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Key words: Gastric cancer; Epigenetics; DNA methylation; Histone modification; Gene expression

Core tip: The pathogenesis of gastric, or stomach cancer has long been a topic of extensive research, and these research efforts have resulted in tremendous improvements in the diagnosis and treatment of gastric cancer patients. However, research on gastric cancer has been focused on the genetic and environmental determinants of its formation and progression while the role of regulators, another important set of contributors to gastric cancer, has just begun to be elucidated. In this review, we highlight our current understanding of the epigenetic mechanisms by which gastric cancer arises and progresses and discuss future research directions.

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INTRODUCTION

Gastric cancer is the fourth most frequently occurring cancer worldwide and the second leading cause of cancer-related death^[1]. The occurrence of gastric cancer varies with geographic area, with the highest incidence rate of gastric cancer in East Asia, especially South Korea, Mongolia, Japan and China. Although the mortality rate

has dramatically decreased due to improvements in endoscopy and surgical technology, the survival rate is still less than 15% once gastric cancer metastasizes.

Gastric cancer can arise from precursor lesions or *de novo* and is commonly categorized into two main subtypes, diffuse-type gastric cancer and intestinal-type gastric cancer. Research in the past decades has provided us with insights into the molecular mechanisms that drive gastric cancer tumorigenesis and progression. As in other types of cancer, genetic, epigenetic and environmental factors in combination contribute to gastric cancer tumorigenesis and progression. Previous research has mainly focused on genetic factors such as the inheritance of gastric cancer susceptibility genes and environmental factors including *Helicobacter pylori* infection, salt consumption, stress, smoking *etc.* In recent years, however, the epigenetic mechanisms governing gastric cancer have been at the center of gastric cancer research.

EPIGENETIC REGULATION OF GENE EXPRESSION

In multi-cellular organisms, different gene expression patterns determine the fates of cells, causing them to differentiate into various cell types. Therefore, it is critical to precisely coordinate the gene expression pattern based on cell types during developmental processes. Furthermore, gene expression patterns need to be maintained throughout the life span once established. Each cell appears to “memorize” the genetic information to be expressed and precisely passes this memory on to its daughter cells after cell division. This process is referred to as “epigenetic cellular memory”. Dysregulation of epigenetic memory causes developmental defects, cancers and neurodegenerative diseases.

In the nucleus, DNA is packaged into a higher order structure called chromatin, the physiological template for transcription. Alteration of chromatin structure *via* the various modifications described below is the major factor that controls gene expression in a temporal and spatial manner, resulting in the establishment and maintenance of epigenetic cellular memory.

Regulation by DNA methylation

Chromatin structure is modified and altered in several layers. First of all, DNA itself is methylated, and this event mostly occurs at cytosines in CpG-rich regions. DNA methylation at promoter regions generally occludes the binding of transcription factors or recruits methyl-DNA-binding proteins, leading to the inactivation of gene expression, with few exceptions in which DNA methylation can be involved in preventing gene repression^[2]. DNA methylation is a stable epigenetic mark that is inherited by offspring or daughter cells once established.

Two different classes of DNA methyltransferases (DNMTs) are responsible for establishing and maintaining DNA methylation. DNMT1 maintains DNA methylation

through its substrate preference for hemimethylated DNA at CpG regions. DNMT3 family members, DNMT3A, DNMT3B and DNMT3L, are involved in establishing *de novo* DNA methylation patterns, although DNMT3L is catalytically inactive and might cause gene repression independent of DNA methylation^[3].

DNA methylation was once believed to be a permanent epigenetic mark. So far, no enzyme has been discovered that directly removes the methyl group from methylcytosine. However, TET family proteins were identified to oxidize 5-methylcytosine to 5-hydroxymethylcytosine, eventually leading to the removal of the methyl group from methylcytosine^[4]. TET family proteins are involved in regulating transcription during embryonic development. The tight regulation of writing and erasing methyl marks on DNA is required for proper gene expression, and the imbalance between writing and erasing is implicated in various cancers.

Regulation by histone modifications

The nucleosome, which is composed of 146 bp of DNA and a histone octamer (dimers of H2A, H2B, H3 and H4) is the fundamental repeating unit of chromatin structure and a major target of chromatin regulation^[5]. Histone proteins have long flexible N-terminal tails that are subject to several covalent modifications including acetylation, methylation, phosphorylation, ubiquitylation, ADP-ribosylation, crotonylation and glutarylation^[6].

The combinations of different types and locations of histone modifications, also known as histone codes, are the main determinants of gene repression or activation. Covalent modifications are regulated by a trio of writers, erasers and readers. Writers and erasers add and remove covalent modifications, respectively, while readers recognize specific modifications with specialized domains, resulting in the recruitment of transcriptional machinery or transcription-repression complexes. More detailed descriptions are given in the sections below.

Regulation by histone lysine acetylation and deacetylation

The first covalent modification identified was the acetylation of lysine (Lys or K) residues of histones by histone acetyltransferases (HATs), more specifically called histone lysine acetyltransferases (KATs). Many Lys residues of histones are involved in interacting with DNA, and this acetylation neutralizes the positive charge of Lys, leading to the weakening of the DNA-histone interaction and subsequent activation of transcription^[7]. In addition, acetyllysine recruits other chromatin modifiers containing a bromodomain that recognizes an acetyllysine to activate transcription^[8].

Histone deacetylases (HDACs), the erasers of acetylation, have been shown to be directly involved in cancer pathogenesis *via* transcriptional repression of tumor suppressor genes^[9]. Some HDAC inhibitors are currently in use to treat certain types of cancer or in clinical trials^[9].

Regulation by histone lysine methylation and demethylation

Several Lys residues of histones can also be mono-, di- or trimethylated. The different locations and levels of histone methylation add another layer of complexity to covalent modification of histones. Among histone lysine methylations, those of histone H3 Lys4 (denoted as H3K4) and histone H3 Lys27 (H3K27) are particularly interesting because H3K4 and H3K27 methylations are directly implicated in transcriptional activation and repression, respectively^[10].

Methylation of H3K4 and H3K27 is catalyzed by multi-subunit protein complexes. For example, KMT2A (K-specific methyltransferase 2A, commonly called MLL), which methylates H3K4 using its SET domain, is complexed with WDR5, RBBP5 and ASH2L^[11]. H3K27 is methylated by PRC2 (polycomb repressive complex 2) composed of EED, EZH2, SUZ12 and RBBP4^[12].

It is not clearly understood how H3K4 or H3K27 methylation regulates transcriptional activation or repression, respectively. However, it has been shown that H3K4 methylation recruits the BAF chromatin remodeling complex *via* its chromodomain to activate transcription^[13]. In regard to H3K27 methylation, another polycomb repressive complex, PRC1, recognizes trimethylated H3K27 (denoted as H3K27me3) *via* the chromodomain-containing protein CBX1 (chromobox homolog 1) and induces the compaction of chromatin, resulting in transcriptional repression, although the requirement of H3K27me3 for PRC1 function is controversial^[14-16].

Because histone methylation status is critical for gene expression, the removal of histone methylation is highly regulated by several histone Lys-specific demethylases (KDMs). H3K4 is demethylated by KDM1 (commonly known as LSD1) and KDM5B (JARID1), whereas H3K27 is demethylated by KDM6A (UTX) and KDM6B (JMJD3)^[10]. Because the balance between methylation and demethylation of histones is critical for coordinating gene expression, the disruption of this balance is found in many cancers.

Regulation by histone arginine methylation

Arginine (Arg or R) residues in histones are also targets for methylation. Arg methylation affects gene expression by activating or repressing transcription depending on the methylated sites^[17]. Arg can be monomethylated, symmetrically dimethylated, or asymmetrically dimethylated, although the different biological consequences of symmetric *vs* asymmetric Arg dimethylation are unclear.

The methylation of Arg functions in at least two different ways. The methylation of Arg near a Lys in histones can block the Lys methylation^[18]. Specifically, methylation of histone H3 Arg2 (denoted as H3R2) represses transcription by blocking H3K4 methylation, which is critical for transcriptional activation^[19]. In addition, methylarginine may serve as a site-specific docking stage for methylarginine-binding proteins, which recruit other transcriptional regulators^[20].

Regulation by other histone modifications and nucleosome variants

Other covalent modifications such as ubiquitylation, crotonylation and glutarylation are also involved in regulating gene expression. However, the downstream pathways of these modifications are not well studied.

A canonical nucleosome is composed of histones H3, H4, H2A and H2B. There are also several histone variants such as H3.3, H2A.Z, CENP-A and macroH2A, which are incorporated into nucleosomes with other histones to execute their specific functions. For example, histone H3.3 is not only found in transcriptionally active genes but is also involved in recruiting PRC2. H2A.Z functions in the cell cycle, and the improper incorporation of H2A.Z is implicated in various cancers^[21].

Regulation by chromatin remodeling

The immediate consequence of forming a nucleosome is to limit DNA accessibility by protein factors. Therefore, cells have developed an elaborate system to remodel nucleosomes using ATP as an energy source. ATP-dependent chromatin remodeling complexes are classified into five families depending on the type of ATPase subunit of the complexes and are known as the SWI/SNF, ISWI, CHD, INO80 and SWR1 families.

Each ATP-dependent chromatin remodeling family is believed to remodel nucleosomes *via* a distinct mechanism and is involved in a distinct biological pathway, such as gene repression, gene activation, histone exchange and the DNA-damage response^[22]. Due to the importance of the roles of ATP-dependent chromatin remodelers in various physiological processes, mutations and over-expression of the remodelers are often found in several cancers.

Regulation by long noncoding RNAs

The most interesting but least studied epigenetic regulator is long noncoding RNAs (lncRNAs), which are defined as transcripts that are generally longer than 200 nucleotides and do not code for proteins. Most lncRNAs are synthesized by RNA polymerase II, capped at the 5' end and polyadenylated at the 3' end^[23]. Although the role of lncRNAs in chromatin regulation was first identified in X-chromosome inactivation several decades ago, the significance of lncRNAs in chromatin regulation was not fully recognized until the recent discovery that many lncRNAs interact with chromatin modifiers and directly control gene expression^[24].

Although exact mechanisms of lncRNAs are not well understood, it is believed that lncRNAs function through their binding partners in several different ways^[25]. They function as a scaffold for multi-subunit protein complex formation or recruit chromatin modifying complexes to a specific locus leading to transcriptional activation or repression. For example, HOTAIR (HOX antisense intergenic RNA) transcribed from a *HOXC* locus interacts with PRC2 and LSD1 *via* its 5' and 3' ends, respectively, to repress gene expression.

CANCER AND EPIGENETICS

Numerous studies have unraveled complex networks of epigenetic regulation in several types of cancer. In this section, we will highlight some of the epigenetic mechanisms that contribute to tumorigenesis and tumor progression, mainly focusing on DNA methylation, histone acetylation, histone methylation and lncRNAs.

DNA methylation in cancer

In general, cancer cells exhibit hypermethylation of the CpG islands of some genes, including tumor suppressor genes, *BRCA1* (breast cancer 1, early onset), *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *MLH1* (mutL homolog 1) and *VHL* (von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase)^[26-28]. In contrast, cancer cells exhibit global hypomethylation at many genomic sequences, which can result in chromosomal instability as well as activation of proto-oncogenes^[29-31].

DNA methyltransferase genes have been shown to be mutated in certain cancers. For example, *DNMT3A* gene is mutated in acute myelogenous leukemia, myeloproliferative disease and myelodysplastic syndrome^[32]. In addition, the recently identified TET1 (tet methylcytosine dioxygenase 1) and TET2 (tet methylcytosine dioxygenase 2) proteins in the Tet (ten-eleven translocation) family of DNA hydroxylases are involved in DNA demethylation and found to be mutated in acute myelogenous leukemia, myeloproliferative disease, myelodysplastic syndrome and chronic myelomonocytic leukemia. Furthermore, *TET1* gene is also fused with the histone methyltransferase *MLL* (myeloid/lymphoid or mixed-lineage leukemia) gene in some cases of acute myelogenous leukemia^[33,34].

Histone modifications in cancer

As described above, histone modification status is finely regulated by modification writers and erasers. Disruption of this balance can cause aberrant histone modifications, resulting in dysregulation of gene expression. In cancer cells, one of the best-established changes in histone modifications is a global decrease in the acetylation of H4K16 and trimethylation of H4K20^[35]. Recent findings regarding the roles of histone modifications in various types of cancer are summarized in the sections below.

Histone acetylation modifiers in cancer

In cancer cells, mutations or changes in the expression of HATs and HDACs are frequently observed. For example, some genes that encode HATs such as CREBBP (alternatively called CBP), EP300 (p300), KAT6A (MOZ), KAT6B (MORF) and MOXD1 (MOX) have been shown to be mutated, translocated, or overexpressed in solid and hematological tumors^[35-38]. In addition, altered expression of HDACs has been observed in a variety of cancers, while somatic mutations are rarely found. Moreover, the recruitment of HDACs to certain target genes *via* chimeric fusion proteins, which can occur in leukemia, has been shown to be another mechanism of gene repression^[39].

In addition to HATs and HDACs, proteins called acetylation readers, which contain a bromodomain that recognizes acetylated histones and recruits other complexes, have been reported to undergo mutations or translocations in certain tumor types, suggesting that modification readers also contribute to tumorigenesis.

Histone lysine methylation writers in cancer

Histone methylation can take place at lysine, histidine or arginine residues. However, we will mainly discuss lysine and arginine methylation in this section. With the discovery of histone lysine demethylases (KDMs), the contribution of aberrant histone methylation status to tumorigenesis and cancer progression has received renewed attention in the field of cancer epigenetics in recent years.

Alteration of histone methylation status can be a consequence of translocation, amplification, deletion, overexpression or repression of histone methyltransferase or demethylase genes. The best-studied methyltransferase that undergoes chromosomal translocation is KMT2A (commonly known as MLL). This H3K4 methyltransferase is often fused with another protein, such as AFF1 (AF4/FMR2 family, member 1), ELL (elongation factor RNA polymerase II, alternatively called ELL1), MLLT1 (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 1, or ENL) or MLLT3 (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 3, or AF9)^[36,39,40]. MLL-fusion proteins can cause aberrant H3K4 methylation of target genes including *HOXA7* (homeobox A7) and *HOXA9* (homeobox A9)^[41]. Intriguingly, several MLL-fusion proteins have been reported to recruit other histone methyltransferase such as DOT1L (DOT1-like histone H3K79 methyltransferase) in leukemia^[42]. Elevated expression of another H3K4 methyltransferase, SMYD, was found in breast cancer.

In contrast, overexpression of another H3K27 methyltransferase gene, *EZH2*, has been observed in a wide variety of solid tumors and exhibits a strong association with tumor stage and aggressiveness. An inactivating mutation of *EZH2* has also been found in lymphoid, myeloid and T-cell acute lymphoblastic leukemia (T-ALL)^[43]. Specifically, NOTCH1 antagonizes PRC2, thus driving the formation of T-ALL. Another histone methyltransferase that undergoes mutation, translocation or repression is the H3K36-specific methyltransferase NSD1^[44,45].

Histone lysine methylation erasers in cancer

In addition to histone methyltransferases, the role of histone demethylases in cancer has been highlighted in recent studies. The activity of histone demethylases can be dysregulated by somatic mutations or changes in their expression in cancer cells. So far, somatic mutations have been found in *KDM5A* (commonly known as *JARID1A*), *KDM5C* (*JARID1C*) and *KDM6A* (*UTX*)^[28,46]. Specifically, mutations in *UTX*, a histone H3K27 demethylase gene, have been found in 12 different types of cancer,

indicating a tumor-suppressive role of UTX in various cancers. This concept was supported by a recent finding showing that UTX controls the cell cycle by targeting the RB1 (Rb) protein network^[47].

In contrast, the role of KDM6B (JMJD3), another H3K27 demethylase, seems to vary depending on the type of cancer. For example, JMJD3 has been shown to function in oncogene-induced senescence, suggesting a tumor-suppressive role of the protein^[48]. However, upregulation of JMJD3 in metastatic prostate cancer indicates a potential role of the protein in the progression of prostate cancer^[48]. In addition, overexpression of the histone H3K4 demethylase gene *KDM1A* (*LSD1*) has been associated with the recurrence of prostate cancer^[49]. Furthermore, LSD1 has been identified as a positive regulator of neuroblastoma and breast tumors^[50,51].

Other enzymes with altered expression in cancer include KDM2B (JHDM1B), KDM4C (JMJD2C), KDM5A (RBP2) and KDM5B (PLU1)^[52-57]. JMJD2C, a member of the JMJD2 H3K9 demethylase family, has been shown to be upregulated in various tumors including breast cancer, prostate cancer, esophageal squamous cell carcinoma and desmoplastic medulloblastoma^[54,55,58-60]. In addition, its potential role as an oncogene has been suggested by a recent study using immortalized mammary epithelial cells^[61].

Histone lysine methylation readers in cancer

Like acetylation readers, methyllysine readers play a pivotal role in cancer. For example, ING (inhibitor of growth) family proteins, which can bind di- and trimethylated H3K4, have been found to be mutated or downregulated by the loss of heterozygosity, supporting their role as tumor suppressors in several types of cancer^[62-64]. Another example of a methylated H3K4 binding protein, NUP98 (nucleoporin 98 kDa), is often fused with several subunits of histone lysine methyltransferases, thereby contributing to hematopoietic cancer^[65].

Histone arginine methylation in cancer

Although histone arginine methylation has not received as much attention as lysine methylation, numerous studies have implicated the function of protein arginine methyltransferases (PRMTs) in cancers. The best-studied PRMT1 has been reported to be overexpressed and/or aberrantly spliced in various types of cancer, including breast, prostate, lung and colon cancers^[66-69]. A recent study demonstrated that H4R3 methylation has a strong positive correlation with tumor stages in prostate cancer^[67].

In addition to PRMT1, other PRMTs, such as PRMT2, PRMT5 and PRMT6, are overexpressed in breast, gastric, colon and lung cancers, while elevated PRMT3 activity without changes in its expression level has been reported^[70-73]. Furthermore, somatic mutations in PRMT8 were found in ovarian and skin cancers. Finally, a non-PRMT family arginine methyltransferase CARM1 (coactivator-associated arginine methyltransferase 1) has been shown to be overexpressed in breast,

prostate and colon cancers^[74-77].

The mechanisms by which the aforementioned PRMTs contribute to tumorigenesis and metastasis have been studied by several groups. For example, PRMT1 and CARM1 are involved in the activation of WNT signaling, a well-known tumor-promoting signaling pathway^[78,79]. In addition, elevated activity of PRMTs *via* the various mechanisms mentioned above can affect cell growth and migration and the tumor microenvironment.

lncRNAs in cancer

It has become clear that lncRNAs have fundamental roles in tumorigenesis and tumor progression. One of the best-studied lncRNAs is HOTAIR. HOTAIR has been shown to be overexpressed in breast and colon cancers and esophageal squamous cell carcinoma and functions *via* altering PRC2 target-gene occupancy^[25,80-83]. In addition, several lncRNAs have been implicated in cancer with oncogenic functions. They include CDKN2B-AS1 (CDKN2B antisense RNA 1, or ANRIL), H19 (imprinted maternally expressed transcript), MALAT1 (metastasis associated lung adenocarcinoma transcript 1), PCAT1 (prostate cancer-associated transcript 1), PCBP2-OT1 (PCBP2 overlapping transcript 1, or TUC338), PCGEM1 (prostate-specific transcript), PRNCR1 (prostate cancer associated non-coding RNA 1) and SPRY4-IT1 (SPRY4 intronic transcript 1). These lncRNAs are often found to be upregulated in several types of cancer and exert their oncogenic effects *via* promoting cell proliferation or inhibiting apoptosis and senescence.

The mechanisms by which some of these lncRNAs execute their oncogenic functions have been uncovered. For instance, CDKN2B-AS1 functions by causing aberrant recruitment of the PRC2 complex to *CDKN2A* (cyclin-dependent kinase inhibitor 2A, or INK4A) or *CDKN2B* (cyclin-dependent kinase inhibitor 2B, or INK4B), thus suppressing their expression^[84,85], whereas PCAT1 inhibits *BRC42* (breast cancer 2, early onset) expression^[86]. In contrast, other lncRNAs, such as GAS5 (growth arrest-specific 5), MEG3 (maternally expressed 3), PTENP1 (phosphatase and tensin homolog pseudogene 1) and LincRNA-p21, have been suggested to have tumor-suppressive effects^[87-92]. GAS5 induces the expression of the proapoptotic protein BIRC3 (baculoviral IAP repeat containing 3, or cIAP2) and has been found to be downregulated in breast cancer^[87,88], while LincRNA-p21 induces apoptosis by affecting the TP53 (p53) pathway^[92].

DNA METHYLATION IN GASTRIC CANCER

As in other types of cancer, numerous studies have shown that key players in gastric cancer are regulated by changes in DNA methylation patterns at their promoter CpG islands, *i.e.*, hyper- or hypomethylation (Table 1). These genes include tumor-suppressor genes, oncogenes, and genes that are involved in tumor progression and

Table 1 Examples of epigenetic alterations found in gastric cancer

| Alteration source | Expression | Alteration target | Ref. |
|------------------------|------------|--|-----------|
| DNA hypermethylation | Down | Signal pathway mediator genes (<i>ADAMTS9</i> , <i>BCL2L10</i> , <i>BCL6B</i> , <i>BNIP3</i> , <i>CXCL12</i> , <i>DAPK</i> , <i>DKK1</i> , <i>DKK3</i> , <i>DLL1</i> , <i>FBLN1</i> , <i>GATA4</i> , <i>HOXD10</i> , <i>LMX1A</i> , <i>OPCML</i> , <i>PCDH10</i> , <i>RELN</i> , <i>SFRP</i> proteins, <i>SOCS1</i> , <i>SOX17</i> , <i>TIMP3</i> , <i>VEZT</i>) | [100-120] |
| | Down | Chromatin-modifying enzyme genes (<i>MGMT</i> , <i>SMARCA5</i>) | [93,140] |
| | Down | MicroRNA genes (<i>Let-7f</i> , <i>MIR10B</i> , <i>MIR34C</i> , <i>MIR137</i> , <i>MIR155</i> , <i>MIR182</i> , <i>MIR195</i> , <i>MIR200B</i> , <i>MIR200C</i> , <i>MIR210</i> , <i>MIR212</i> , <i>MIR338</i> , <i>MIR375</i> , <i>MIR378</i> , <i>MIR429</i> , <i>MIR449</i>) | [127-139] |
| DNA hypomethylation | Up | <i>ALDH2</i> , <i>ASCL2</i> , <i>MTHFR</i> , <i>SULF1</i> , <i>SULF2</i> , <i>TERF2</i> | [122-126] |
| | Up | MicroRNA gene (<i>MIR93</i>) | [83] |
| H3/H4 hyperacetylation | Up | <i>MYC</i> | [107] |
| H3/H4 deacetylation | Down | <i>GATA</i> , <i>RND3</i> | [149,150] |
| H3 dephosphorylation | Down | <i>c-JUN</i> , <i>HSP70</i> | [151] |
| MicroRNA function | Down | Chromatin-modifying enzyme genes (<i>DNMT1</i> , <i>DNMT3A</i> , <i>DNMT3B</i> , <i>UHRF1</i>) | [141,142] |

metastasis. In addition, recent findings demonstrating changes in the DNA methylation patterns of microRNA genes in gastric cancer patient samples have revealed more complexity in the epigenetic regulation of gastric cancer.

DNA hypermethylation in gastric cancer

Hypermethylation of CpG islands results in the silencing of neighboring genes, and promoters of tumor-suppressor genes are often methylated in gastric cancer patient samples. Widely studied genes with methylated promoters include *CDKN2A*, *TP53* (tumor protein p53), *MLH1*, *CDH1* (cadherin 1, or E-cadherin), *RUNX3* (runt-related transcription factor 3), *APC* (adenomatous polyposis coli) and *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1)^[93-99]. In addition, recent studies have identified numerous hypermethylated genes encoding pro-apoptotic or anti-growth proteins (*BCL2L10*, *BCL6B*, *BNIP3*, *DAPK* and *FBLN1*), transcription factors (*GATA4*, *HOXD10*, *LMX1A* and *SOX17*), enzymes (*KL*), cell-cell interaction or migration-related proteins (*ADAMTS9*, *OPCML*, *PCDH10*, *RELN*, *TIMP3* and *VEZT*), DNA-repair proteins (*XRCC1*), signaling molecules (*CXCL12*, *DKK1*, *DKK3*, *DLL1*, *SFRP* proteins and *SOCS1*), an RNA binding-protein (*QKI*) and others (*NDRG2*)^[100-121].

Hypermethylation of the aforementioned genes generally promotes gastric cancer tumorigenesis and/or metastasis *via* several mechanisms. DNA methylation of tumor-suppressor genes endows gastric cells with the ability to overcome oncogene-induced senescence as well as apoptosis. For example, downregulation of *DKK1* (dickkopf WNT signaling pathway inhibitor 1) and *SOCS1* (suppressor of cytokine signaling 1) reactivates the WNT and STAT3 pathways, respectively^[116,119,122].

DNA hypomethylation in gastric cancer

Hypomethylation causes derepression of target genes; several genes involved in tumorigenesis, progression, and metastasis of gastric cancers have been found to be hypomethylated. For example, Kwon *et al.*^[122] demonstrated that the promoter of *ASCL2* (achaete-scute family bHLH transcription factor 2), which encodes a basic helix-loop-

helix transcription factor, shows hypomethylation in gastric cancer samples compared to normal tissues, and high expression levels of this gene are correlated with poor survival of gastric cancer patients. In addition, the promoter of the well-known oncogene *MYC* has been shown to undergo hypomethylation in gastric cancer with lymph node metastasis^[123]. Yashiro *et al.*^[124] showed that demethylation in *TERF2* (telomeric repeat binding protein 2, or TRF2) and *ERAS* (ES cell expressed Ras) promoters causes reactivation of these genes in gastric cancer^[124,125].

A recent study by Balassiano *et al.*^[126] reported that gastric cancer patient samples contain hypomethylated promoters of two cancer-associated genes, *ALDH2* (aldehyde dehydrogenase 2 family) and *MTHFR* (methylene tetrahydrofolate reductase). Furthermore, overexpression of *SULF1* (sulfatase 1) and *SULF2* (sulfatase 1), members of the sulfatase family, caused by promoter hypomethylation has been shown to be an independent prognostic marker for lymph node metastasis. Finally, an interesting study by Yuasa *et al.*^[127] showed an association between hypomethylation of blood leukocyte DNA and the risk of gastric cancer, indicating that changes in the DNA methylation pattern in non-tumor cells in addition to tumor cells themselves can be used as potential prognostic markers in gastric cancer.

MicroRNA promoter methylation in gastric cancer

MicroRNAs (miRNAs) are small noncoding RNAs that can regulate the expression of target genes at the post-transcriptional level. Because a single miRNA can target several messenger RNAs, dysregulation of miRNAs can effectively affect multiple signaling pathways leading to tumor formation and metastasis. As in other types of cancer, recent studies have identified several miRNAs as frequent targets of DNA methylation in gastric cancer (Table 1). For example, the suppression of several miRNA genes, such as *MIR137*, *MIR210*, *MIR375* or *MIR449*, *via* promoter methylation has been shown to prevent apoptosis by alleviating the miRNA-induced inhibition of pro-survival pathways such as MAPK1 (by *MIR137* and *MIR210*) and PDK1 (by *MIR375*) or by inhibiting pro-apoptotic pathways (by *MIR449*)^[128-130].

In some cases, downregulation of miRNAs *via* methylation activates tumor growth-promoting pathways such as CDK6-VEGF (by MIR195 and MIR378), c-MYC (by MIR212 and MIR429), cAMP response element (by MIR182) and MAPRE1 (by MIR10B)^[28,131-133]. Thus, methylation of the aforementioned miRNAs causes overall growth of gastric cancer.

In addition to regulating gastric cancer cell survival and growth, DNA methylation of some miRNAs promotes the ability of gastric cancer cells to invade and migrate, thus increasing their metastatic potential. Examples of these include Let-7f, MIR155 and MIR338, which exert their effects by altering the expression of *MYH9* (myosin, heavy chain 9, non-muscle), *SMAD2* (SMAD family member 2) and *SSX2IP* (synovial sarcoma, X breakpoint 2 interacting protein), respectively^[134-136].

Downregulation of *MIR9* *via* hypermethylation in gastric cancer has also been found to increase not only proliferation but also cell migration and invasion, a prerequisite for the formation of successful metastasis, although their target genes have not been identified yet^[137]. Finally, dysregulation of MIR34C can cause drug resistance by affecting MAPT (microtubule-associated protein tau)^[138], and dysregulation of the *MIR200BC/429* cluster can do so by altering the expression of BCL2 (B-cell CLL/lymphoma 2) and XIAP (X-linked inhibitor of apoptosis)^[139].

Hypomethylation of miRNAs has also been studied. For example, the loss of methylation at the promoter of the *MIR196* gene and upregulation of this miRNA are frequently found in primary gastric cancer, indicating the tumor-suppressive role of MIR196^[83]. In addition, the upregulation of several oncogenic miRNAs such as MIR9, MIR93, MIR106B and MIR222 in gastric cancer have been reported, and their role in proliferation, anti-apoptosis and metastasis has been studied in gastric cancer cell lines^[137,140,141]. However, the question of whether the upregulation of the aforementioned miRNAs is a consequence of DNA hypomethylation has yet to be answered.

Promoter methylation of chromatin-modifying enzyme genes in gastric cancer

Chromatin-modifying enzymes (CMEs) can affect the DNA methylation and histone modification status of target genes, thus causing changes in chromatin structure. Alteration at the level of CMEs can initiate several epigenetic cascades that affect diverse pathways involved in tumorigenesis and the progression and metastasis of gastric cancer.

In gastric cancer, several CMEs are also the targets of DNA methylation (Table 1). For example, Gigeck *et al.*^[142] found that SMARCA5 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5), which has helicase and ATPase activity, was often downregulated in gastric cancer patient samples compared to normal tissue and as a consequence of its promoter methylation. MGMT (O-6-methylguanine-DNA methyltransferase) has also been frequently found to be

absent in gastric cancer due to promoter methylation^[93].

Moreover, the expression of several CMEs is regulated by miRNAs. For example, upregulation of *UHRF1* (ubiquitin-like with PHD and ring finger domains 1) expression *via* downregulation of MIR146A and MIR146B causes aberrant DNA methylation in *CDH1*, *RUNX3* and *SLIT3* (slit homolog 3) genes^[143]. Furthermore, DNMT1, DNMT3A and DNMT3B proteins are downregulated *via* overexpression of *MIR200B* and *MIR200C* in gastric cancer, and this may be a cause of global DNA hypomethylation in gastric cancer cells^[144].

HISTONE MODIFICATIONS IN GASTRIC CANCER

Histone modifications including acetylation, methylation, phosphorylation and ubiquitylation can directly alter gene expression. Several histone modifiers show aberrant expression patterns or mutations during tumorigenesis and cancer progression as explained above. The mechanisms by which alterations of histone modifications contribute to tumorigenesis and metastasis have been intensively studied in several types of cancer. In contrast, studies of histone modifications in gastric cancer are lacking (Table 1).

Histone-modifying enzymes in gastric cancer

Most epigenetic studies of gastric cancer have been focused on DNA methylation. Thus, scientists only recently started to investigate histone modifiers in gastric cancer. Recent findings on the role of histone modifiers in gastric cancer have shed light on the complex epigenetic mechanisms governing the development and progression of gastric cancer.

For example, histone H3K4 demethylase KDM1A (LSD1) is upregulated in some gastric cancer cells, and treatment of these cells with LSD1 inhibitors exerts cytotoxic effects as well as inhibitory effects on the migration and invasion of these cells, suggesting an important role for LSD1 in gastric cancer^[145]. In addition, it has been shown that the histone deacetylase SIRT1 (sirtuin 1) plays a tumor-suppressive role in gastric cancer development *via* inhibition of NF- κ B signaling and is downregulated in gastric cancer^[146].

In contrast, the H3K9/K36 demethylase KDM4B (commonly called JMJD2B) was recently discovered to be a potent activator of cell proliferation as well as the epithelial-mesenchymal transition (EMT) and correlated with lymph node/distant metastasis^[147,148]. Another H3K9 demethylase, JMJD1C, is also upregulated in gastric cancer. In addition, the H3K27 methyltransferase EZH2 has been shown to promote gastric cancer tumorigenesis in various model systems and exhibits significant association with patient survival as well as lymph node metastasis^[149]. Furthermore, the expression of the histone lysine acetyltransferase KAT5 (TIP60) has been shown to be reduced in gastric cancer and to have a significant correlation with lymph node metastasis^[150].

Genes deregulated by histone modifications in gastric cancer

Several recent studies identified genes whose expression is regulated by various histone modifications. These include the H3/H4 hyperacetylation of the *MYC* promoter *via* FOXO6/HNF4 axis, the repression of *GATA* by deacetylation of histone H3/H4 at the promoter, and the downregulation of *RND3* (*RboE*)^[107,151,152]. In addition, dephosphorylation of histone H3 serine 10 on *c-JUN* and *HSP70* genes has been shown to cause altered expression of these genes^[153].

Combinatorial modifications of DNA and histones in gastric cancer

DNA methylation events are often accompanied by histone modifications and *vice versa* to tightly regulate gene expression. Several studies also discovered that the expression of several genes in gastric cancer can be regulated by DNA methylation and histone modification simultaneously. For example, Meng *et al.*^[154] showed that the promoter of the *CDKN2A* gene undergoes both DNA methylation and histone H3K9 dimethylation. Lee *et al.*^[97] showed that hypoxia silences *RUNX3*, which is known to be suppressed by DNA methylation, *via* modification of histones during the progression of gastric cancer.

Overexpression of *LAMB3* (laminin, beta 3) affects several malignant phenotypes in gastric cancer cell lines, and these genes not only undergo demethylation at CpG islands but also exhibit an increase in H3K4 trimethylation^[155]. *MYO5B* (myosin VB) gene is suppressed by DNA methylation as well as histone deacetylation, causing persistent c-MET signaling in gastric cancer^[156]. A study by Ma *et al.*^[157] demonstrated that DNA hypermethylation and histone hypomethylation of *PDX1* (pancreatic and duodenal homeobox 1) causes downregulation of this gene in gastric cancer. Finally, gene expression of *PRDM5* (PR domain containing 5), a member of the kruppel-like zinc finger family, is downregulated *via* DNA methylation and H3K27 trimethylation, alleviating the cell growth suppressive effect of *PRDM5*^[158].

LncRNAs in gastric cancer

LncRNAs, once thought to be junk in cells, have now become a center of attention in various fields from developmental biology to the study of human diseases. However, there are few studies of the role of lncRNAs in gastric cancer. Arita *et al.*^[159] have examined several lncRNAs previously shown to be involved in other cancers, including H19, HOTAIR and MALAT1, and showed that the plasma level of H19 was higher in gastric cancer patients than in healthy controls, raising the possibility of using lncRNA as a tumor marker in gastric cancer. Another study of H19 showed its role in the proliferation of gastric cancer cells^[160].

Cao *et al.*^[161] compared the expression profiles of almost 10000 lncRNAs in gastric cancer and normal tissue samples and identified TUG1 (taurine upregulated 1), UCA1 (urothelial cancer associated 1), PVT1 (Pvt1

oncogene), SNHG1 (small nucleolar RNA host gene 1), LINC00152 (long intergenic non-protein coding RNA 152) and LINC00261 (long intergenic non-protein coding RNA 1261) as differentially expressed lncRNAs in gastric cancer. Studies by several groups revealed that the expression of *HOTAIR* is positively associated with gastric cancer development and plays a role in invasion and the epithelial-mesenchymal transition of gastric cancer cells^[159,162,163].

DISCUSSION

Due to tremendous research efforts, it has become clear that epigenetic modification is a major contributor to the formation and metastasis of most, if not all, of cancers, including gastric cancer. Epigenetic changes including DNA methylation and histone modifications can be caused by mutations and/or altered expression of writers, erasers and readers of these modifications. These deregulated modifiers, in turn, facilitate uncontrolled expression of oncogenes and metastasis-promoting genes while keeping that of tumor- and metastasis-suppressor genes silenced.

The focus of epigenetic research in cancer has shifted from mere identification of changes in chromatin modifications to distinguishing epigenetic modifications that truly drive cancer formation from bystanders. These types of research are imperative to the design and development of effective anti-cancer therapeutic drugs. In contrast to the extensive studies on the epigenetic dysregulation of other types of cancer such as breast cancer, similar studies on gastric cancer are still lagging behind, calling for more vigorous research on this subject.

In particular, our understanding of the histone modifications in gastric cancer is very limited compared to that of other cancer types and to DNA methylation. In contrast to the few types of DNA modifications, histone modifications are more diverse, adding more layers of complexity to the epigenetic mechanisms involved in cancer. Thus, a better understanding of the network of histone modifications in gastric cancer will provide not only a complete picture of gastric cancer but also an opportunity to develop anti-gastric cancer therapeutics.

Another player whose importance in gastric cancer has only recently been identified is noncoding RNA, such as miRNAs and lncRNAs. Whereas miRNAs regulate protein-coding RNAs *via* direct binding, lncRNAs work through guiding chromatin modifiers to the target genes. Studies on the role of lncRNAs in gastric cancer have only recently begun, and we are just starting to understand their functions in gastric cancer. There is no doubt that further studies on noncoding RNAs will reveal a new paradigm in the field of gastric cancer research.

One of the remaining important needs in understanding gastric cancer is to gain insights into the diversity of epigenetic drivers in different types of gastric cancer. As in genetic modifications, the types of epigenetic changes that contribute to the formation of tumors vary depend-

ing on cancer types and subtypes even within tumors originating from the same organ. For example, a given modification contributing to intestinal-type gastric cancer may not be the key factor for diffuse-type gastric cancer development. Thus, it is crucial to understand cancer type-specific epigenetic modifications in order to develop personalized anti-gastric cancer therapeutics.

Finally, for cancer cells to grow and metastasize, they must acquire abilities to exploit surrounding stroma, emphasizing the importance of distinction between alterations in tumor cells and those in stromal cells. However, most previous studies of gastric patient samples have been performed on whole tumor tissues without separating tumor cells and surrounding stromal cells, making it hard to interpret the results. Very recently, several research groups have utilized elegant methods including fluorescence-activated cell sorting and laser capture microdissection to separate stromal cells from tumor cells.

They made the very intriguing discovery that stroma cells also undergo alterations in gene expression profiles, likely caused by epigenetic modifications. These stroma-specific changes might have been masked by the tumor cell gene expression profile if whole tumor tissue had been used. Scientists argue that targeting the tumor stroma might be a safer and more effective way to treat cancer due to the relatively stable and homogeneous features of stromal cells compared to heterogeneous and rapidly evolving tumor cells. To this end, it is imperative to accurately characterize stroma- and tumor cell-specific epigenetic changes, particularly in the case of gastric cancer.

As a final comment, the primary unmet needs for gastric cancer are the development of an accurate way to predict patients at high risk for metastasis and the generation of therapeutic drugs that effectively treat gastric cancer. This will reduce unnecessary gastrectomy, thus improving the quality of patients' life and moving us one step closer to conquering gastric cancer in the near future.

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WJG 20th Anniversary Special Issues (8): Gastric cancer

Epigenetic dysregulation in Epstein-Barr virus-associated gastric carcinoma: Disease and treatments

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Core tip: Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) comprises nearly 10% of gastric carcinoma cases worldwide. In the present review, we critically discuss the role of EBV in gastric carcinogenesis, summarising the role of viral proteins and microRNAs with respect to aberrant methylation in EBVaGC. Given the role of epigenetic dysregulation in tumorigenesis, epigenetic modifiers may represent a novel therapeutic strategy.

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Abstract

Epstein-Barr virus (EBV)-associated gastric carcinoma (EBVaGC) comprises nearly 10% of gastric carcinoma cases worldwide. Recently, it was recognised to have unique clinicopathologic characteristics, including male predominance, lower rates of lymph node involvement, and better prognosis. EBVaGC is further characterised by abnormal hypermethylation of tumour suppressor gene promoter regions, causing down-regulation of their expression. In the present review, we critically discuss the role of EBV in gastric carcinogenesis, summarising the role of viral proteins and microRNAs with respect to aberrant methylation in EBVaGC. Given the role of epigenetic dysregulation in tumorigenesis, epigenetic modifiers may represent a novel therapeutic strategy.

INTRODUCTION

Epstein-Barr virus (EBV) infection is ubiquitous, and is accepted as a causative microorganism for various malignancies including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma, and gastric carcinoma (GC). EBV-associated GC (EBVaGC) accounts for approximately 10% of cases worldwide^[1,2], and is characterised by unique clinicopathologic features including a relatively favourable prognosis (Table 1)^[1-4]. In recent years, the molecular mechanisms underlying EBV-related carcinogenesis have become increasingly understood. EBV may contribute to tumorigenesis through the expression of viral proteins and microRNAs (miRNAs). Previous studies have also reported that promoter methylation was observed more

Table 1 Clinical and pathological features of Epstein-Barr virus-associated gastric carcinoma

| Clinical and pathological features | |
|------------------------------------|---|
| Age | Younger ¹ |
| Gender | Male predominance |
| Associations | Smoking |
| Prevalence | 10% of gastric carcinoma cases |
| Location | Gastric body/cardia Remnant stomach |
| Clinical | Multiple carcinomas ¹ Thickening of gastric wall Ulcerated (saucer-like) neoplasm Lower rate of lymph node involvement ¹ |
| Histology | Lymphoepithelioma-like Lymphocytic infiltration in various degrees Atrophic gastritis Lace pattern within the mucosa |
| Prognosis | Moderate to poorly differentiated adenocarcinoma Longer survival ¹ |

¹Items are controversial and subject to on-going research.

frequently in EBVaGC. Hence another method by which EBV contributes to gastric carcinogenesis is through aberrant DNA methylation and histone modification. Thus EBVaGC is characterised by distinct variations on genomic, epigenomic, and transcriptomic levels^[5]. Here, we review the mechanism by which EBV infection causes aberrant methylation, transformation, cancer development, and its associated therapeutic implications.

MECHANISM OF EBV INFECTION

EBV may infect host gastric epithelial cells directly or indirectly (Figure 1). In direct infection, the viral envelope glycoprotein BMRF-2 interacts with cellular $\beta 1$ integrins. Subsequently, viral protein gH/gL attaches to cellular $\alpha \nu \beta 6/8$ integrins, and triggers fusion of the viral envelope with the epithelial cell membrane^[6]. EBV preferentially infects B lymphocytes, which then mediates subsequent infection to epithelial cells^[7]. In B cell invasion, EBV envelope glycoproteins gp350/220 bind to B cell receptors CD21 and/or CD35^[8,9]. Simultaneously, viral glycoprotein gp42 interacts with Human Leukocyte Antigen (HLA) class II molecules on the B cell membrane to trigger the core fusion complex, enabling EBV entry into the B cell (Figure 2)^[8,10]. Through direct cell-to-cell contact, EBV-infected B cells may subsequently infect epithelial cells^[11]. The exact mechanism of epithelial cell invasion is unclear, but involves CD21-mediated co-capping of EBV and integrins on B cells, as well as conjugate formation between EBV-infected B cells and epithelial cells *via* the capped adhesion molecules^[11]. Once EBV enters epithelial cells, the viral capsid dissolves and the viral genome is transported to the cell nucleus.

LATENCY, VIRAL PROTEINS, AND CARCINOGENESIS

Following infection, EBV typically persists in a latent

Table 2 Latent gene expression patterns in Epstein-Barr virus infected malignancies

| Genes | Latency I a | Latency I b | Latency II | Latency III |
|----------------|----------------------------------|-------------|---|--|
| <i>EBNA1</i> | + | + | + | + |
| <i>EBNA2</i> | - | - | - | + |
| <i>EBNA3a</i> | - | - | - | + |
| <i>EBNA3b</i> | - | - | - | + |
| <i>EBNA3c</i> | - | - | - | + |
| <i>EBNA-LP</i> | - | - | + | + |
| <i>LMP1</i> | - | - | + | + |
| <i>LMP2A</i> | - | + | + | + |
| <i>LMP2B</i> | - | - | + | + |
| <i>EBER1</i> | + | + | + | + |
| <i>EBER2</i> | + | + | + | + |
| <i>BARTs</i> | + | + | + | + |
| Disease | EBVaGC, Burkitt's lymphoma | EBVaGC | NPC, Hodgkin's lymphoma, NK/T-cell lymphoma | AIDS- associated B-cell lymphomas, Pyothorax- associated lymphoma |

EBVaGC: Epstein-Barr virus-associated gastric carcinoma; NPC: Nasopharyngeal carcinoma; AIDS: Acquired-immunodeficiency-syndrome.

stage. During latency, the viral genome is largely silenced by host-driven methylation of CpG island motifs. Based on the subset of viral genes which are expressed, tumours may be classified into four types; latency I a, I b, II, and III (Table 2). EBVaGC belongs to latency type I, where the viral genes EBV nuclear antigen 1 (EBNA1), EBV-encoded small RNA (EBER1/2), BamHI-A rightward transcripts (BARTs), and latent membrane protein 2A (LMP2A) may be expressed^[12,13]. Notably, the expression of latency genes is associated with malignancy. For example, EBER1 up-regulates the expression of insulin-growth factor-1, an autocrine growth factor which accelerates cell proliferation in EBVaGC^[14].

Half of all EBVaGCs also express LMP2A. LMP2A plays a critical role in the oncogenic processes in EBVaGC, and thus EBV latency patterns should be further subdivided into Ia or Ib based on the absence or presence of LMP2A^[12,15]. LMP2A not only inhibits apoptosis through up-regulation of the cellular survivin gene *via* the NF- κ B pathway^[16], but induces expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), which causes up-regulation of DNA methyltransferase DNMT1^[16] and DNMT3B^[17] in EBV-infected GC cells. DNA methyltransferases play important roles in controlling DNA methylation. The subsequent overdrive of CpG methylation and silencing of tumour suppressor genes such as PTEN, p16, and p73 leads to the transformation of EBV-infected cells. Hence epigenetic dysregulation plays an important role in gastric carcinogenesis.

EBVaGC AND EPIGENETIC ALTERATIONS

Epigenetics refer to functionally relevant and heritable changes in gene expression that occur without alteration of the underlying DNA sequence. The two primary mechanisms which may produce this change are DNA

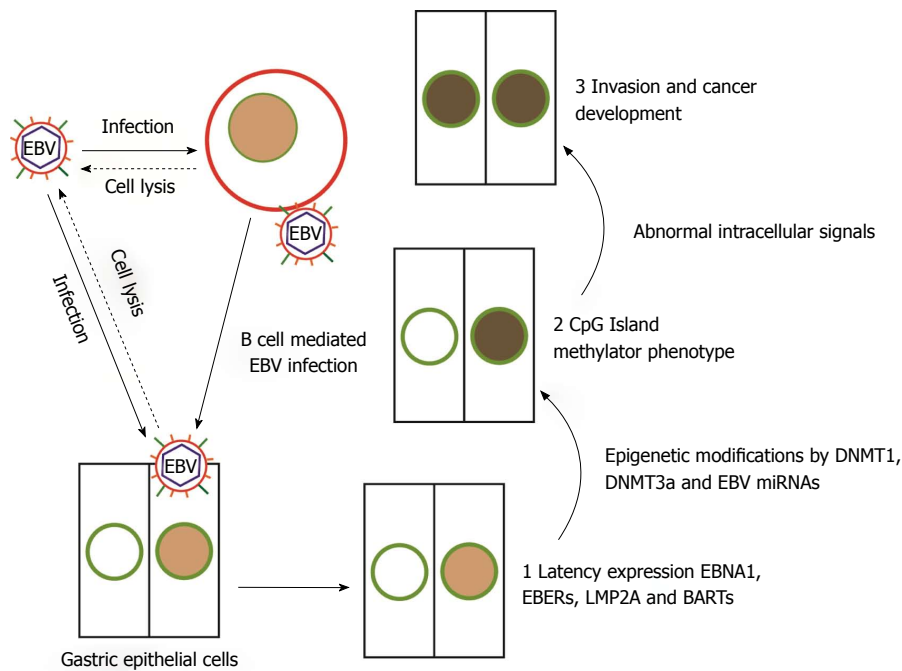


Figure 1 Epstein-Barr virus infects host gastric epithelial cells through direct and indirect mechanisms. Epstein-Barr virus (EBV) preferentially infects B lymphocytes, which subsequently infects gastric epithelial cells through direct cell-to-cell contact. EBV infection causes expression of latency 1a and/or 1b proteins in gastric epithelial cells. EBV infection also up-regulates genes including *EBNA1*, *EBER*, *LMP2A*, and *BART*, altering expression of DNMTs and miRNAs. Collectively, the abnormal intracellular signals lead to carcinogenesis and tumour development.

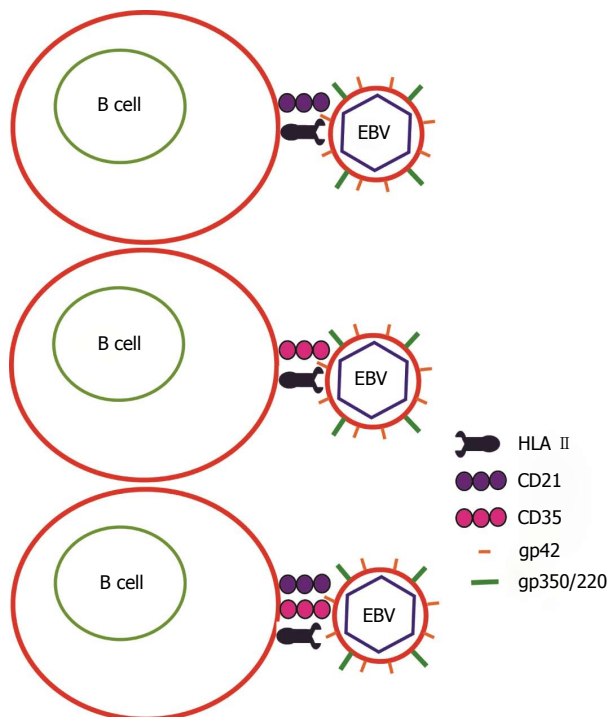


Figure 2 Mechanism of Epstein-Barr virus infection in B lymphocytes. Epstein-Barr virus (EBV) envelope glycoproteins gp350/220 bind to B lymphocyte receptors CD21 and/or CD35. Simultaneously, viral glycoprotein gp42 interacts with HLA II on the B cell membrane to trigger the core fusion complex, enabling EBV entry into the B cell.

methylation, and histone modification. According to the epigenetic progenitor model, tumour-progenitor genes promote the polyclonal epigenetic disruption of stem

cells as a first step in the development of cancer^[18]. This epigenetic plasticity causes genomic instability, and collectively drives tumour progression^[19].

The CpG island methylator phenotype was first observed in EBVaGC in 1999^[20]. EBV infection was shown to induce extensive methylation and repression of tumour suppressor genes over 18 wk in MKN7, a low methylation GC cell line^[21]. Subsequent studies confirmed that EBVaGC has higher rates of aberrant DNA methylation than EBV non-associated GC (EBVnGC)^[21,22]. Nevertheless, the mechanisms by which EBV induces aberrant DNA methylation and histone modification remain poorly understood.

EBV AND microRNA

Viral encoded miRNAs play a pivotal role in alterations to DNA methylation status in host cells. The expression of EBV miRNAs vary under different latency programs (Table 3)^[23]. For example, miR-BART1-5p, 6, and 17-5p suppresses LMP1 expression^[24], whilst miR-BART-22 regulates expression of LMP2A^[25]. EBV miRNAs further repress cellular proteins, including PUMA, DICER1, and BIM. EBV infection may also affect host cell miRNA expression. Specifically, miR-200a and miR-200b are down-regulated in EBVaGC compared to EBVnGC and adjacent mucosa. This down-regulation may be mediated by viral proteins such as BRAF0, EBER, and LMP2A, as well as by aberrant DNA methylation following EBV infection^[26]. More recently, miRNA sequencing studies have revealed that EBV-infection mediates down-regulation of tumour suppressor miRNAs including the Let-7 family.

Table 3 Epstein-Barr virus-driven miRNAs and their target genes

| Gene name | Gene targets in EBV | Gene targets in host cell |
|--------------|------------------------------|---|
| miR-BHRF1-1 | - | <i>GUF1</i> ^[58] , <i>SCRN1</i> ^[58] |
| miR-BART1-5p | <i>LMP1</i> ^[24] | <i>CLEC2D</i> ^[58,59] , <i>LY75</i> ^[58,59] , <i>SP100</i> ^[58,59] , <i>DICER1</i> ^[58,59] , <i>MICB</i> ^[58,59] |
| miR-BART1-3 | - | <i>CXCL11</i> ^[60] |
| miR-BART2-5p | <i>BALF5</i> ^[61] | <i>MICB</i> ^[62] |
| miR-BART3 | - | <i>DICER1</i> ^[58] , <i>MICB</i> ^[58] |
| miR-BART3-3p | - | <i>IPO7</i> ^[63] |
| miR-BART5 | <i>LMP1</i> ^[59] | <i>PUMA</i> ^[66] |
| miR-BART6 | <i>LMP1</i> ^[24] | <i>DICER1</i> ^[65] |
| miR-BART10 | <i>BHRF1</i> ^[59] | - |
| miR-BART13 | - | <i>CAPRN2</i> ^[59] |
| miR-BART16 | <i>LMP1</i> ^[24] | <i>TOMM22</i> ^[63] |
| miR-BART17-p | <i>LMP1</i> ^[24] | - |
| miR-BART19 | <i>LMP1</i> ^[59] | - |
| miR-BART22 | <i>LMP2A</i> ^[25] | - |
| miR-BARTs | - | <i>BIM</i> ^[66] |

EBV: Epstein-Barr virus.

Further research is required to elucidate their role in tumorigenesis^[27].

ABERRANT DNA METHYLATION IN EBVaGC

Currently, GC is subdivided into three subtypes based on CpG-island methylator phenotype (CIMP). Defined as high (CIMP-H), low (CIMP-L), or none (CIMP-N), the classification is based on the number of methylated loci (≥ 4 , 1-3, and 0 respectively) in the promoter regions of five genes (LOX, HRASLS, FLNC, HAND1, and THBD)^[28]. It was previously shown that promoter methylation of cancer-related genes was seen more frequently in EBVaGC than EBVnGC. EBVaGC is thus classified as CIMP-H^[29].

In a genome-wide study comparing promoter methylation between EBV-infected and EBV non-infected GC cell lines, hundreds of genes involved in cancer pathways such as cell adhesion molecules, wnt signalling pathway, and mitogen-activated protein kinase signalling were observed to be hypermethylated following EBV infection^[17]. Further investigation through epigenomic and transcriptomic sequencing revealed that 216 genes were down-regulated by promoter hypermethylation. Significantly, hypermethylation of tumour suppressor genes, including p14, p15, p16, APC, E-cadherin, and PTEN were noted in EBVaGC, but not EBVnGC^[30,31]. All studies unanimously agreed that p16 was significantly more hypermethylated in EBVaGC^[29-35]. P16 is a tumour suppressor gene which acts in the G1 phase of the cell cycle to phosphorylate the retinoblastoma gene product (pRb). Loss of p16 leads to uncontrolled cell growth^[36], and is thus commonly found in tumours^[37,38].

Another important cellular abnormality in EBVaGC is its resistance to apoptosis. The frequency of apoptosis is significantly lower in EBVaGC than in EBVnGC^[38].

Table 4 Hypermethylated genes verified in Epstein-Barr virus-associated gastric carcinoma tissue

| Function | Hypermethylated genes |
|------------------------|---|
| Apoptosis | <i>DAPK</i> ^[30] , <i>BNIP3</i> ^[29] , <i>FAM3B</i> ^[5] , <i>HRK</i> ^[29] , <i>IL15RA</i> ^[17] , <i>MINT31</i> ^[32] , <i>p16</i> ^[29-35] , <i>p73</i> ^[30,32-34] , <i>PTEN</i> ^[31,67] , <i>RASS-FIA</i> ^[31] |
| Cell adhesion | <i>EPHB6</i> ^[17] , <i>FLNC</i> ^[30] , <i>FSD1</i> ^[34] , <i>REC8</i> ^[17] , <i>CSPG2</i> ^[29] |
| Cell-cell interactions | <i>MDGA2</i> ^[17] , <i>THBS1</i> ^[31] |
| Cell cycle regulation | <i>APC</i> ^[31] , <i>p15</i> ^[30] , <i>p16</i> ^[29-35] , <i>p57</i> ^[29] , <i>p73</i> ^[30,32-34] |
| Cell invasion | <i>E-Cadherin</i> ^[30,68,69] |
| Cell migration | <i>EPHB6</i> ^[17] |
| Cell proliferation | <i>E-Cadherin</i> ^[30,68,69] , <i>HRASLS</i> ^[30] , <i>IL15RA</i> ^[17] , <i>MINT31</i> ^[32] , <i>NKX3.1</i> ^[34] , <i>RUNX3</i> ^[32] , <i>TIMP2</i> ^[21] , <i>TIMP3</i> ^[30] |
| Cell signalling | <i>14-3-3Sigma</i> ^[31] , <i>CSPG2</i> ^[29] , <i>MINT1</i> ^[31] , <i>MINT2</i> ^[31,32] , <i>PLXND1</i> ^[21] |
| Differentiation | <i>HAND1</i> ^[30] |
| Dna repair | <i>hMLH1</i> ^[32,43,53] , <i>MGMT</i> ^[31] |
| Exocytosis | <i>SCRN1</i> ^[34] |
| Metastasis | <i>E-Cadherin</i> ^[30,68,69] , <i>LOX</i> ^[30] |
| Other | <i>BCL7A</i> ^[34] , <i>BLU</i> ^[34] , <i>CHFR</i> ^[29] , <i>CXXC4</i> ^[21] , <i>GSTP1</i> ^[30,31,40] , <i>HLTF</i> ^[29] , <i>HOXA10</i> ^[70] , <i>IHH</i> ^[5] , <i>MARK1</i> ^[34] , <i>MINT25</i> ^[31] , <i>PAX5-β</i> ^[29] , <i>SCARF2</i> ^[17] , <i>SSTR1</i> ^[17,39] , <i>THBD</i> ^[30] , <i>WNT5A</i> ^[71] |

It may be caused by hypermethylation of SSTR1 and GSTP1; both genes are frequently hypermethylated in NPC and GC infected EBV tissues, and regulate cell migration, proliferation, and apoptosis^[30,31,39-42]. Notably, EBV infection also up-regulates expression of FAM3B and IHH^[5]. FAM3B is associated with invasion^[43], and Indian Hedgehog (IHH) with increased metastatic potential through angiogenesis and Snail protein expression, as well as a decrease in e-cadherin and tight junctions^[44,45]. Table 4 shows a comprehensive list of hypermethylated genes and their role in carcinogenesis. Hence aberrant DNA methylation plays an important role in gastric carcinogenesis.

IMPLICATIONS FOR TREATMENT

Current treatment guidelines from the National Institute for Health and Clinical Excellence (NICE) for the management of GC depends on the stage of disease. Broadly, the mainstay for cure is surgical excision with clearance of adjacent lymph nodes. Radiotherapy, and chemotherapeutic agents including cisplatin, docetaxel, epirubicin, and 5-fluorouracil (5-FU) may be used as adjuvants or in the palliative setting. Notably, no differentiation is made between the distinct subtypes of GC in the treatment guidelines.

Research has established that EBVaGC represents a distinct entity of GC, characterised not only by unique genomic aberrations, but also by clinicopathologic features such as less lymph node involvement, and significantly better prognosis^[2]. Naturally, there are associated therapeutic implications, as evidenced by resistance to docetaxel and 5-FU in EBV-positive, but not EBVnGC cell lines^[46,47]. The chemoresistance is mediated by EBV-lytic gene expression, which induces expression of Bcl-2

Table 5 Selection of epigenetic therapeutics in cancer chemotherapy

| Target | Drug name | Status | Ref. |
|----------------------|---|----------------|---------|
| DNA methylation | Azacitine (5-Aza-CdR) | Approved | [72] |
| | Decitabine (5-Aza-CdR) | Approved | [72] |
| | Hydralazine | Phase II / III | [73] |
| | Epigallocatechin-3-gallate (EGCG) | Phase II | [74-76] |
| | 5-Fluoro-deoxycytidine (FdCyd/FDAC) | Phase I / II | [77] |
| | 5-fluoro-2'-deoxycytidine (FCdR) | Phase I / II | [78] |
| | Procainamide | Phase I | [79] |
| | Procaine | Phase I | [80] |
| | Psammaplin A | Phase 0 | [81,82] |
| | RG108 | Phase 0 | [83-86] |
| Histone deacetylases | Zebularine | Phase 0 | [87-89] |
| | Vorinostat | Approved | [90] |
| | Romidepsin | Approved | [90] |
| | Panobinostat | Phase II | [91] |
| | SEN196 | Phase II | [92] |
| | Phenyl butyrate | Phase I / II | [93-95] |
| | Valporic acid | Phase I | [93-95] |
| | Compound 6j (R = -C ₄ H ₉) | Phase 0 | [96] |

and survivin whilst simultaneously suppressing p21 to inhibit apoptosis^[48]. In support of this hypothesis, silencing of EBV-lytic gene LMP1 through specific small interfering RNA (siRNA) enhanced chemosensitivity of cancer cells to bleomycin and cisplatin^[49]. Since epigenetic dysregulation is implicated in the expression of EBV-lytic genes and consequent tumour progression, we believe that epigenetic processes are a rational therapeutic target in EBVaGC.

Crucially, aberrant DNA methylation in cancer is reversible. Thus the enzymes which regulate epigenetic modifications are attractive targets for pharmacological intervention. Current epigenetic therapies may be classified into histone acetyltransferase (HAT), histone deacetylase (HDAC), and DNA methyltransferase (DNMT) inhibitors (Table 5). Broadly, they facilitate demethylation and re-expression of epigenetically silenced tumour suppressor genes, lowering the apoptotic threshold to sensitise tumour cells to chemotherapy and radiotherapy. Consequently, there has been an emphasis on investigating the clinical value of epigenetic therapies in combination with conventional cytotoxic agents and radiation.

It was previously reported that the combination of irradiation and 5-aza-CdR significantly decreased growth activity compared with irradiation alone in OCUM-2M, OCUM-12, and MKN-45 GC cell lines ($P < 0.05$). The cell cycle arrest and increased apoptotic rate may be partly mediated by enhanced expression of p53, RASSF1, and DAPK gene families by 5-aza-CdR^[50]. The use of epigenetic therapies in conjunction with targeted therapies such as gefitinib in lung cancer, imatinib in chronic myeloid leukemia, and trastuzumab in breast cancer cell lines and *in vivo* tumour models also had synergistic effects on the induction of apoptosis^[51,52]. More recently,

epigenetic modifiers and ZEB1 inhibitors have been used to induce lytic transformation of EBV-infected gastric cancer cells. Expressed only in the lytic form of infection, virally encoded kinases convert ganciclovir into its active form, potentiating its cytotoxic effects^[53,54]. Hence epigenetic modifiers may be a useful therapeutic strategy in EBVaGC.

However, several problems must be considered. Firstly, methylation is reversible, so re-methylation and re-silencing after cessation of drug therapy may occur^[55]. Moreover, there have been numerous concerns raised regarding the systemic effects of non-specific gene activation in non-cancerous cells by epigenetic therapies. Conflicting evidence exists in the literature regarding the effect of epigenetic therapies on normal cells. Some studies have demonstrated that 5-Aza and decitabine increases mutation frequency, causes chromosomal rearrangements, and decreases fertility in mice. Conversely, no increase in chromosomal integrity was observed following administration of low dose 5-aza-CdR in patients with myelodysplastic syndrome^[56]. Additionally, treatment of 41 leukemia patients with 5-aza-CdR showed only mild effects on global genomic de-methylation, as measured by changes in Alu methylation^[57]. Few adverse effects were observed, and original methylation levels were regained within two weeks after therapy. No development of secondary malignancies were recorded. Consequently, further studies are needed to investigate the long term effects of epigenetic therapies.

CONCLUSION

EBVaGC is a unique type of GC. The characteristic global hypermethylation of the promoter region in tumour-suppressor genes may be due to overexpression of DNMTs by viral latent proteins, miRNAs, and various epigenomic changes. However, the precise role of EBV in the multifactorial etiology of GC is still not fully understood. Further studies are needed to elucidate the intricate relationship between EBV infection, environmental factors, genetic backgrounds, and aberrant DNA methylation in GC. A better understanding of the role of EBV in gastric carcinogenesis will enable discovery of novel therapeutic targets and strategies.

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WJG 20th Anniversary Special Issues (11): Cirrhosis

Virus-related liver cirrhosis: Molecular basis and therapeutic options

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Abstract

Chronic infections with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) are the major causes of cirrhosis globally. It takes 10-20 years to progress from viral hepatitis to cirrhosis. Intermediately active hepatic inflammation caused by the infections contributes to the inflammation-necrosis-regeneration process, ultimately cirrhosis. CD8⁺ T cells and NK cells cause liver damage *via* targeting the infected hepatocytes directly and releasing pro-inflammatory cytokine/chemokines. Hepatic stellate cells play an active role in fibrogenesis *via* secreting fibrosis-related factors. Under the inflammatory microenvironment, the viruses experience mutation-selection-adaptation to evade immune clearance. However, immune selection of some HBV mutations in the evolution towards cirrhosis seems different from that towards hepatocellular carcinoma. As viral replication is an important driving force of cirrhosis pathogenesis, antiviral treatment with nucleos(t)ide analogs is generally effective in halting the progression of cirrhosis, improving liver function and reducing the morbidity of decompensated cirrhosis caused by chronic HBV infection. Interferon- α plus ribavirin and/or the direct acting antivirals such as Vaniprevir are effective for compensated

cirrhosis caused by chronic HCV infection. The standard of care for the treatment of HCV-related cirrhosis with interferon- α plus ribavirin should consider the genotypes of IL-28B. Understanding the mechanism of fibrogenesis and hepatocyte regeneration will facilitate the development of novel therapies for decompensated cirrhosis.

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Key words: Liver cirrhosis; Hepatitis B virus; Hepatitis C virus; Evolution; Immune cells; Signaling pathway; Hepatic stellate cells; Antiviral therapy

Core tip: Hepatic inflammation caused by viral infections contributes to the inflammation-necrosis-regeneration process, ultimately cirrhosis. Immune selection of some hepatitis B virus mutations in the evolution towards cirrhosis seems different from that towards hepatocellular carcinoma. Hepatic stellate cells and macrophages are important for the fibrogenesis. Antiviral treatment is generally effective in reducing the morbidity of decompensated cirrhosis. The standard of care for the treatment of hepatitis C virus-related cirrhosis with pegylated interferon- α and ribavirin should consider the genotypes of IL-28B. Stem cell-based therapy can be an option for the treatment of decompensated cirrhosis patients who fail to respond to antiviral treatment.

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INTRODUCTION

Liver cirrhosis represents a worldwide public health

problem and its mortality rate increases stably these years. Alcoholic hepatitis, non-alcoholic steatohepatitis, and chronic infection with hepatitis C virus (HCV) are the major causes of cirrhosis in Western countries, while chronic infection with hepatitis B virus (HBV) contributes greatly to cirrhosis in HBV-endemic areas. Chronic HBV and HCV infections account for 57% of cirrhosis cases globally^[1]. There are about 300 million subjects chronically infected with HBV and 130-170 million subjects chronically infected with HCV worldwide. About one million people die from diseases related to chronic HBV and/or HCV infections each year, mostly end-stage liver diseases, namely, decompensated cirrhosis, liver failure, and hepatocellular carcinoma (HCC). In East Asia where HBV genotypes B and C are endemic, HBV genotype B is more apt to cause acute infection in young people and to be cleared than genotype C; whereas HBV genotype C leads to higher persistence following an acute course and is more apt to cause cirrhosis and HCC compared to genotype B^[2-5]. HBV genotype C is associated with a lower rate of spontaneous hepatitis B e antigen (HBeAg) seroconversion than HBV genotype B. Cirrhosis is specifically found in HBeAg-negative subjects and more frequently found in subjects with genotype C than in those with genotype B. In HBeAg-negative subjects, high viral load is frequently associated with abnormal alanine aminotransferase (ALT) level, while ALT abnormality is more frequent in those with cirrhosis than those without^[6]. In addition, older age (longer duration of infection), high levels of HBV DNA, habitual alcohol consumption, and concurrent infection with HCV, hepatitis D virus or human immunodeficiency virus are significantly associated with an increased rate of cirrhosis^[7]. Most (70%-80%) HCV infections may persist and about 30% of individuals with persistent HCV infection will develop end-stage liver diseases, including cirrhosis and HCC. According to the most recent classification criteria, HCV variants are classified into 6 genotypes, of which HCV-1b is the most common one worldwide^[8]. HCV genotype 1b has been associated with an increased risk of HCC in both European and Asian populations^[9,10], but it remains uncertain if this genotype of HCV is specifically related to fibrosis and cirrhosis. An interesting cross-sectional study carried out in mainland China indicated that co-infection with HBV, low HCV viral load, low serum ALT, high serum aspartate aminotransferase, diabetes, and high pickled food consumption were significantly associated with the risk of cirrhosis in HCV-infected patients^[11].

Since both HBV and HCV are preferentially hepatotropic, not directly cytopathic, virus-caused liver damage is attributed to immune-mediated mechanisms. Under inflammatory microenvironment caused by the infections, the continuous infiltration of immune cells and the secreted inflammatory cytokines result in liver damage. The hepatic lobule reconstruction following the damage promotes hepatic fibrosis and eventually leads to cirrhosis. Cirrhosis represents a consequence of wound-

healing response to chronic stimulation. It is generally believed that cirrhosis is the outcome of interaction between liver damage and tissue repair. Hepatic satellite cells (HSCs) play a critical role in the progression of cirrhosis. However, the mechanisms by which HSCs modulate fibrogenesis and cirrhosis are less clarified. Active viral replication and intermediate inflammatory reactions facilitate hepatic fibrogenesis but the evolution of fibrogenesis might depart from HCC evolution^[12]. In this paper, we focus on virus-related cirrhosis and summarize recent progress on its molecular basis. Moreover, we also discuss possible therapeutic options for hepatic fibrosis, early cirrhosis, and even decompensated cirrhosis.

MOLECULAR BASIS OF VIRUS-RELATED CIRRHOSIS

Immune response and liver damage

Roles of immune cells and cytokines: The immune system is a complicated dynamic network constituted by various immune cells and cytokines. T and B lymphocytes, macrophages (macrophages residing in the liver are also called Kuffer cells), NK cells, neutrophils, HSCs, NKT cells, dendritic cells (DCs), and mast cells are all important in the maintenance of chronic inflammation. CD8⁺ cytotoxic T lymphocytes (CTL) and CD4⁺ T helper lymphocyte subpopulations [Th1, Th2, Th17, and regulatory T (Treg) cells] also play key roles in maintaining chronic inflammation. The immune effectors not only play critical roles in the HBV and HCV clearance, but also participate in liver damage. Toll-like receptors (TLR)-3 and -7 can recognize the viruses and induce the production of type I interferon (IFN) (IFN- α/β), proinflammatory cytokines and chemokines to inhibit the viruses^[13-15], whereas TLR-4 activation by lipopolysaccharides (LPS) in HSCs enhances TGF- β signaling and hepatic fibrosis^[16]. TGF- β , a pleiotropic cytokine produced by immune and non-immune cells, has receptors on several cell types. It induces fibrosis *via* increasing Th17 and HSCs and reducing NK cell activation^[17]. A study using TLR-9-deficient mouse model has demonstrated that TLR-9 is also involved in liver fibrosis^[18]. As important antiviral cytokines activated in the initial immune response, IFN- α/β can inhibit viral replication and lead to death of the infected hepatocytes by inducing the expression of multiple IFN-stimulated genes (ISG), including protein kinase R (PKR), Mx proteins, ISG-15, RnaseL/2',5'-oligoadenylate synthetase (2',5'-OAS) and RNA helicases^[19,20]. Additionally, it may activate the neighboring immune cells, including macrophages, NK cells, NKT cells and DCs^[21-23]. Both NK cells and CD8⁺ CTLs exert immunoregulatory functions *via* direct, non-MHC-restricted cytotoxic mechanisms and cytokine production^[24,25]. IFN- γ and tumor necrosis factor- α (TNF α) produced by NK cells and CD8⁺ CTLs can not only inhibit the viruses, but also cause liver damage through TNF-related apoptosis-inducing ligand

(TRAIL)-mediated death of hepatocytes^[25,26]. Moreover, CD8⁺ CTLs can exert the bystander killing effect through perforin, Fas/Fas ligand and TNF α pathways^[27], thus causing a wide range of hepatocellular apoptosis. IFN- γ -induced chemokines such as CXC chemokine ligands CXCL9, CXCL10, and CXCL11 can induce the migration of nonspecific mononuclear cells into the liver^[28], which are unable to control the infection but result in sustained liver damage. In addition, the antibody response has also been associated with the extent of liver injury during HCV chronic infection, and anti-E2 antibodies can mediate liver damage *via* antibody-dependent cell-mediated cytotoxicity (ADCC)^[29], but it just occurs after a prolonged HCV infection. The contributions of natural HCV antibodies to liver damage and fibrosis progression still need to be determined.

Dysfunction of T cells: In the phase of chronic HBV and HCV infections, specific T cells present a condition of dysfunction due to the persistent exposure to high levels of viral antigens that exceed the capacity of host immunity. It may be induced by the following mechanisms. First, genetic predisposition of immune-related genes contributes to persistence of HBV and/or HCV infections. Genetic polymorphism of class II human leukocyte antigen (HLA) has been associated with HBV^[30] and HCV persistence/clearance^[31], possibly because this genetic predisposition affects T cell function upon HBV infection. Genetic polymorphisms of other immune-related genes, notably, interleukin-28B (IL28B), have closely been associated with HCV clearance, progression, and treatment response^[32,33]. Second, antigen presentation by DCs and macrophages may be impaired, resulting in ineffective priming of T cells or deficient maintenance of antigen-experienced T cells^[34]. Additionally, dysfunctional specific T cells can express the inhibitory receptor programmed death-1 (PD-1), which inhibits immune activity and induces the apoptosis of T cells^[35-37]. Moreover, HCV core protein can inhibit T cell proliferation by binding to the complement receptor gC-1qR^[38,39]. During the prolonged infection, the expression of chemokine (C-C) motif ligand (CCL) 17 and CCL22, attractors for Tregs^[40,41], can inhibit the proliferation and function of T cells and other immune cells^[42]. The dysfunctional T cells can hardly eliminate HBV or HCV. On the contrary, the consistent but insufficient immune responses break up several balances between the immune cells or cytokines, such as Th1/Th2 cells or Th17/Treg cells, neutrophils/lymphocytes, neutrophils/CD8⁺ T cells, and Th1/Th2 cytokines^[43,44], which induces a high immune pressure and results in the evolution of the viruses during chronic infection.

Role of inflammatory signaling pathways

There are multiple inflammatory signaling pathways and molecules involved in the sustained inflammation caused by chronic infection, including nuclear factor- κ B (NF- κ B), Wnt/ β -catenin, TGF- β /Smad, RAF/MEK/

ERK, JAK/STAT, PI3K-AKT/PKB, Ras-MAPK, and Vitamin A^[45-48]. NF- κ B as a dimeric transcription factor can be activated by proinflammatory stimuli, such as TNF α or interleukin-1 β (IL-1 β)^[48]. The activated NF- κ B signaling pathway thereafter induced the expression of a series of growth factors and cytokines to regulate the inflammatory response. IL-6 can be released by macrophages and regulate the proliferation and differentiation of liver fibroblasts^[49]. The PI3K-AKT/PKB and Ras-MAPK pathways are also important because they are involved in the activation of HSCs. Platelet-derived growth factor (PDGF) can lead to the Ras-MAPK activation by binding its receptor, and the activation of protein kinase C (PKC) family through PI3K-AKT/PKB eventually induces cell proliferation and HSC activation^[50]. These signaling pathways and molecules may play an active role in the pathogenesis of HBV- and HCV-related cirrhosis, thus serving as therapeutic targets and prognostic markers.

HBV and HCV escape strategies in chronic infection

Spontaneous clearance of the viruses can be achieved in some infected subjects by an efficient immune response. However, following the early immune response, 70%-80% of HCV infected patients will develop chronic infection^[51] and 8.5% of adults with acute hepatitis B will develop chronic infection^[2]. Chronic infection of HBV occurs frequently in those who acquired the infection perinatally (90%) or during childhood (20%-30%), when the immune system is thought to be immature^[12]. It implies that HBV and HCV can produce a series of strategies to evade the clearance. Under immune pressure, HBV and HCV can evade the immune clearance through persistent viral replication and mutations, making themselves preferably adapt to the inflammatory microenvironment. However, HBV and HCV have different mutation patterns conforming to their distinct viral structures. HCV replicates at a rate of 10^{10} - 10^{12} virions per day. The RNA-dependent RNA polymerase lacks a proofreading function, favoring the Darwinian selection of viral variants by humoral and cellular immune responses^[52]. During the phase of acute infection, there is a high rate of nonsynonymous and synonymous substitutions due to the high levels of selective pressure exerted by antibodies and activated T cells, but the rate decreases at continuous infection stage^[53]. The HCV E2 glycoprotein is thought to be a major target for HCV-specific antibody. The nucleotide substitutions at the hypervariable HCV E2 region 1 during anti-HCV seroconversion in acutely HCV-infected individuals are intensively correlated with the viral evasion. HBV exists in the form of covalently closed circular DNA (cccDNA) in the nuclei of hepatocytes and exhibits higher frequencies of mutations than other DNA viruses due to the spontaneous error rate of reverse transcriptase. The rate of HBV mutation in HBeAg-positive patients is 1.5×10^{-5} - 5×10^{-5} nucleotide substitutions/site per year^[54], and is even higher in HBeAg-negative patients. Under

immune pressure, hypermutation provides viruses a choice not only for growth advantage, but also for long-term survival. APOBEC3, an important member of the APOBEC family of cellular cytidine deaminases, can inhibit HBV through a series of editing-dependent and -independent mechanisms. However, it is also involved in the viral genetic diversification and evolution^[12]. The expression of activation induced deaminase, an APOBEC3 paralog, has been observed in a variety of chronic inflammatory syndromes including HCV infection. Introduction of exogenous APOBEC3G into HCV-infected Huh7.5 human hepatocytes inhibits HCV replication; knockdown of endogenous APOBEC3G enhances HCV replication^[55]. Some APOBEC3 genes in primary human hepatocytes are up-regulated by IFN- α and IFN- γ . Up-regulation of APOBEC3 by inflammation inhibits HCV genome synthesis *via* viral editing. Two to five APOBEC3 genes are significantly up-regulated in cirrhotic livers in following order: HCV \pm HBV-related cirrhosis > HBV-related cirrhosis > alcoholic cirrhosis, compared to normal livers. In HBV-related cirrhosis, HBV genome is particularly edited by APOBEC3G, and APOBEC3G is the dominant deaminase *in vivo* with up to 35% of HBV genomes being edited^[56]. Hepatic inflammation might provide a “fertile field” to facilitate “viral mutation-selection-adaptation” evolutionary process *via* activating a series of enzymes including APOBEC3 and promote immune escape of HBV and HCV during chronic infection.

Association of HBV mutations with cirrhosis

HBV DNA consists of four overlapping open reading frames that encoding the envelope (pre-S/S), core (precore/core), polymerase (P), and X proteins, respectively. HBV preS region consists of preS1 and preS2 domains and they are essential for the immune responses because they contain several epitopes for T and/or B cells. In our previous studies, we defined wild-type nucleotides and mutations of HBV genotypes B and C, respectively. A nucleotide with the highest frequency in the sequences of HBV from asymptomatic HBsAg carriers (ASCs) seropositive for HBeAg was termed a wild-type nucleotide because HBeAg-positive HBV has been traditionally treated as a wild-type strain^[6,57,58]. Under this definition, we found some of the cirrhosis-related HBV mutations (compared to ASCs and CHB patients) and HCC-related HBV mutations (compared to HBV-infected subjects without HCC) in the preS and enhancer II (Enh II)/basal core promoter (BCP)/precore regions. In the preS region, 81.0% of preS1 wild-type nucleotide hotspots of HBV genotype C are significantly associated with an increased risk of cirrhosis, as compared with CHB; 90.5% of preS1 mutations of HBV genotype C are significantly associated with an increased risk of HCC, compared to cirrhosis. Furthermore, wild-type nucleotides A2964C and T3116C are independently associated with an increased risk of cirrhosis, whereas their mutation counterparts C2964A and

C3116T are independently associated with an increased risk of HCC^[57]. In the Enh II/BCP/precore region of HBV genotype C, C1673T, A1726C, A1727T, C1730G, C1766T, T1768A, C1773T, and C1799G are significantly associated with an increased risk of cirrhosis, compared to CHB; whereas these mutations are inversely associated with HCC risk, compared to cirrhosis. T1768A, A1762T/G1764A, and A1846T are independently associated with an increased risk of cirrhosis^[57]. The frequencies of G1652A, T1673C, and G1730C increase successively from the ASC state to CHB to cirrhosis, but significantly decrease in HCC^[58]. A study conducted in Taiwan has demonstrated that multiple HBV mutations including preS deletion, T1762/A1764, T1766 and/or A1768 are more often found than a single mutation patterns in the process of progression to cirrhosis^[59]. The evidence indicates that immune selection of some viral mutations in HBV evolution towards cirrhosis seems different from that in the evolution towards HCC. Even though, several important HCC-related HBV mutations, namely, preS deletion, C1653T, T1753V, and A1762T/G1764A successively increase in their frequencies during HBV evolution from ASC state, CHB, and cirrhosis to HCC^[58,60]. This reflects the robustness of HBV preS deletion, C1653T, T1753V, and A1762T/G1764A in predicting the entire evolutionary process from ASC state to HCC. Although cirrhosis is an important risk factor of HCC, most of the virus-related cirrhosis patients do not develop HCC. The difference in immune selection of viral mutants between evolutionary processes towards cirrhosis and HCC should be further elucidated.

Our previous study has demonstrated that genetic predisposition to HLA-DP function play an important role in immune selection of cirrhosis-related HBV mutations and a significant effect of HBV mutations on cirrhosis is selectively evident in those with HLA-DP genotypes that promote HBV persistence^[58]. HLA class II loci are mainly associated with spontaneous clearance of HBV and HCV. Interestingly, genetic polymorphisms within HLA II loci have been frequently associated with chronic HBV or HCV hepatitis, hepatic fibrosis, and the development of HCC^[61]. Thus, HLA-DP affects HBV persistence, regulates immune selection of viral mutations, and influences cirrhosis risk contributed by HBV mutations.

Cirrhosis-related HBV mutations biologically affect the progression from chronic hepatitis to cirrhosis. Approximately 30.9% of S gene mutations occur in the major hydrophilic region of HBs antigen, which can change the epitopes of the three-dimensional structure, and lead to immune escape of HBV. C1766T/T1768A, an independent risk factor of cirrhosis in HBeAg-negative patients, can increase the pre-genome mRNA encapsidation and then promote viral assembly^[62]. In addition, the X gene mutations such as G1386M, C1485T, and C1653T, can regulate the NF- κ B signaling pathway which plays a crucial role in the progression to cirrhosis or HCC^[63].

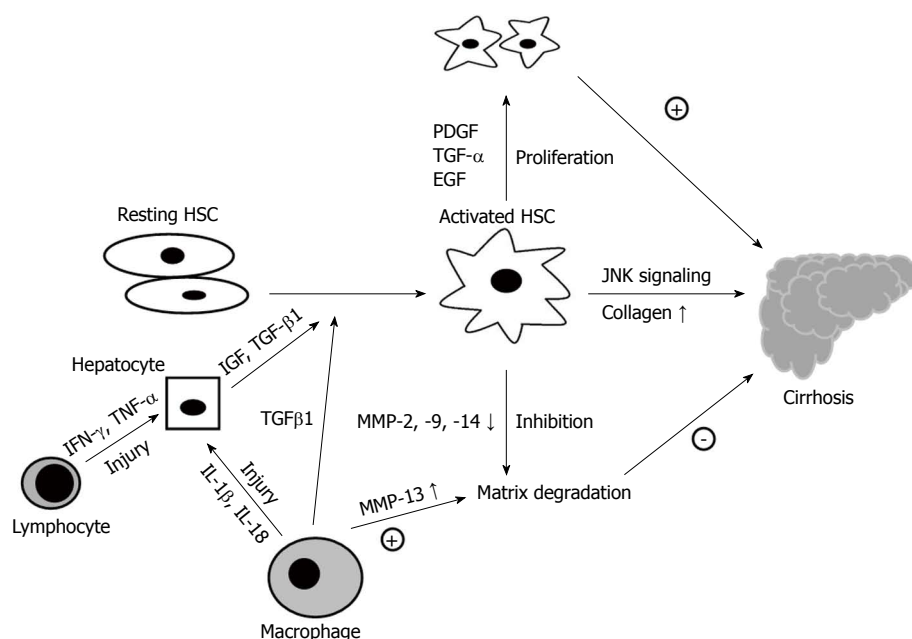


Figure 1 Process of hepatic satellite cells induced fibrosis. HSC: Hepatic satellite cell; IGF: Insulin-like growth factor; TGF: Transforming growth factor; PDGF: Platelet-derived growth factor; EGF: Epidermal growth factor; MMP: Matrix metalloproteinase; TNF: Tumor necrosis factor.

Tissue repair and liver fibrosis

Tissue repair following liver damage as a consequence of the inflammation through the accumulation of extracellular matrix (ECM) proteins mainly secreted by HSCs will eventually result in the occurrence of fibrosis. PDGF is the most potent mitogen for HSCs *via* the Ras-MAPK and PI3K-AKT/PKB signaling pathways^[50]. Moreover, TGF α and epidermal growth factor (EGF) can also stimulate the proliferation of HSCs. The “inactive” HSCs should undergo a process of unidirectional activation, contributing to differentiate into myofibroblast-like phenotypes during fibrosis^[64]. TGF- β 1, the most important profibrogenic cytokine known for activated HSCs, is mediated by intracellular signaling *via* Smad proteins. Smad2 and Smad3 proteins are associated with the activated receptor of TGF- β 1, whereas Smad7 is an effective inhibitor for TGF- β 1 signaling. In addition, TGF- β 1 also can increase α 1 collagen mRNA stability *via* p38 MAPK. HSCs also can trigger an activated process *via* phagocytizing the apoptotic bodies induced by virus infection^[65]. The c-Jun N-terminal kinase-1 (Jnk1) as a profibrotic kinase in HSCs, but not in hepatocytes, significantly contributes to liver fibrosis development^[66]. JNK is involved in HSC activation and fibrogenesis and represents a potential target for antifibrotic treatments^[67,68]. These fibrosis-promoting proteins will increase the secretion of fibrillar collagens, resulting in the deposition of excess fibrotic matrix. The activated HSCs also can inhibit the expression of matrix metalloproteinases (MMPs)-2, -9 and -14 which play a role because of proteolytic activity towards ECM, *via* promoting the expression of tissue inhibitors of metalloproteinases (TIMPs), thus inhibit the matrix degradation^[69]. The characteristics of HSC-mediated fibrogen-

esis result in disruption of the original architecture and liver dysfunction (Figure 1).

Macrophages also are critical for both liver damage and fibrosis. The proinflammatory cytokines interleukin-1 β (IL-1 β) and IL-18, which induced by macrophages in HCV-infected patients, can promote the inflammation *via* the NF- κ B signaling pathway, resulting in liver damage^[70]. Activated macrophages release growth factors, cytokines and chemokines which induce the recruitment of monocytes, thus affecting the function of HSCs and fibroblasts^[71]. In particular, TGF- β 1 and insulin-like growth factor can activate the fibroblasts and promote a switch in fibroblast gene expression to initiate matrix remodeling^[72]. Macrophages display a key role in the different stages of fibrosis and these characteristics may be induced by either different cytokines in the microenvironment or the populations of macrophages^[73]. On the other hand, macrophages can exert antifibrotic activity and promote the resolution of fibrosis by producing interstitial collagenases like MMP13^[74]. In addition, the phagocytosis of apoptotic hepatocytes has been shown to inhibit the development of fibrosis. Understanding the mechanism of fibrogenesis may pave the way for the treatment of cirrhosis.

THERAPEUTIC OPTIONS FOR VIRUS-RELATED LIVER CIRRHOSIS

Without appropriate therapy for cirrhosis caused by HBV and/or HCV infections, liver injury may persist, thus facilitating the development of decompensated cirrhosis and HCC, especially in those with active viral replication. As viral replication is an important driving force of cirrhosis development, antiviral treatment should be

carried out before progression into decompensated cirrhosis^[75,76]. In addition, as some signaling systems, such as JNK, have been recently associated with the formation of cirrhosis^[66-68], targeting these signaling systems might be an important option for the treatment of liver fibrosis and cirrhosis.

Antiviral treatment of HBV-related compensated cirrhosis

Around 30%-70% of the patients with compensated liver cirrhosis still have active viral replication, and this active viral replication promotes the progression of liver injury. Successful antiviral treatment prior to the development of cirrhosis is able to greatly reduce the morbidity and mortality of HBV-related end-stage liver diseases. Currently approved nucleos(t)ide analogs (NAs) for the treatment of HBV-related diseases include lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (TBV), and tenofovir disoproxil fumarate (TDF)^[77,78]. Antiviral therapy using newer NAs with lower resistance rates can suppress replication and re-activation of HBV, improve liver function, and restore many patients to a state of well compensated cirrhosis^[79,80]. Long-term antiviral therapy can prevent the development of liver decompensation in patients with compensated cirrhosis^[77]. The antiviral treatment to halt the progression of compensated cirrhosis should be carried out as early as the diagnosis has been confirmed.

Antiviral treatment of HBV-related decompensated cirrhosis

About 2%-5% of patients with HBV-related compensated cirrhosis developed decompensation every year^[81]. The prognosis of patients with decompensated cirrhosis is usually poor, with a 5-year survival rate of 14%-35%^[82]. All oral antivirals can prevent viral replication efficaciously and improve biochemical and clinical parameters in patients with viral-related decompensated cirrhosis. The selection of antivirals with high efficiency and a low rate of resistance is necessary to attain fast and enduring viral suppression. The use of LAM or TBV is restricted by drug resistance. ADV is restricted by its slower initiation of effect and potential risk of renal injury, which is fatal in decompensated patients. Furthermore, with the more use of TBV, serum creatinine phosphokinase levels frequently increase. Therefore, TBV should be used as a second-line drug for patients with decompensated cirrhosis because of the safety is not guaranteed. A meta-analysis of clinical trials on NA-naïve patients with HBeAg-positive CHB has demonstrated that TDF is associated with the highest probability of achieving undetectable HBV DNA at 1 year of all NAs considered^[83]. A randomized open-label study has shown that ETV has a virological efficacy precede that of ADV in HBV-related hepatic decompensation^[84]. TDF and ETV are well tolerated in these patients, with an improvement in virological, biochemical and clinical parameters^[85]. Recently, a meta-analysis showed that

LAM and TBV significantly decrease the mortality rate and disease severity in patients with decompensated cirrhosis. Also, both of them promote HBeAg seroconversion in these patients^[86]. NAs with low rates of inducing drug-resistant mutations and powerful and rapid HBV suppressive function, such as ETV or TDF, could be regarded as the first-line drugs for NAs-naïve patients with decompensated cirrhosis for long-term therapy^[84-91]. Even with low doses, the application of IFN- α in patients with HBV-related decompensated cirrhosis can facilitate clinical decompensation and increase the risk of bacterial infection^[92]. IFN is contraindicated in patients with HBV-related decompensated cirrhosis in the era of NAs. The studies using antivirals for the treatment of cirrhosis are listed in Table 1.

Antiviral therapy using newer NAs could delay or obviate liver transplantation in some patients^[76]. Some clinical guidelines suggest that the clinical improvement in some wait-listed patients with antiviral therapy could lead to their retreat from the transplant list^[93,94]. However, if the decompensation is caused by superimposed viral infection, the effect of anti-HBV therapy would be limited. In this situation, liver transplantation should be the most immediate option. Standard of therapy for patients with HBV-related decompensated cirrhosis in accordance with their clinical manifestations, including control of ascites, infection or encephalopathy, should be instituted quickly and sufficiently^[93]. Monitoring of HCC and timely transferring consultation for liver transplantation are also mandatory^[93,94].

Antiviral treatment of HCV-related cirrhosis

The antiviral treatment of HCV-related cirrhosis poses much greater challenges. IFN remains an essential element of HCV antiviral treatment, but has reduced efficacy and significant toxicity at this stage of cirrhosis^[79]. The current standard of care for HCV patients is the therapy with pegylated interferon (Peg-IFN) and ribavirin (RBV), leading to 45% of cure for genotype 1 HCV patients and approximately 80% for patients infected with HCV genotypes 2 and 3^[95]. The current standard of care for chronic hepatitis C is the combination of PEG-IFN α and RBV^[96]. A recent study provides solid evidence that anti-HCV treatment using recombinant or pegylated IFN plus RBV is equally effective in compensated cirrhosis both before and after liver transplantation^[97]. Several clinical trials have demonstrated that the new direct acting antivirals, such as Sofosbuvir, Daclatasvir, Asunaprevir, ABT-450, Faldaprevir, Simeprevir, and Deleobuvir, interact with several of vital components of HCV (NS3/4, NS5A, NS5B polymerase, *etc.*) and have a chance of viral eradication of 80%-90%, with a few negligible side effects^[98]. The combination of vaniprevir (a NS3/4A protease inhibitor) with Peg-IFN plus RBV significantly increase rates of sustained virologic response (SVR) among treatment-experienced patients with chronic HCV genotype 1 infection, compared to re-treatment with Peg-IFN plus RBV alone. Vaniprevir

Table 1 Current regimes of antiviral treatment for virus-related cirrhosis

| Medicine used | Number of patients | Virological responses | Survival | Ref. |
|---|--|--|---|--------------|
| HBV-related cirrhosis | | | | |
| ETV (1.0 mg/d) or ADV (10 mg/d) | 100 subjects treated with ETV, 91 subjects treated with ADV | 57% and 20% of subjects achieve HBV DNA undetectable after 48 wk (ETV and ADV, respectively) | Overall 1-yr patient survival rates were 84% and 83% (ETV and ADV, respectively) | [83] |
| TBV (600 mg/d) or LAM (100 mg/d) | 114 in the TBV group, another 114 in LAM group | 49.1% and 39.5% of subjects achieve HBV DNA undetectable after 104 wk (TBV and LAM, respectively) | Overall 1-yr patient survival rates were 94% and 88% (TBV and LAM, respectively) | [85] |
| ETV (0.5 mg/d) | 144 compensated and 55 decompensated cirrhosis patients treated with ETV | 78.5% and 89.1% of subjects achieve HBV DNA undetectable after 12 mo (compensated and decompensated, respectively) | Not analyzed | [86] |
| TDF (300 mg/d) or TDF (300 mg/d) + FTC (200 mg/d) or ETV (0.5 mg/d) ADV (10 mg/d) | 45 in TDF group, 45 in TDF plus FTC group and 22 in ETV group | 70.5%, 87.8% and 72.7% achieve HBV DNA undetectable after 48 wk (TDF, TDF plus FTC, ETV, respectively) | Not analyzed | [84] |
| | 226 wait-listed and 241 post-LT patients | 59% and 40% achieve HBV DNA undetectable after 48 wk (wait-listed and post transplantation, respectively) | Overall 1-yr patient survival rates were 86% and 91% (wait-listed and post transplantation, respectively) | [87] |
| HCV-related cirrhosis | | | | |
| Group 1: PegIFN-2a + RBV ² Group 2: PegIFN-2b + RBV ³ Group 3: PegIFN-2a + placebo ⁴ Treated: IFN ⁵ Untreated: placebo | 453 in group 1, 444 in group 2 and 224 in group 3 72 patients in both treated group and untreated group | 69%, 52% and 59% achieve SVR ¹ after 24 wk (group 1, group 2 and group 3, respectively) Not analyzed | Not analyzed 5-yr overall survival is 50% and 39% in treated and untreated group, respectively | [81] [89] |
| Non-LT and LT cirrhotic patients are all treated for PEG-IFN a2a or a2b plus RBV ⁶ | 43 HCV non-LT cirrhotic patients and 17 LT HCV related-cirrhotic patients | 69.8% and 47.1% achieve EVR and 41.9% and 29.4% achieve SVR (non-LT group and LT group, respectively) | None of the non-LT cirrhotic patients died; LT cirrhotic patients with survival rates of 87% at 1 yr and of 76% at 3 and 5 yr after the treatment | [97] |

¹Undetectable HCV RNA in serum after 24 wk of post-treatment follow-up; ²Once-weekly injections of 180 mcg of PegIFN-2a plus RBV (1000 mg/d);

³Thrice-weekly injections of 3 million units of PegIFN-2b plus RBV (1000 mg/d); ⁴Once-weekly injections of 180 mcg of PegIFN-2a plus daily placebo;

⁵Treatment started with 1 mega unit three times weekly for three months then increased every three months to 3, 6, and 9 mega units; ⁶Peg-IFN-2a or -2b: at the dose of 180 mcg/wk or 1.5 mcg/kg per week respectively; and RBV 800-1200 mg, based on body weight. HBV: Hepatitis B virus; ETV: Entecavir; TBV: Telbivudine; LAM: Lamivudine; ETV: Entecavir; ADV: Adefovir; TDF: Tenofovir disoproxil fumarate; FTC: Emtricitabine; HCV: Hepatitis C virus; SVR: Sustained virological response; PegIFN: Peginterferon; RBV: Ribavirin; CHB: Chronic hepatitis B; IFN: Interferon; LT: Liver transplanted; EVR: Early virological response.

is generally well-tolerated for up to 48 wk in those with compensated cirrhosis^[99]. Eltrombopag (an oral, non-peptide, thrombopoietin receptor agonist) can significantly increase platelet numbers in thrombocytopenic patients with HCV-induced cirrhosis, allowing otherwise ineligible or marginal patients to begin and maintain antiviral therapy and leading to significantly increased rates of SVR^[100]. It has been well-established that the CC genotype of the genetic polymorphism rs12979860 located at 3 kilobases upstream of the *IL28B* gene, encoding IFN-lambda-3, is associated with spontaneous clearance of HCV infection and an approximately 2-fold change in response to treatment with Peg-IFN plus RBV^[101,102]. The frequency of C allele of rs12979860 is 80.3% in subjects of European ancestry and 56.2% in those of African ancestry^[101], which might be one of reasons why the European population is more apt to eradicate HCV than the African population. The frequency of rs12979860 CC genotype is 84.1% in Chinese HCV-positive patients^[111], indicating that HCV-related cirrhosis in Chinese HCV carriers should not be as common as

in HCV carriers of African ancestry. Subsequent studies have also demonstrated that the IL-28B rs8099917 genotype TT significantly predict SVR in patients chronically infected with HCV genotype 1 to PEG-IFN-RBV therapy^[96,103]. The prediction of nonresponse to the treatment is mandatory to avoid side effects and reduce costs^[104]. Genotyping the IL-28B rs12979860 and/or rs8099917 should be considered before the treatment of HCV-related cirrhosis. The baseline mean model of end stage-liver disease (MELD) score predicts the risk of hepatic decompensation during antiviral therapy^[105], and should be considered for the treatment of HCV-related cirrhosis with peg-IFN and RBV. Thus, antiviral treatment with PEG-IFN+RBV and/or DAAs is recommended to prevent the progression of HCV-related cirrhosis in patients with the CC genotype of rs12979860 and/or the TT genotype of rs8099917 polymorphisms.

Potential stem cell treatment for decompensated cirrhosis

Liver transplantation is the currently last option for the

treatment of virus-related decompensated cirrhosis patients who fail to respond to antiviral treatments. Due to the lack of donors, surgical complications, rejection reactions, and high cost for liver transplantation, other strategies have been considered for the treatment of decompensated cirrhosis. Of those, stem cell-based treatments can be expected to be an alternative for patients with liver failure or decompensated cirrhosis because it may improve scarring and supplement hepatocytes^[106,107]. Repopulation induced hepatic stem cells (iHepSCs) can become hepatocyte-like cells in the injured liver of fumarylacetoacetate hydrolase (Fah)-deficient mice^[108]. Mesenchymal stem cells (MSCs) produce inhibitory cytokines or induce the development of regulatory T cells in the inflammatory and fibrotic processes, therefore they play an immunomodulatory role in this process although many details remain unknown^[109]. Interestingly, MSC therapy seems to be effective in regulating the immune response in liver injury, transplantation, and autoimmunity in both patients in clinical trials and animal models of liver disease^[110]. MSCs can directly suppress the activation of the main cell source of ECM, HSCs, *via* MSC-derived IL-10 and TNF- α , and may also induce HSC apoptosis *via* the Fas/FasL pathway^[111]. Therefore, MSCs are considered to work through multiple mechanisms to harmonize a dynamic, integrated response to liver inflammation and fibrosis, which prevents the progressive distortion of hepatic architecture.

Another actual objective of MSC treatment is to substitute impaired hepatocytes in patients with liver failure or decompensated cirrhosis with exogenous functional hepatocytes^[106]. For this reason, induced pluripotent stem (iPS) cells and embryonic stem (ES) cells have been shown to be the most competent, producing large numbers of functional hepatocyte-like cells (HLCs) in both animal models and humans. However, ethical issues and indeterminacy about their reaction *in vivo* in a proper homeostatic manner have limited their clinical applications^[112]. It is currently unknown whether MSC therapy could induce side effects such as hepatic artery dissection, fibrogenesis, and even tumorigenesis. The long-term clinical significance and safety of stem cell-based therapies should be confirmed in large-scale randomized controlled trials. Thus, the co-transplantation of iPS/ES-derived MSCs and HLCs may offer the potency for a series of new therapeutic interventions for liver diseases^[106]. It will be highly important to tailor future stem cell therapies to specific patient types due to the mutable feature of different stem cells (ES, iPS, and MSCs).

Regenerative therapies have the potential to provide minimally invasive procedures with few complications. The potential for stem cells in bone marrow (BM) to differentiate into hepatocytes and intestinal cells was confirmed through detection of Y chromosome-containing cells in samples from female recipients of BM cells (BMCs) from male donors^[113-115]. Recent studies showed that use of whole bone marrow as a cell therapy in a rodent model with chronic liver disease led to the evolu-

tion of hepatic fibrosis^[116]. These studies suggest that BMCs are effective sources for regenerative liver therapy. A study group have found that targeting androgen receptor, which is a key factor in male sexual phenotype in bone marrow mesenchymal stem cells (BM-MSCs), can improve the therapeutic efficacy of transplantation for liver fibrosis^[117]. Autologous BMC infusion (ABMI) in patients with cirrhosis is one of regenerative therapies. Serum albumin levels and Child-Pugh scores significantly improved after ABMI therapy, and the most important is lack of adverse effects^[118]. Thus, ABMI therapy should be developed as a hopeful option for the treatment of decompensated cirrhosis.

CONCLUSION

The process of virus-induced cirrhosis is a dynamic, multifaceted network. Inflammation provides a suitable microenvironment for the evolution of viral mutation-selection-adaptation, which in turn causes disease-specific viral mutation pattern. The repeated liver damage and tissue repair eventually progress to fibrosis and cirrhosis. In this process, both HSCs and macrophages are important for the fibrogenesis *via* excessive accumulation of ECM, and macrophages also promote the formation of fibrosis. Oral NAs can prevent viral replication efficiently in viral-related decompensated cirrhosis, cause the stabilization or improvement of liver function, and improve survival. Antiviral treatment should be started as early as the diagnosis has been confirmed. Targeting key signaling pathway should be effective in halting the progression of cirrhosis. Moreover, stem cell-based treatments could be an option for patients with liver failure or decompensated cirrhosis. Future studies should focus more on insight into the cross-link between the mechanisms and therapeutic options.

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Selection of a TIPS stent for management of portal hypertension in liver cirrhosis: An evidence-based review

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Abstract

Nowadays, transjugular intrahepatic portosystemic shunt (TIPS) has become a mainstay treatment option for the management of portal hypertension-related complications in liver cirrhosis. Accumulated evidence has shown that its indications are being gradually expanded. Notwithstanding, less attention has been paid for the selection of an appropriate stent during a TIPS procedure. Herein, we attempt to review the current evidence regarding the diameter, type, brand, and position of TIPS stents. Several following recommendations may be considered in the clinical practice: (1) a 10-mm stent may be more effective than an 8-mm stent for the management of portal hypertension, and may be superior to a 12-mm stent for the improvement of survival and shunt patency; (2) covered stents are superior to bare stents for reducing the development of shunt dysfunction; (3) if available, Viatorr stent-grafts may be recommended due to a higher rate of shunt patency; and (4) the placement of a TIPS stent in the left portal vein branch may be more reasonable for decreasing

the development of hepatic encephalopathy. However, given relatively low quality of evidence, prospective well-designed studies should be warranted to further confirm these recommendations.

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Key words: Transjugular intrahepatic portosystemic shunt; Portal hypertension; Liver cirrhosis; Variceal bleeding; Hepatic encephalopathy; Shunt dysfunction

Core tip: This review suggests the following: first, a 10-mm stent may be more effective than an 8-mm or 12-mm stent for the management of portal hypertension in liver cirrhosis; second, Viatorr covered stents may be recommended for maintaining the shunt patency; finally, the placement of a transjugular intrahepatic portosystemic shunt stent in the left portal vein branch may be more reasonable for decreasing the development of hepatic encephalopathy.

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INTRODUCTION

Transjugular intrahepatic portosystemic shunt (TIPS) refers to an interventional creation of a shunt between the portal vein and the hepatic vein or inferior vena cava by deploying an expandable stent, thereby reducing the portosystemic pressure gradient^[1,2]. Compared with the traditional surgical portosystemic shunt, the major advantages of TIPS include local anaesthesia and less invasive-

Table 1 Transjugular intrahepatic portosystemic shunt for the prevention of variceal rebleeding: An overview of meta-analyses

| Ref. | Design | No. trials | Comparative arms | Target population | Efficacy of TIPS | Encephalopathy | Survival or death |
|---|-----------------------|------------|--|----------------------------------|---|--|--|
| Zheng <i>et al</i> ^[6] | Meta-analysis of RCTs | 12 | TIPS <i>vs</i> endoscopic treatment | Variceal rebleeding in cirrhosis | Variceal rebleeding: TIPS was lower ($P < 0.00001$) | The frequency of HE: TIPS was higher ($P < 0.00001$) | Death due to all causes: NS |
| Khan <i>et al</i> ^[7] | Meta-analysis of RCTs | 22 | Portosystemic shunts (surgical or TIPS) <i>vs</i> endoscopic therapy | Variceal rebleeding in cirrhosis | Rebleeding: shunt was lower | Acute or chronic HE: shunt was higher | Mortality: NS |
| Burroughs <i>et al</i> ^[8] | Meta-analysis of RCTs | 13 | TIPS <i>vs</i> endoscopic treatment | Variceal rebleeding in cirrhosis | Recurrent bleeding: TIPS was lower | Encephalopathy: TIPS was higher | Survival: NS |
| Papatheodoridis <i>et al</i> ^[9] | Meta-analysis of RCTs | 11 | TIPS <i>vs</i> endoscopic treatment | Variceal rebleeding | Variceal rebleeding: TIPS was lower ($P < 0.001$) | Encephalopathy: TIPS was higher ($P < 0.001$) | Overall mortality: NS; sensitivity analyses: NS |
| Luca <i>et al</i> ^[10] | Meta-analysis of RCTs | 11 | TIPS <i>vs</i> endoscopic treatment with or without propranolol | Recurrent bleeding in cirrhosis | Recurrent bleeding: TIPS was lower | Encephalopathy: TIPS was higher | Death due to all causes: NS; death due to bleeding: NS |

HE: Hepatic encephalopathy; NS: Not significant; RCT: Randomized controlled trial; TIPS: Transjugular intrahepatic portosystemic shunt.

Table 2 Transjugular intrahepatic portosystemic shunt for the treatment of refractory ascites: An overview of meta-analyses

| Ref. | Design | No. trials | Comparative arms | Target population | Efficacy of TIPS | Encephalopathy | Survival or death |
|---------------------------------------|--|------------|--|---------------------------------------|---|--|--|
| Chen <i>et al</i> ^[25] | Meta-regression and Trial Sequential Meta-analysis | 6 | TIPS <i>vs</i> large-volume paracentesis | Refractory ascites in liver cirrhosis | Ameliorate refractory ascites: TIPS was better ($P < 0.05$) | Frequency of HE: TIPS was higher ($P < 0.01$) | Overall mortality: NS; subgroup mortality (patients with better hepatic and renal function): TIPS was lower ($P < 0.05$) |
| Salerno <i>et al</i> ^[26] | Meta-analysis of individual patient data | 4 | TIPS <i>vs</i> large-volume paracentesis | Refractory ascites in liver cirrhosis | Tense ascites recurrence: TIPS was lower ($P < 0.0001$) | Average number of HE episodes: TIPS was higher ($P = 0.006$) | Transplant-free survival: TIPS was better ($P = 0.035$) |
| Saab <i>et al</i> ^[27] | Meta-analysis of RCTs | 5 | TIPS <i>vs</i> paracentesis | Refractory ascites in liver cirrhosis | Re-accumulation of ascites: TIPS was lower ($P < 0.01$) | Frequency of HE: TIPS was higher ($P < 0.01$) | 30-d mortality: NS; 24-mo mortality: NS |
| D'Amico <i>et al</i> ^[28] | Meta-analysis of RCTs | 5 | TIPS <i>vs</i> paracentesis | Refractory ascites in liver cirrhosis | Recurrence of ascites: TIPS was lower ($P < 0.05$) | Frequency of HE: TIPS was higher ($P < 0.05$) | Mortality: NS |
| Albillos <i>et al</i> ^[29] | Meta-analysis of RCTs | 5 | TIPS <i>vs</i> paracentesis | Refractory ascites in liver cirrhosis | Ascites recurrence: TIPS was lower ($P < 0.05$) | Risk of HE: TIPS was greater | Overall mortality: NS; subgroup mortality (patients with recidivant ascites): TIPS was lower ($P < 0.05$) |
| Deltenre <i>et al</i> ^[30] | Meta-analysis of RCTs | 5 | TIPS <i>vs</i> large-volume paracentesis | Refractory ascites in liver cirrhosis | Control of ascites: TIPS was better ($P < 0.001$) | HE: TIPS was higher ($P < 0.001$) | Survival: NS |

HE: Hepatic encephalopathy; NS: Not significant; RCT: Randomized controlled trial; TIPS: Transjugular intrahepatic portosystemic shunt.

ness. Since its first clinical application, TIPS has been widely used for the treatment of portal hypertension-related complications in liver cirrhosis for nearly 25 years^[3]. Existing and well-established evidence supports the following indications for TIPS^[4,5]. First, TIPS should be recommended as the second-line treatment option for the prevention of variceal rebleeding in liver cirrhosis^[4]. This recommendation is mainly based on the results of 5 meta-analyses^[6-10] and 12 randomized controlled trials^[11-22] (Table 1). Although TIPS significantly reduces the incidence of variceal rebleeding in liver cirrhosis, it cannot improve the survival with a significantly higher rate of hepatic encephalopathy and shunt dysfunction. Second,

TIPS should be used as the rescue treatment for acute variceal bleeding that is not responsive to medical and/or endoscopic therapy in liver cirrhosis^[4]. However, a recent multi-center randomized trial has shown a significant survival benefit of early TIPS with covered stents for the treatment of acute variceal bleeding in high-risk cirrhotic patients^[23], which potentially challenges the current recommendation^[24]. Third, TIPS should be used for the treatment of refractory ascites that is not responsive to large volume paracentesis^[4]. This recommendation primarily originates from the results of 6 meta-analyses^[25-30] and 6 randomized controlled trials^[31-35] (Table 2). Notably, the subgroup meta-analyses have shown that TIPS

can significantly reduce the mortality in patients with recidivant ascites^[29] and those with better hepatic and renal function^[25]. More importantly, a meta-analysis of individual data has revealed that TIPS can significantly improve the transplant-free survival^[26]. This positive conclusion is also confirmed by our recent meta-analysis using hazard ratios (our unpublished data). However, due to a high incidence of post-TIPS hepatic encephalopathy, it is still regarded as the second-line therapy of choice. Apart from these classical indications, emerging evidence has attempted to establish the novel indications for TIPS, such as the management of gastric variceal bleeding^[36,37], ectopic variceal bleeding^[37-39], hepatic hydrothorax^[40-42], hepatorenal syndrome^[42-44], portal vein thrombosis^[45-49], and Budd-Chiari syndrome (BCS)^[50-52].

Generally, accumulated evidence has witnessed the essential role of TIPS for the management of portal hypertension in liver cirrhosis. Notwithstanding, the technical details remain controversial, such as the selection of stents and puncture position, necessity of adjunctive variceal embolization (see a recent meta-analysis^[53]), and benefit of postoperative anticoagulation or anti-platelets (see previous randomized controlled trials^[54,55]). In this paper, we focus on reviewing the current evidence regarding the diameter, type, brand, and position of TIPS stents. Other issues are beyond the scope of this review.

DIAMETER OF TIPS STENTS: 8-MM, 10-MM VS 12-MM

Theoretically, a larger diameter of TIPS stent can reach the target portosystemic pressure gradient more effectively and rapidly. However, the excessive shunting of portal blood flow can induce the development of hepatic dysfunction and encephalopathy. Therefore, it is important to choose an appropriate diameter of stent to balance between the efficacy and complications of TIPS.

An early retrospective study compared the outcomes of TIPS between cirrhotic patients receiving 10-mm ($n = 23$) and 12-mm ($n = 23$) Wallstents^[56]. The 1-d occlusion rate was significantly higher in the 12-mm stent group than in the 10-mm stent group (17% *vs* 0%). But the long-term primary and secondary patency rates were similar between the two groups. Additionally, the 1-mo mortality rate was higher in the 12-mm stent group than in the 10-mm stent group (26% *vs* 4%). More importantly, the survival time was significantly shorter in the 12-mm stent group than in the 10-mm stent group ($P < 0.03$) over the course of the study.

Recently, an Italian, single-center, randomized controlled trial compared the outcomes of TIPS between cirrhotic patients with variceal bleeding or refractory ascites receiving 8-mm ($n = 22$) and 10-mm ($n = 23$) PTFE-covered stents^[57]. The 10-mm stents were more effective than the 8-mm stents for reducing the portosystemic pressure gradient after TIPS (6.5 ± 2.7 mmHg *vs* 8.9 ± 2.7 mmHg, $P = 0.007$). Accordingly, the 10-mm stent group was also superior to the 8-mm stent group for decreasing

the 1-year rate of remaining free of recurrence and/or persistence of complications due to portal hypertension (82.9% *vs* 41.9%, $P = 0.002$, by Log-Rank test). In details, the difference was statistically significant in patients treated for refractory ascites, but was slight in those treated for variceal bleeding. In spite of its advantages in the improvement of portal hypertension, the 10-mm stent group was similar to the 8-mm stent group for the 1-year rate of remaining free of post-TIPS hepatic encephalopathy (46.7% *vs* 42.6%, $P = 0.48$, by Log-Rank test) and 1-year cumulative survival rate (79.6% *vs* 79.1%, $P = 0.20$, by Log-Rank test).

On the basis of these findings, it might be recommended that the 10-mm stent, rather than 12-mm or 8-mm stent, was more appropriate for TIPS procedure. Notably, the latter clinical trial was prematurely stopped due to the side effects of treatment failure from the 8-mm stent group^[57]. The behavior might influence the weight of these conclusions. In this case, the statistical difference in the incidence of post-TIPS hepatic encephalopathy as the primary endpoint could not be reached. Additionally, the subgroup analysis of this trial did not show any significant improvement of variceal rebleeding in the 10-mm stent group^[57]. Due to the potential limitations, a randomized controlled trial (ClinicalTrials.gov: NCT01410591) is ongoing to primarily compare the incidence of shunt dysfunction as the primary endpoint in cirrhotic patients with at least one episode of variceal bleeding receiving 10-mm and 8-mm covered stents.

TYPE OF STENTS: COVERED VS BARE

In the era of bare stents, a high incidence of shunt dysfunction is one of the most severe complications of TIPS. Since the introduction of covered stents, numerous comparative studies^[45,58-69] (Table 3) and case series^[70-77] (Table 4) have shown their remarkable benefit in the improvement of shunt patency. However, only one of these studies was randomized controlled trial^[68]. In this European, multi-national, randomized controlled trial, 80 cirrhotic patients were assigned to the covered ($n = 39$) and bare ($n = 41$) stent groups^[68]. The preliminary analysis confirmed a lower incidence of shunt dysfunction (5/39 *vs* 18/41, $P < 0.001$) and clinical relapse (3/39 *vs* 12/41, $P < 0.05$) in the covered stent group. Subsequently, an extended follow-up analysis further demonstrated a higher actuarial rate of remaining free of hepatic encephalopathy (67% *vs* 51%, $P < 0.05$) in the covered stent group^[69]. But no survival benefit from the covered stents was found^[68,69]. Thus, the wide application of covered stents during a TIPS procedure was greatly prompted by these promising findings. But the potentially lethal complication associated with covered stents should not be neglected, such as segmental liver ischemia due to the obstruction of hepatic venous outflow caused by covered stents^[78-80].

Recently, a meta-analysis of 6 studies, including 346 and 929 patients receiving covered and bare stents, re-

Table 3 Comparison of outcome after transjugular intrahepatic portosystemic shunt between covered and bare stents: An overview of comparative studies

| Ref. | Period | Target population | No. patients (covered/bare) | Efficacy of TIPS (covered/bare) | Shunt dysfunction or patency (covered/bare) | Post-TIPS encephalopathy (covered/bare) | Survival or death (covered/bare) |
|---------------------------------------|-----------------|--|-----------------------------|--|--|--|--|
| Luca <i>et al</i> ^[45] | 2003.1-2010.2 | Cirrhotic patients with non-tumoural PVT | 70 (57/13) | NA | 12-mo shunt dysfunction rate: 21%/38%; 24-mo shunt dysfunction rate: 29%/85% | NA | NA |
| Sommer <i>et al</i> ^[58] | 2001.2-2011.1 | Patients with elective TIPS procedures | 174 (58/116) | Clinical success rate: ascites: 90.5%/81.3%; ascites + bleeding: 85.7%/73.7%; bleeding: 90.0%/86.2% (NS) | 12-mo primary shunt patency rate: 62.4%/43.9% ($P < 0.05$) | Overall rate: 36.5%/37.5% (NS) | 12-mo survival rate: 79.1%/75.6%; overall survival time: 835.25 ± 823.0 (9-3200)/805.6 ± 868.4 (6-3290) d (NS) |
| Clark <i>et al</i> ^[59] | 2001-2010 | Patients with PH | 246 (176/70) | NA | Overall shunt dysfunction rate: 22% / 57% ($P = 0.05$) | NA | Survival time: 33/31 mo ($P = 0.5$) |
| Maleux <i>et al</i> ^[60] | 1992-2006 | Cirrhotic patients with refractory ascites | 222 (126/96) | Rate of clinically significant residual ascites 1 mo after TIPS: 35.5%/55.6% ($P = 0.003$) | 1-yr shunt dysfunction rate: 19%/49% ($P < 0.0001$) | 1-yr rate: 22%/56% ($P < 0.0001$) | 6-mo survival rate: 73.2%/62.8%; 1-yr survival rate: 65.5%/55.0% ($P = 0.0071$) |
| Wu <i>et al</i> ^[61] | 2007.4-2009.4 | Patients with PH | 60 (30/30) | Number of rebleeding: 1/6 ($P = 0.04$) | Number of shunt dysfunction: 0/9 ($P = 0.002$) | Number: 5/6 ($P = 0.74$) | Number of death: 0/4 ($P = 0.038$) |
| Bandi <i>et al</i> ^[62] | 2006.3-2009.3 | Patients with PH | 66 (33/33) | Clinical relapse number (rate): 8 (26%)/15 (45%) ($P < 0.05$) | Number of shunt dysfunction: 5/15 ($P < 0.05$) | Overall rate: 22%/33% (NS) | Overall survival rate: 66%/37% ($P < 0.05$) |
| Jung <i>et al</i> ^[63] | 1996.6-2006.2 | Patients who received de novo TIPS | 81 (51/30) | Bleeding group: 3-mo clinical success rate: 100%/58% ($P = 0.03$); 12-mo clinical success rate: 67%/18% ($P = 0.046$). Ascites group: 3-mo clinical success rate: 77%/70% ($P = 0.2$); 12-mo clinical success rate: 64%/33% ($P = 0.18$) | 3-mo primary patency rate: 94%/63% ($P = 0.03$); 6-mo primary patency rate: 67%/8% ($P = 0.47$); 12-mo primary patency rate: 38%/24% ($P = 0.65$) | Overall rate: 15%/14% ($P = 0.7$) | Bleeding group: 30-d mortality rate: 40%/33% ($P = 0.69$); overall mortality rate: 40%/50% ($P = 0.57$). Ascites group: 30-d mortality rate: 6%/27% ($P = 0.13$); overall mortality rate: 13%/55% ($P = 0.02$) |
| Pan <i>et al</i> ^[64] | 2001.1- 2005.12 | Patients with variceal bleeding and refractory ascites | 128 (57/71) | NA | 30-d shunt dysfunction rate: 1.8%/4.2% ($P = 0.4$); 6-mo shunt dysfunction rate: 5.2%/25.3% ($P = 0.003$); 1-yr shunt dysfunction rate: 5.2%/30.9% ($P = 0.004$); overall shunt dysfunction rate: 8.7%/40.8% ($P = 0.004$) | NA | 6-mo mortality rate: 10.5%/16.9% ($P = 0.3$); 1-yr mortality rate: 14%/23.9% ($P = 0.2$); overall mortality rate: 21.1%/35.2% ($P = 0.08$) |
| Tripathi <i>et al</i> ^[65] | 1991.7- 2004.12 | Patients with variceal bleeding, ascites, portal hypertensive gastropathy, hepatic hydrothorax | 473 (157/316) | 2-yr cumulative rebleeding rate: 6%/17% ($P < 0.05$) | 2-yr cumulative shunt dysfunction rate: 11%/74% ($P < 0.001$); overall shunt dysfunction rate: 8%/48% | 2-yr cumulative rate: 23%/38% ($P < 0.05$) | 2-yr cumulative mortality rate: 49%/50% |

| | | | | | | | |
|--|-----------------|--|------------|--|---|--|--|
| Gandini <i>et al</i> ^[66] | 1994.1- 2003.11 | Patients with BCS | 13 (7/6) | Clinical relapse rate: 100%/0% | 6-mo primary patency rate: 100%/16.7%; 12-mo primary patency rate: 85.7%/0% ($P < 0.001$, Log-Rank) | Overall rate: 0%/0% | NA |
| Barrio <i>et al</i> ^[67] | 1998.9-2002.5 | Cirrhotic patients with PH related complications | 70 (20/50) | Rate of clinical recurrence of portal hypertension related complications: 0%/22% ($P = 0.085$) | 6-mo shunt dysfunction rate: 0%/32%; 12-mo shunt dysfunction rate: 0%/82% ($P = 0.03$, Log-Rank) | 1-mo rate: 41%/20%; 3-mo rate: 44%/34%; 9-mo rate: 44%/40% ($P = 0.5$, Log-Rank) | 6-mo survival rate: 67%/88%; 12-mo survival rate: 67%/81% ($P = 0.11$, Log-Rank) |
| Bureau <i>et al</i> ^[68,69] | 2000.2-2002.4 | Patients with cirrhosis and uncontrolled bleeding, recurrent bleeding, or refractory ascites | 80 (39/41) | Clinical relapse rate: 7.7%/29.3% | 1-yr primary patency rate: 85.6%/46.6%; 2-yr primary patency rate: 80.2%/18.6% ($P = 0.0005$, Log-Rank) | 1-yr rate: 22%/41% ($P = 0.0586$) | 1-yr survival rate: 70.9%/59.5%; 2-yr survival rate: 64.5%/40.5% |

BCS: Budd-Chiari syndrome; NA: Not available; NS: Not significant; PH: Portal hypertension; PVT: Portal vein thrombosis; TIPS: Transjugular intrahepatic portosystemic shunt.

spectively, showed not only a significant improvement of primary patency (HR = 0.28) and a significant reduction of risk of hepatic encephalopathy (HR = 0.65) but also a significant decrease of mortality in the covered stent group (HR = 0.76)^[81]. In addition, the heterogeneity among studies was not significant in all analyses. But it should be noted that the indication for TIPS was heterogeneous among these included studies.

Taken together, covered stents should be recommended for the TIPS procedure. More importantly, because bare stents were employed in all previous randomized controlled trials comparing the outcome between cirrhotic patients with portal hypertension receiving TIPS and those receiving other treatments, the role of TIPS with covered stents in the management of portal hypertension should be reconsidered in future trials^[82]. Until now, one completed trial (Current Controlled Trials number: ISRCTN58150114) has shown positive results that the early use of TIPS with covered stents can significantly reduce the treatment failure and mortality of acute variceal bleeding in high-risk cirrhotic patients^[23]. Additionally, several ongoing trials have attempted to further update the indications of TIPS, as follows: (1) whether TIPS with coated stents or paracentesis plus albumin administration is better for the treatment of refractory ascites in patients with cirrhosis (ClinicalTrials.gov: NCT00222014); (2) whether TIPS with covered stents or endoscopic band ligation is better in cirrhosis with recurrent variceal bleeding non-responding to medical therapy (ClinicalTrials.gov: NCT00570973); (3) whether TIPS endoprosthesis or large volume paracentesis is better for the treatment of ascites in patients with portal hypertension (ClinicalTrials.gov: NCT01236339); (4) whether early TIPS with covered stents or non-selective beta blocker plus endoscopic treatment is better for acute variceal bleeding in high-risk cirrhotic patients (ClinicalTrials.gov: NCT01370161); and (5) whether TIPS with covered

stents or conventional treatment is better for the prevention of variceal rebleeding in cirrhotic patients with portal vein thrombosis (ClinicalTrials.gov: NCT01326949).

As for BCS patients, the benefit of covered stents appears to be controversial. In 17 retrospective case series focusing on the outcome of BCS treated with TIPS alone^[52], the rate of shunt dysfunction is 18%-100%, which is higher in patients with BCS than in those with cirrhotic portal hypertension. This phenomenon may be attributed to the hypercoagulability and more complex anatomy in BCS patients. Although most of studies support the use of covered stents for improving the shunt patency^[50,66,83-88], a large study reports a similar shunt patency rate (bare stents: 81% *vs* covered stents: 85%)^[89]. More recently, our retrospective study of 51 BCS patients treated with TIPS, by using Cox regression, demonstrated no significant association between the type of stents (bare *vs* covered) and the development of shunt dysfunction (HR = 1.14, 95%CI: 0.46-2.82, $P = 0.775$)^[51]. Certainly, the results should be cautiously interpreted, due to a relatively small number of patients, a short follow-up time, the retrospective nature of this study, and the use of Fluency stents.

BRAND OF COVERED STENTS: FLUENCY VS VIATORR

Currently, the Viatorr stent-graft (Gore WL and Associates, Flagstaff, AZ, United States), which is produced as the specialized TIPS endoprosthesis, is commercially available in the United States and Europe. Alternatively, Fluency covered stent (Angiomed GmbH Co. subsidiary of C.R. Bard, Inc.), which is mainly employed for the treatment of iliac artery diseases, can be purchased in some other countries, such as China mainland. They have different designs. The former mainly includes a 4 to 8-cm-long intra-hepatic region covered by PTFE inside a

Table 4 Outcome of transjugular intrahepatic portosystemic shunt with covered stents: An overview of case series

| Ref. | Period | n | Indication for TIPS | Liver function | Follow-up time ¹ | Patients with shunt dysfunction (n) | Cumulative shunt dysfunction or patency rate | HE (n) | No. Pts death (n) |
|--|-----------------|-----|--|---------------------|-----------------------------|---|---|-----------|----------------------|
| Sajja <i>et al</i> ^[70] | 2001.1- 2011.12 | 59 | Ascites (16), variceal bleeding (31), both (12) | MELD score: 12.5 | 654 ± 341 d (253-1584) | 6-mo: 8; overall: 14 | NA | 15 | 7 |
| Wu <i>et al</i> ^[71] | NA | 114 | Pure esophageal variceal disruption hemorrhage (92), pure refractory cirrhotic ascites (8), esophageal variceal disruption hemorrhage with refractory ascites (14) | CPC A/B/C: 29/68/34 | NA | 16 | 1-yr dysfunction rate: 13.3%; 2-yr dysfunction rate: 24.8% | 23 | NA |
| Wu <i>et al</i> ^[72] | 2008.1- 2011.12 | 150 | Gastroesophageal variceal bleeding (134), refractory ascites (16) | CPC A/B/C: 30/81/39 | 24.1 ± 8.8 mo | 17 | NA | 23 | 18 |
| Rössle <i>et al</i> ^[73] | 2000.4-2004.10 | 100 | Variceal bleeding (41); refractory ascites, hydrothorax, or hepatorenal syndrome (59) | CPC A/B/C: 21/58/21 | 22 ± 15 (0.8-47) mo | 6-mo: 6; 1-yr: 7; 2-yr: 11; overall: 16 | NA | NA | 22 |
| Vignali <i>et al</i> ^[74] | 2001.2-2003.12 | 114 | Variceal bleeding (49), refractory ascites (52), hypertensive gastropathy (10), BCS (1), hepatorenal syndrome (2) | CPC A/B/C: 8/60/46 | 11.9 ± 10.2 (0-38) mo | 15 | 6-mo dysfunction rate: 8.1%; 1-yr dysfunction rate: 20.1%; 2-yr dysfunction rate: 24.1% | 27 | 35 |
| Maleux <i>et al</i> ^[75] | 2000.8-2003.5 | 56 | Upper variceal bleeding (18), refractory ascites (23), variceal bleeding with refractory ascites (10), refractory ascites with hydrothorax (4), hydrothorax (1) | CPC A/B/C: 8/13/35 | 337 (4-962) d | 1 | NA | 10 | 30-d: 3; overall: 16 |
| Charon <i>et al</i> ^[76] | 2000.7-2003.1 | 100 | Variceal bleeding (81), refractory ascites (19) | CPC A/B/C: 20/46/34 | 261 (45-837) d | 11 | 1-yr patency rate: 84% | Acute: 13 | 45 |
| Hausegger <i>et al</i> ^[77] | 1999.9-2002.3 | 71 | Refractory ascites (44), recurrent esophageal bleeding (27) | CPC A/B/C: 10/43/18 | NA | 9 | 6-mo patency rate: 87.4%; 1-yr patency rate: 80.8% | 18 | 30-d: 7; overall: 20 |

¹Data are expressed as absolute mean ± SD (range) or mean (range). BCS: Budd-Chiari syndrome; CPC: Child-Pugh class; HE: Hepatic encephalopathy; MELD: Model for end-stage liver disease; NA: Not available.

stent and a 2-cm-long portal-vein region uncovered. The latter is fully covered by PTFE inside and outside a bare stent without a bare segment at the portal vein end of the stent. Thus, the placement of a Fluency stent should not be extended into the main portal vein trunk. Otherwise, the hepatic perfusion from the portal vein blood flow would be affected.

In a retrospective study, the investigators compared the outcome of TIPS between patients receiving Viatorr stents only ($n = 28$) and those receiving Fluency stents only ($n = 93$)^[90]. Although the major encephalopathy rate was not significantly different between the two groups (3.6% *vs* 4.3%, $P = 1.0$), the Viatorr stent group showed a higher hemodynamic success rate (98% *vs* 90%) and primary unassisted patency rate (6-mo: 95% *vs* 87%; 12-mo: 89% *vs* 81%, $P = 0.03$) than the Fluency stent group. No-

tably, the development of shunt dysfunction was primarily attributed to the stenosis of the portal and hepatic vein end in the Fluency and Viatorr stent groups, respectively. The difference in the causes of shunt dysfunction might be explained by the different design of the two stents.

In a retrospective case series regarding the outcome of TIPS for the treatment of BCS, Fluency covered stents elevated the incidence of post-TIPS hepatic encephalopathy than bare stents^[51]. This might be explained by the possibility that fully covered stents decreased hepatic perfusion, thereby preventing the liver from removing toxic substances from the body. However, the retrospective nature and a small sample size of this study might limit the generalization of this finding.

Collectively, the Viatorr stent may be superior to the Fluency stent in reducing the incidence of shunt

dysfunction. Certainly, the Fluency stent should be an alternative choice due to the limited availability of Viatorr stent in some regions. In addition, a combined Wallstent/Fluency stent may be considered to further improve the shunt patency^[90].

POSITION OF STENT PLACEMENT: LEFT VS RIGHT PORTAL VEIN BRANCH

As for the proximal (*i.e.*, hepatic vein) end of the stent placement, the optimal position is the confluence of the hepatic vein and the inferior vena cava^[91]. This is primarily because venous intimal hyperplasia would develop due to the increased high-velocity blood flow after TIPS insertion and thereby lead to hepatic vein stenosis^[92], if a stent did not cover the proximal end of the hepatic vein. As for the distant (*i.e.*, portal vein) end, the stent placement into the right portal vein branch is preferred during a TIPS procedure. This is mainly because it is relatively easier to puncture from the hepatic vein to the right portal vein branch in routine clinical practice. However, whether the placement of TIPS stents into the left or right portal vein branch is more beneficial has been rarely recognized. In a recent randomized controlled trial, 72 advanced cirrhotic patients undergoing TIPS were assigned to the left and right portal vein branch groups^[93]. The findings of this trial were impressive that the placement of stents into the left portal vein branch led to a significantly lower incidence of overall hepatic encephalopathy (7/36 *vs* 14/32, $P = 0.036$) and *de novo* encephalopathy (4/36 *vs* 12/32, $P = 0.012$) after TIPS insertion. Accordingly, the proportion of patients re-admitted to the hospital at least once was significantly lower in the left portal vein branch group than in the right portal vein branch group (16/36 *vs* 24/32, $P = 0.015$). Also, the total cost per patient within the first 2 years was significantly lower in the left portal vein branch group than in the right portal vein branch group. But the position of stent placement did not significantly impact the reduction of portosystemic pressure gradient after TIPS (10.2 ± 1.6 *vs* 10.4 ± 1.4 , $P = 0.889$), the prevention of variceal rebleeding (6/36 *vs* 5/32, $P = 0.907$), and the control of ascites persistence or recurrence (11/36 *vs* 15/32, $P = 0.167$).

This randomized study suggests the rationality of placing a stent into the left portal vein branch during a TIPS procedure. This may be explained by the anatomy of the portal venous system. In the normal circumstance, 30% and 70% of the blood from the main portal vein is drained into the left and right portal vein branch, respectively. Thus, as the stent is placed in the right portal vein branch, a larger amount of blood will be bypassed from the right liver lobe that is nearly 6 times larger than the left liver lobe, thereby greatly decreasing the hepatic perfusion and inducing the development of liver dysfunction and hepatic encephalopathy. By comparison, the stent placement into the left portal vein branch may produce a lower risk of hepatic encephalopathy.

Notably, this conclusion needs to be balanced in the

real-world clinical situations. An occlusive intrahepatic portal vein branch is considered an important factor for TIPS failure in patients with portal vein thrombosis^[46]. Thus, to increase the rate of TIPS success, the stent should be placed in a patent vessel, regardless of left or right portal vein branch. In addition, an ideal position of stent placement is often difficult to be achieved in BCS patients with hepatic vein thrombosis and hepatic enlargement and congestion, because the stent is often placed through a long distance between the IVC and portal vein.

CONCLUSION

Selection of an appropriate stent during a TIPS procedure is very important for the shunt function and treatment efficacy. By reviewing the current evidence, several following recommendations may be considered in the clinical practice: (1) a 10-mm stent may be superior to an 8-mm or 12-mm stent for the management of portal hypertension and the improvement of shunt patency; (2) covered stents are better than bare stents for decreasing the shunt dysfunction; (3) if available, Viatorr stent-grafts may be superior to Fluency stent-grafts for the improvement of shunt patency; and (4) the placement of a stent in the left portal vein branch may improve the hepatic perfusion and decrease the incidence of hepatic encephalopathy. However, we have to acknowledge that these recommendations are based on a majority of retrospective studies. Therefore, prospective well-designed studies should be warranted to confirm them.

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Historical overview and review of current day treatment in the management of acute variceal haemorrhage

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decades with improved survival from an often-terminating event in recent past.

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Key words: Variceal haemorrhage; Oesophageal varices; Gastric varices; Portal hypertension

Core tip: This review article focuses on how the management of variceal haemorrhage, has changed and evolved over the decades. A novel historical approach detailing changes per decades is taken - with a review of each therapies and its impact on outcome.

Abstract

Variceal haemorrhage is one of the most devastating consequences of portal hypertension, with a 1-year mortality of 40%. With the passage of time, acute management strategies have developed with improved survival. The major historical treatment landmarks in the management of variceal haemorrhage can be divided into surgical, medical, endoscopic and radiological breakthroughs. We sought to provide a historical overview of the management of variceal haemorrhage and how treatment modalities over time have impacted on clinical outcomes. A PubMed search of the following terms: portal hypertension, variceal haemorrhage, gastric varices, oesophageal varices, transjugular intrahepatic portosystemic shunt was performed. To complement this, Google™ was searched with the aforementioned terms. Other relevant references were identified after review of the reference lists of articles. The review of therapeutic advances was conducted divided into pre-1970s, 1970/80s, 1990s, 2000-2010 and post-2010. Also, a summary and review on the pathophysiology of portal hypertension and clinical outcomes in variceal haemorrhage was performed. Aided by the development of endoscopic therapies, medication and improved radiological interventions; the management of variceal haemorrhage has changed over recent de-

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INTRODUCTION

Gastro-oesophageal variceal haemorrhage is a life-threatening complication of portal hypertension. Historically, overall mortality rates have been reported up to 30%-50%^[1] and 1-year mortality as high as 70%^[2]. Chronic liver disease of any aetiology can result in portal hypertension, the key event leading to formation of portosystemic collaterals including gastro-oesophageal varices. An increase in portal pressure is the most important risk factor for the development of varices^[2]. The onset of portal hypertension can not only cause variceal haemorrhage, but also herald the development of other complications of liver cirrhosis such as ascites formation and hepatic encephalopathy. Therapies to reduce portal hypertension, along with improved resuscitation techniques and the advent of broad-spectrum antibiotics in variceal haemorrhage have improved outcomes^[1]. Novel endoscopic and

radiological therapies have also improved outcomes and now play a pivotal role in the management of variceal haemorrhage. Injection sclerotherapy with sclerosant agents have been largely superseded by endoscopic variceal band ligation (EVBL) for oesophageal variceal haemorrhage whilst for gastric variceal haemorrhage, tissue adhesives have become increasingly used and incorporated into consensus guidelines as 1st line therapies^[2]. In addition to direct endoscopic therapies, measures have been introduced such as increased access to endoscopy including 24-h “bleeding rotas” performed by skilled endoscopists. These have coincided with the decline in use of tamponade equipment such as the Minnesota, Linton-Nachlas and Sengstaken-Blakemore tubes, and virtual extinction of emergency surgical procedures such as oesophageal transection or portocaval shunt formation, which had high associated mortality^[1,3]. With all the pharmacological, radiological and endoscopic developments, mortality has fallen in the last 3 decades, and in one study mortality rates fell from 42%^[4] in 1981 to recent actual rates ranging from 6%-12%^[3]. New radiological procedures such as transjugular intrahepatic portosystemic stent-shunts (TIPSS) and balloon retrograde transvenous obliteration (BRTO) have a role in acute variceal haemorrhage often as “rescue therapy” when endoscopic therapies have failed. The emerging role of TIPSS in an “early” setting, within 72 h after haemostasis following the index bleed in high-risk patients has been recently studied^[4]. The excellent results could lead to new paradigm in the utility of TIPSS following variceal bleeding.

This article aims to focus on the outcomes following variceal bleeding and how, over time, these have improved with the advent of new medical therapies and endoscopic and radiological therapies. A PubMed search was performed using the following keywords: portal hypertension, variceal haemorrhage, gastric varices, oesophageal varices, transjugular intrahepatic portosystemic shunt, TIPS and TIPSS. From this search 37431 articles were found, however 127 articles/abstracts were studied for the writing of this review article. This search was complemented by a search of the keywords using www.google.comTM.

PATHOPHYSIOLOGY OF PORTAL HYPERTENSION AND UTILITY OF HEPATIC VENOUS PRESSURE GRADIENT

Portal hypertension results from 3 principal events. The first is of a purely mechanical obstruction due to fibrosis or regenerative nodules resulting in increased resistance to flow. The second mechanism accounts for 20%-30% of increased intrahepatic resistance to portal inflow. There is contraction of sinusoidal and extra sinusoidal contractile cells (stellate cells and VSMCs) with intrahepatic imbalance between vasoconstrictors (such as endothelin-1 and angiotensin) and vasodilators (such as nitric oxide and glucagon). This imbalance leads to reduced

intrahepatic eNOS activity. This second event is modifiable with medications such as including beta-blockers and nitrates. These events together result in the development of the portosystemic collateral circulation with the aim of decompressing the portal circulation. However, the opposite occurs, with splanchnic vasodilatation in response to a relatively ischaemic liver or extrahepatic excess of NO, with sGC-PKG signalling and smooth muscle cell relaxation^[3]. The increased portal blood flow maintains portal hypertension. A hyperdynamic circulation results due to these haemodynamic changes in cirrhosis and portal hypertension. This manifests as high cardiac output with low systematic vascular resistance and arterial hypotension^[5].

Portal pressure can be derived from the hepatic venous pressure gradient (HVPG), which is normally in the range 1-5 mmHg. This is performed by advancing a catheter until it is wedged into a hepatic vein thus gaining a wedged hepatic vein pressure (WHVP)^[6].

Initial studies on estimation of portal pressure from an occluded hepatic venule date as far back as 1951^[2]. $HVPG = WHVP - \text{free hepatic venous pressure (FHVP)}$ where HVPG represents the gradient between portal and caval pressure. FHVP cancels out variations in abdominal pressure and acts as an internal zero. Sinusoidal and post sinusoidal, but not pre-sinusoidal portal hypertension results in a raised HVPG as the resistance to flow extends from the hepatic venous system to the portal vein. It has been demonstrated that varices are more likely to develop if the HVPG is $> 10 \text{ mmHg}$ ^[7].

CLINICAL VARIABLES OF OUTCOME IN VARICEAL HAEMORRHAGE

Variceal haemorrhage is a life-threatening emergency, with a mortality of up to 20% at 6-wk^[2,8]. It is now considered that any death occurring within 6 wk from a hospital admission for variceal haemorrhage be considered a variceal bleed-related death^[2]. Other end-points are the advent of rebleeding after 1st variceal bleed (index bleed) or failure to control bleeding, which are often used to define outcomes. Rebleeding is an important predictive factor of mortality and a good indicator of the success of intervention directly targeted at upper gastrointestinal (GI) bleeding^[9]. The factors contributing to outcome often from an upper GI bleed in patients with cirrhosis can be broadly divided into those correlating to severity of bleed and then also those relating to severity of liver disease.

The most applicable measurement of portal hypertension is the HVPG, which has been shown to be of prognostic benefit in patients having an acute variceal haemorrhage. Moitinho *et al*^[10] found HVPG the only independent predictor of 5-d treatment failure after variceal bleed (rebleeding or death) with the best cut-off of HVPG of 20 mmHg. HVPG has also been found to be an independent predictor of 6-wk and 1 year mortality (38% *vs* 5% in those with HVPG < 20 , and 65% *vs* 20%

Table 1 Predictors of day 5 treatment failure

| Variable | OR | 95%CI |
|-----------------------------|------|-----------|
| Transfusion in 24 h (units) | 1.35 | 1.13-1.61 |
| CTP class | 2.27 | 1.22-4.22 |
| AST (per 10 U increase) | 1.03 | 1.01-1.06 |
| PV thrombosis | 2.75 | 1.25-6.04 |

Adapted from D'Amico *et al*^[14]. AST: Aspartate aminotransferase; PV: Portal vein; CTP: Child-Turcotte-Pugh.

at 1 year)^[10,11]. A single HVPG measurement 2 wk after a variceal bleed has been shown to be an independent predictor for survival^[12] with those patients having a measurement < 16 mmHg having a 35% 2 year survival (compared to 15% in those with HVPG > 16 mmHg). In those patients on vasoactive therapy, a HVPG response to treatment (*i.e.*, > 20% drop from baseline of to < 12 mmHg)^[13] are independent predictors of survival.

The severity of liver disease can also be measured by a number of easily clinically accessible scoring systems including the Child-Pugh Turcotte (CPT) score/grade and the MELD scores. In an Italian study of 465 patients^[14], prognostic parameters for 6-wk mortality and also day 5 failure (*i.e.*, uncontrolled bleeding, rebleeding or death) were studied in patients with cirrhosis and an upper gastrointestinal bleed (Tables 1 and 2). The variables in this study could be divided into three variables: (1) severity of underlying liver disease (CTP and its components); (2) specific features of liver disease (HCC and portal vein thrombosis); and (3) severity of bleeding (transfusion requirement and rise in aspartate aminotransferase as reflected by hypotension causing ischaemic hit to liver).

In another study by Carbonell *et al*^[3] patients presenting to a centre with variceal bleeding were studied over 2 decades with 523 episodes of GI bleeding encountered in 468 patients with cirrhosis (319 episodes of variceal bleeding in 295 patients). On multivariate analysis, independent predictors of survival were: younger age ($P = 0.04$), antibiotic prophylaxis ($P = 0.01$), endoscopic therapy ($P = 0.008$), lower CPT score ($P < 0.0001$) and absence of hypovolemic shock ($P = 0.005$). In this same study, persistent bleeding at admission and absence of endoscopic therapy were independent predictors of rebleeding ($P = 0.004$ and $P = 0.01$ respectively). Interestingly, mortality fell from 9%-0% in CPT-A patients and 46%-0% in CPT-B over 20 years. Even in the patients CPT-C disease, mortality fell from 70% to 32%.

The advent of infection, encephalopathy and acute kidney injury (AKI) have been shown to be important late prognostic markers after the 1st index bleed^[15] with AKI, rebleeding, HCC and encephalopathy all independent predictors of mortality in 403 patients presenting with an upper GI bleed in liver cirrhosis (of which 187 episodes were from varices). In this retrospective study, predictors of rebleeding included CPT class ($P < 0.001$) and severity of bleeding ($P < 0.005$) with rebleeding more common in those with oesophageal varices (OR = 4.3, 95%CI: 2.6-7.2). In a retrospective study by Thomopou-

Table 2 Predictors of 6-wk mortality

| Variable | OR | 95%CI |
|-------------------------------|------|-----------|
| Albumin (per 1 g reduction) | 2.33 | 1.32-4.00 |
| Bilirubin (per 1 mg increase) | 1.23 | 1.10-1.37 |
| Transfusion total (units) | 1.40 | 1.19-1.66 |
| Hepatocellular carcinoma | 3.44 | 1.64-7.24 |
| Encephalopathy | 2.30 | 1.39-3.70 |

Adapted from D'Amico *et al*^[14].

los *et al*^[16] identified clinical predictors for early and late mortality in patients with variceal haemorrhage. Child-Pugh C (and haemodynamic shock - another marker of severity of bleed) on admission were independent predictors of 6-wk mortality ($P = 0.003$ and 0.0037 respectively). Predictors of 1 year mortality at initial admission included: Child-Pugh C ($P = 0.028$), presence of hepatocellular carcinoma ($P = 0.04$) and partial thromboplastin time ($P = 0.021$) Mortality however in this series was not affected by the presence of active bleeding at endoscopy or infection. Thus with set parameters in measuring outcomes from acute variceal bleeding - in severity of liver disease and also severity of haemorrhage; different therapeutic strategies over the years have evolved, improving outcomes in this potentially life threatening condition.

MANAGEMENT STRATEGIES AND THEIR INFLUENCE ON OUTCOME

Pre-1970s

Sclerotherapy for the management of oesophageal varices was described initially by Crafoord and Freckner^[17] in 1939 with injection of Quinine. However, it was not until later in the 20th century that this therapy became commonplace in the management of variceal haemorrhage, especially with the advent of fibre-optic endoscopy. Surgery was the mainstay of therapy for variceal haemorrhage prior to the 1970s. Surgical techniques such as oesophageal stapling or oesophagectomy were used, but with high mortality rates from complications such as sepsis, liver failure and renal failure^[18]. In patients with portal hypertension, devascularisation procedures were shown to reduce variceal bleeding and mortality in primary prophylaxis in the 1980s, although there was heterogeneity in one such study by Inokuchi *et al*^[19] with recruitment from a total of 22 centres. Shunt formation such as a splenorenal shunt was also performed with rebleeding rates varying from 5%-40%^[20,21]. The role of splenectomy was and continues to be useful in patients with segmental portal hypertension secondary to an isolated splenic vein thrombosis. However, this surgical procedure was established later in the 20th century. Surgical therapies in present guidelines are reserved for patients who fail endoscopic therapies, and have been superseded by either TIPSS as rescue therapy or early TIPSS post index variceal bleeding, which will be discussed later^[2].

Another method used prior to the advent of endo-

scopic therapy pre-1970s was balloon tamponade. The Sengstaken-Blakemore tube's use was first described in 1950 by Sengstaken and Blakemore^[22] although the role of balloon tamponade was initially described in 1930^[23]. Its place has largely been superseded by endoscopic therapies, however 21st century guidelines^[24] still suggest a role for balloon tamponade, being used in massive haemorrhage as a bridge until definitive treatment can be instituted (for a maximum of 24 h). Although developed pre-1970s, its role in variceal haemorrhage was secured later in the century with effectiveness in controlling acute bleeding in up to 90% of patients, however with up to 50% rebleeding rates when the balloon was deflated^[25]. Complications of balloon tamponade include aspiration pneumonia (often compounded by variceal haemorrhage event itself in encephalopathic patients) and oesophageal ulceration or rupture^[26] in up to 15%-20%.

1970/1980s

The Linton-Nachlas balloon was developed in the 1970s^[27] with a single 600 mL gastric balloon. The safety of this tube compared to Sengstaken Blakemore tube was identified in controlled trial of 79 patients with oesophago-gastric variceal haemorrhage^[28]. Both types of tamponade therapies resulted in primary haemostasis rates of 86%, but when bleeding from oesophageal varices was assessed, the Sengstaken Blakemore tube achieved permanent haemostasis in 52% compared the Linton-Nachlas tube 30%. The latter was more effective at controlling gastric variceal haemorrhage with 50% primary haemostasis rates compared to total failure in the Sengstaken Blakemore arm. The use of balloon tamponade as definitive therapy however was to be revolutionised by the advent of the fibre-optic endoscope and the therapies that could be delivered with it.

Rigid endoscopes were replaced by narrow fibre-optic endoscopes allowing therapy to be deployed through accessory channels. With a new and easier method for not only diagnosis of variceal haemorrhage but also therapeutic manoeuvres, new therapies were developed. The use of the overtube was phased out, patient comfort was improved and twin channel endoscopes were developed. The first reported case series of endoscopic sclerotherapy^[29] was published in the early 1970s with its use becoming more established in the 1980s and thereafter. The concept was that the bleeding varix would "thrombose off" by internal injection of sclerosant causing vascular thrombosis and vascular obliteration^[30]. Ethanolamine oleate, sodium tetradecyl sulphate, polidocanol, sodium morrhuate and ethanol have been used for injection sclerotherapy and successfully used in controlled trials^[31]. In Europe the most common agents used were ethanolamine oleate and polidocanol, whereas in the United States sodium morrhuate was preferred^[32,33]. Paravariceal injection involved injection around the varix causing variceal occlusion by tamponade and subsequent submucosal fibrosis of tissue around the varix, whereas intra-variceal injection induced thrombosis and subsequent occlu-

sion of the lumen^[34]. A meta-analysis by D'Amico *et al*^[1] showed the type and volume of sclerosant did not seem to affect the efficacy.

Another issue of trials using injection sclerotherapy in the late 1980s (and 1990s) was the confounding factor that some trials had patients who were not actively bleeding at the time of initial endoscopy^[1,35]. Furthermore, the optimal doses of sclerosing agents is unknown, with heterogeneity in scheduled follow-up endoscopies, and also differences between para- and intra-variceal injections^[36,37]. There was however no doubt of sclerotherapy efficacy in the role of variceal bleeding. Sclerotherapy was compared to placebo in a controlled trial, with 56 patients having sclerotherapy injection and 60 placebo in patients with variceal bleeding. Survival was significantly better in those treated by sclerotherapy ($P < 0.001$)^[38]. Sclerotherapy was also compared with oesophageal transection in 4 randomised trials^[39-42] with similar mortality rates but rebleeding rates higher in the sclerotherapy arms. Only one trial showed a statistically significant reduction in failure to control bleeding with surgery^[39]. When sclerotherapy was compared to balloon tamponade in 4 trials^[43-46], 2 trials showed significantly higher control of bleeding with sclerotherapy^[43,44].

In 1988, the first human cases were described of the use of EVBL in patients with oesophageal varices, based on the concept of banding haemorrhoids with elastic O-rings^[47]. This technique was initially applied to canine models in the late 1980s^[48,49] and then to patients with portal hypertension by Van Stiegmann *et al*^[50]. EVBL was then successfully incorporated into the management of oesophageal variceal bleeding in the 1990s.

1990s

In the 1990s, further trials were carried out with injection sclerotherapy in not only oesophageal but also gastric variceal haemorrhage. Endoscopic therapy with sclerotherapy was found to control active bleeding from oesophageal varices in more than 90% of patients, and effective in reducing the frequency of rebleeding^[51-53]. Injection sclerotherapy agents were compared, however most studies found them to have similar efficacy, although with some differences in cost^[54,55] and time to obliteration^[54,56]. The choice of sclerosant was dependant often on the operator and availability in the endoscopy units. A meta-analysis of 5 studies (Laine L, personal communication^[24]) of 251 patients, showed significant benefits of sclerotherapy in terms of initial haemostasis rates compared to sham sclerotherapy, vasopressor therapy alone or balloon tamponade. In another meta-analysis, sclerotherapy was found to be the "gold standard" in acute variceal bleeding^[57] with survival benefit seen when used in combination with vasoconstrictors than vasoconstrictors alone. Thus its role in the management of variceal bleeding became established. Injection sclerotherapy use was also extended to the treatment of gastric varices initially by Gotlib and Zimmerman^[58] in 1984. The mechanism of action became clearer in the 1980s and 1990s

Table 3 Comparison of vasoactive pharmacological therapies used in variceal haemorrhage

| | Octreotide | Somatostatin | Terlipressin |
|------------------------------|---|--|---|
| Mode of administration | Bolus followed by IV infusion | Bolus followed by IV infusion | IV bolus |
| Class | Somatostatin analogue | | Synthetic analogue of Vasopressin |
| Indication | Variceal haemorrhage | Variceal haemorrhage | Variceal haemorrhage Hepatorenal syndrome |
| Proposed mechanism of action | Mechanism unclear Inhibition of glucagon-mediated splanchnic vasodilatation and reduction of postprandial gut hyperemia | Amino-acid peptide that reduced splanchnic blood flow (especially azygous). Prevent release of vasoactive peptides | V1 receptors blockade causing splanchnic vasoconstriction |
| Dose | Bolus of 50 µg, followed by an infusion of 50 µg per hour for up to 5 d | Infusion of 250-500 µg/h | 2 mg bolus followed by 1 mg every 4 h for 3-5 d |
| Side effects/cautions | Vomiting, abdominal pain, nausea, hepatitis, abnormal LFTs, diarrhoea, hypoglycaemia. Rarely arrhythmias, dyspnoea, pancreatitis, rash and alopecia | Loss of appetite, nausea, vomiting, abdominal, diarrhoea and fatigue | Vasoconstrictive side-effects: myocardial ischemia, limb ischemia (avoid if peripheral vascular disease), nausea and diarrhoea. Hyponatraemia |

LFTs: Liver function tests.

with reports of gastric variceal endothelial damage with subsequent sclerosis^[58]. Sarin *et al*^[59] reported a 71.6% variceal obliteration rate in patients with gastric variceal haemorrhage treated with sclerotherapy. However, high re-bleeding rates of up to 60%-90% were reported^[26,60]. The combination of ethanolamine sclerosant and cyanoacrylate glue was reported to produce rapid eradication of oesophagogastric varices, with fewer number of injection sessions^[61,62].

The 1990s also saw the role of EVBL developed to the forefront of oesophageal variceal haemorrhage. EVBL however is not without complications including: oesophageal ulceration, chest pain, transient dysphagia and occasionally oesophageal stricturing seen at follow-up endoscopy. EVBL however evolved in the 1990s and into the 21st century as the recommended standard treatment for bleeding oesophageal varices^[24]. In a meta-analysis of 10 randomised controlled trials comparing sclerotherapy with EVBL, there was a non-significant benefit of EVBL in achieving initial haemostasis *vs* sclerotherapy (pooled relative risk of 0.53 with 95%CI: 0.28-1.01)^[63]. In one particular study, HVPG increased significantly immediately after both therapies but remained elevated for the duration of the 5 d in the sclerotherapy group whilst returning to baseline levels by 48 h after EVBL group^[64] thus potentially identifying a rationale for the use of EVBL over sclerotherapy. In another meta-analysis there was no difference in initial haemostasis rates between both modalities (RR = 1.1, 95%CI: 0.4-2.9)^[65], but actively bleeding patients represented only a small subset from larger trials^[24].

To complement endoscopic therapies, pharmacological therapies were developed for optimising outcomes in variceal bleeding (Table 3). The lowering of portal pressure, even prior to endoscopy, if the source of upper gastrointestinal bleeding was suspected to be variceal^[2,66] became an important issue. To that end, vasopressin and terlipressin were developed and deployed in such a setting. Terlipressin (triglycyl-lysine vasopressin) is a

synthetic analogue of vasopressin with longer half-life negating the need for continuous infusion and acts on V1 receptors leading to splanchnic vasoconstriction. This in turn reduces portal inflow and pressure. Consequently there is an improvement in renal blood flow and reduction in portal pressure. Blockade of the V2 receptors can also result in free water absorption in the renal collecting ducts. Vasopressin (mainly used in the United States due to the unavailability in terlipressin) had been shown to achieve haemostasis in 60%-80%^[67] of patients, but compared with terlipressin had less effect on the reduction of early rebleeding and did not improve survival from active variceal haemorrhage. Terlipressin was shown to reduce all-cause mortality when compared to placebo in meta-analyses^[68,69] and guidelines recommend early treatment, which should be continued for up to 5 d^[24] when potential for rebleeding is greatest. Side-effects include peripheral or coronary ischaemia, nausea and diarrhoea. Blocking activation of the V2 receptors of the renal tubules can cause a dilutional hyponatraemia, an effect that reverses rapidly on discontinuation of the drug. When compared to somatostatin analogues such as octreotide, the haemodynamic effects of terlipressin on portal pressure were found to be more sustained^[69] suggesting terlipressin might have a more prolonged benefit in bleeding varices. Thus vasoactive drugs became a key part of the initial therapy in variceal haemorrhage.

One of the major radiological advances in the management of variceal haemorrhage in the 1990's was the advent of TIPSS. Although first described in 1983 by Colapinto *et al*^[70], it was largely in the 1990s and thereafter that its place in the management of portal hypertensive complications was secured. TIPSS involves the placement of a stent between the portal and hepatic vein to reduce portal pressure, thus stemming variceal haemorrhage or preventing rebleeding. Complications of TIPSS include haemorrhage, infection, intravascular haemolysis, liver dysfunction, shunt dysfunction and worsening of hepatic encephalopathy^[71,72]. Initial TIPSS were

bare-metal stents with rebleeding rates of up to 20% at 2 years^[73]. TIPSS was initially used for uncontrolled bleeding with control of bleeding in 90%-95% of patients and a 4-wk survival of 50%-60%^[74]. In a review of 15 studies, immediate haemostasis rates of 93% were found with rebleeding rates of 12%^[75]. In another meta-analysis of 11 randomised controlled trials, although TIPSS reduced risk of rebleeding, TIPSS was found to not affect survival in patients with variceal haemorrhage^[76]. TIPSS was also found to be successful in the management of bleeding gastric varices^[77-79].

2000-2010

With the dawn on the 21st century, pharmacological, endoscopic and radiological therapies for variceal haemorrhage improved outcomes. The role of antibiotics in variceal bleeding became clear in the early 21st century. Primary or secondary bacterial infections are common in cirrhotic patients^[80,81] due to bacterial translocation into the portal system from impaired mucosal integrity and an impaired immune function. Antibiotics were found to reduce bacterial infections, recurrent bleeding and improve mortality in patients bleeding from oesophageal varices^[82-84]. Current guidelines recommend broad-spectrum antibiotic prophylaxis^[2,24,26] in patients with suspected and proven variceal haemorrhage. Local antibiotic policy can vary and a patient's "nil-by mouth" status can influence the choice of antibiotic. However, oral quinolones are recommended, or a 3rd generation cephalosporin in patients who received quinolone prophylaxis, have advanced cirrhosis, or live in areas of high quinolone resistance^[2]. Another area of interest recently in resuscitation has been that of transfusion. In a study by Villanueva *et al.*^[85] the role of over-transfusion in GI bleeding has been explored and its effects on portal pressure. In patients with a liberal transfusion strategy [transfused when haemoglobin (Hb) fell to less than 9 g/dL] there was a significant rise in portal pressure gradient in the 1st five d post bleed compared to patients with a restrictive transfusion strategy (transfused when Hb fell to less than 7 g/dL). Thus it could be argued that patients with a variceal bleed are not as aggressively resuscitated/over-transfused as they may have previously been, however further clarification in this area is required.

Endoscopic therapy developed further in the 21st century, with obturation therapies for gastric variceal bleeding coming of age in the new millennium. Gastric varices account for 10%-30% of variceal haemorrhage, and although less common than oesophageal varices, when bleeding occurs it can often be torrential and associated with a high mortality^[86-89]. Gastric varices can also bleed at a lower portal pressure than oesophageal varices^[86-89]. There is limited data on EVBL in the management of gastric variceal bleeding with high rates of gastric variceal recurrence following EVBL due to a more superficial effect compared with obturation therapy^[86]. Technical difficulty of banding in a retroflexed endoscope position and a theoretical risk of gastric rupture has meant EVBL for gastric varices has largely been superseded by obturation

therapies using thrombin and *N*-butyl-2-cyanoacrylate (HistoacrylTM) injection. *N*-butyl-2-cyanoacrylate is a long-chain cyanoacrylate glue that polymerises and solidifies within seconds following contact with blood in a gastric varix. It is mixed with the oily agent Lipiodol delaying polymerisation. Complications of its use include: endoscope damage due to blockage of the injection channel, sticking of the injection needle into a varix, mediastinitis, local abscesses, and cerebral/pulmonary embolisms or splenic infarcts from glue migration. Immediate haemostasis rates of 92%-100% have been reported with variable re-bleeding rates^[86-93]. Cyanoacrylate glue when compared with ethanol injection in a randomised study had faster rates of variceal obliteration, required smaller injection volumes, had improved efficacy in control of acute gastric variceal bleeding and reduced need for rescue surgery^[92,93]. Thrombin was another obturation therapy developed in the 21st century used in acutely bleeding gastric varices. It is a haemostatic agent converting fibrinogen to a fibrin clot and causing platelet aggregation^[94]. Initially in the late 1980s and 1990s there were small case-series of its use with haemostasis rates between 70%-100% using bovine thrombin^[95-99]. Bovine thrombin was discontinued because of the potential risk of prion transmission. Thus, short-term small uncontrolled studies of human-derived thrombin have demonstrated initial haemostasis rates of 100% but often a high mortality from re-bleeding^[99-101]. A recent retrospective study from Edinburgh, United Kingdom demonstrated in 33 patients treated with human thrombin for gastric variceal bleeding rebleeding rates of 10.8%^[102]. It is worth noting to date there have been no controlled trials with thrombin *vs* other obturation treatments such as *N*-butyl-2-cyanoacrylate) to our knowledge.

Radiological interventions in variceal haemorrhage improved in the new century too. In 2004, the advent of covered TIPSS stent (with an expanded polytetrafluoroethylene cover) was hailed as a breakthrough and approved by the United States Food and Drug Administration. The covered stent improved shunt patency by reducing tissue ingrowth by minimising transmural bile permeation^[103]. The primary patency of covered stents at 1-year were found to be up to 80%-90%^[104-107] with reduction of rebleeding post "index bleed" to less than 10%^[105,107,108]. Other studies confirmed the role of a rescue TIPSS in variceal bleeding which could not be controlled by endoscopy or vasoactive drugs^[67,68]. The early TIPSS placement has been shown to have beneficial effect in patients with a HVPg > 20 mmHg presenting with a variceal bleed^[11]. In this study published in 2004, patients who were considered high risk (HVPg > 20 mmHg) were selected and randomised to early uncovered TIPSS or standard of care within 24 h of presentation. Treatment failure was deemed as failure to control acute variceal bleeding and/or early rebleeding after the first endoscopic therapy. TIPSS reduced rebleeding and treatment failure, and was associated with superior in-hospital and 1-year survival. However, the therapy used in the control arm was endoscopic sclerotherapy alone, which

is not the accepted standard of care. The other major issue translating this study into real world practice was the availability of HVPg in routine clinical practice.

Interventional radiological procedures for the treatment of gastric varices in the 2000s included the advent of BRTO^[109-111] as salvage or rescue therapy when endoscopic obturation therapy fails. BRTO is an interventional radiological technique for gastric variceal bleeding whereby a splenorenal shunt often seen in such patients can be occluded with sclerosant using a balloon catheter approached *via* the left renal vein^[109-111]. BRTO may potentially become an alternative to TIPSS in patients with active gastric variceal bleeding in whom a gastorenal shunt is present^[110]. However, it is not commonly used out-with the Far East or large tertiary referral centres. There is also an increased risk of the development of oesophageal varices after its use^[109]. Its role has not been incorporated in any European or United States guidelines to date^[2,24].

Liver transplantation is the only curative treatment for liver cirrhosis at this point in time, although its role in bleeding variceal haemorrhage has not been established. In some centres it has been proposed as a treatment in patients with advanced liver disease who fail endoscopic therapies^[112]. These studies were however in the era prior to EVBL, combined pharmacological and endoscopic therapies and TIPSS. In a trial by Orloff *et al*^[113] unselected consecutive patients with advanced cirrhosis and bleeding oesophageal varices were studied who had either sclerotherapy ($n = 106$) or emergency direct portocaval shunt ($n = 105$). The 3-, 5-, 10- and 15-year survival rates were significantly higher in the portocaval shunt group ($P < 0.001$). On the follow-up, 6% of patients were referred for liver transplant assessment, 3% listed and only a total of 2% actually underwent liver transplantation for progressive liver failure. A conclusion drawn from the study authors was that transplantation was infrequently required in this setting (even prior to the TIPSS era) and if initial bleeding was controlled (in 100% of the portocaval shunt arm) then survival was similar or better than that following transplant. It should be remembered that such centre-specific data however often differs from centres with less experience in portocaval shunts. However to our knowledge there are no randomised trials of endoscopic therapy with radiological therapy and liver transplantation in the setting for acute variceal haemorrhage and this is certainly not current practice. It could be argued that transplantation should only be reserved in those patients whom combined pharmacological and endoscopic therapy fails along with a trial of radiological intervention such as TIPSS or BRTO, or even surgery. However, patients with poor liver function or in whom liver function does not recover should always be considered at an early stage for liver transplant assessment where appropriate based on local scoring systems such as MELD in the United States and UKELD in the United Kingdom.

Post-2010

Areas of recent interest that required future clarification

and further study include the early role of TIPSS in variceal haemorrhage, oesophageal stents and new agents for haemostasis.

The exact and optimal role of TIPSS in variceal haemorrhage has been particularly under the spotlight recently. In 2004, the early placement of TIPSS was shown to have beneficial effect in patients with a HVPg > 20 mmHg presenting with a variceal bleed^[11]. In a recent seminal multi-centre European study in 2010^[4], 63 cirrhotic patients with oesophageal variceal bleeding were treated with vasoactive drugs plus endoscopic therapy and then randomised to one of two treatment arms if they had Child's C disease or active bleeding and Child's B disease. The first arm was covered TIPSS within 72 h ("early-TIPSS"), and the second arm continuation of vasoactive drugs for 3 to 5 d followed by non-selective beta-blockers and with long-term EVBL (with the insertion of a TIPSS only if required as a rescue therapy). Rebleeding or failure to control bleeding occurred in only one patient in the "early TIPSS" arm, and in 14 patients in the control arm ($P < 0.001$). Overall mortality was lower in the "early-TIPSS" group (12 *vs* 4 patients, $P = 0.01$) with 1-year survival of 61% in the control group *vs* 86% in the "early-TIPSS" group ($P < 0.001$). There was no difference in the incidence of hepatic encephalopathy. A post RCT surveillance study from the same group published last year, aimed to confirm the results in a clinical setting^[114] (Table 4). Patients admitted with acute variceal bleeding and high risk of treatment failure (Child C < 14 or Child B plus active bleeding) were thereafter treated with early covered TIPSS ($n = 45$) or combined pharmacology/endoscopic therapy ($n = 30$). The patients treated with "early-TIPSS" had lower rates of rebleeding or failure to control bleeding than patients receiving combined therapy (3 *vs* 15, $P < 0.001$). There was a tendency also towards reduced mortality in the "early-TIPSS" group ($P = 0.056$). Criticisms of the "early TIPSS" trial however included that recruitment was prolonged (3 years) to recruit 63 patients *via* 9 centres^[4], with a high exclusion rate (296 patients excluded). The second issue is that of the inclusion of patients with ongoing bleeding following index endoscopy. This might arguably be termed a "rescue" TIPSS and although no studies have been done in this area it is intuitive to suggest that survival would be improved if haemostasis has not been achieved. Thirdly, survival at 1 year with "early TIPSS" was remarkably high (86% *vs* 61% in the medical management group)^[4]. Thus the current Baveno V guidelines^[2] suggest considering the "early TIPSS" approach, but clearly further studies are necessary where patients requiring an "early TIPSS" as a rescue therapy are excluded.

Another novel area of interest recently is the use of self-expanding oesophageal stents, which again will require further study to clarify their role in influencing outcome from variceal haemorrhage. The stent acts by applying direct tamponade to the distal oesophageal mucosa and any associated bleeding varices. Such stents were used in a pilot study in 20 patients who failed to

Table 4 Summary of randomized controlled trials and meta-analysis of different therapies over time in variceal haemorrhage

| Ref. | Trial design/therapy | Outcome/results |
|---|--|--|
| Surgical techniques | | |
| Inokuchi <i>et al</i> ^[19] | Randomised controlled trial (RCT)/prophylactic surgical intervention (<i>n</i> = 60) <i>vs</i> non surgical intervention (<i>n</i> = 52) for oesophageal varices | 5-yr cumulative survival rate at 5 yr in the operated group was 72% <i>vs</i> 45% (<i>P</i> < 0.05). 5-yr cumulative variceal bleeding rate at 5 yr was 7% in the operated group <i>vs</i> 46% (<i>P</i> < 0.001) |
| Balloon tamponade Terés <i>et al</i> ^[28] | RCT/comparison of SB <i>vs</i> Linton-Nachlas (LN) | Primary haemostasis rates of 86%. In oesophageal variceal bleeding SB tube achieved permanent haemostasis in 52% <i>vs</i> 30% in LN tube |
| Sclerotherapy | | |
| The Copenhagen esophageal varices sclerotherapy project ^[46] Westaby <i>et al</i> ^[38] | Randomised multicentre trial/187 unselected patients with oesophageal variceal bleed randomly assigned to medical treatment including balloon tamponade or to medical treatment supplemented with paravariceal sclerotherapy RCT of sclerotherapy (<i>n</i> = 56) <i>vs</i> placebo (<i>n</i> = 60) | Overall mortality in the sclerotherapy group (hazard) was 76% (95%CI: 10%-54%) of that in the medical-regimen group (relative mortality in the sclerotherapy group was 63% of that in the medical-regimen group) Survival was significantly better in those treated by sclerotherapy (<i>P</i> < 0.001) |
| Burroughs <i>et al</i> ^[39] | Randomised trial/a comparison of sclerotherapy (<i>n</i> = 5) with staple transection (<i>n</i> = 51) of the oesophagus for the emergency control of bleeding from oesophageal varices | Total mortality did not differ significantly between the two groups. Mortality at six wk was 44% among those assigned to sclerotherapy and 35% assigned to staple transection. Complication rates were similar for the two groups |
| D'Amico <i>et al</i> ^[126] | Cochrane database systematic/meta-analysis of 17 trials, assessing the benefits of sclerotherapy <i>vs</i> vasoactive drugs in patients with variceal bleeding | Authors concluded no convincing evidence to support the use of emergency sclerotherapy as the first, single treatment when compared with vasoactive drugs |
| Thakeb <i>et al</i> ^[62] | Randomised controlled trial/assess the role of the combined N-butyl-2-cyanoacrylate and ethanolamine oleate (<i>n</i> = 58) <i>vs</i> ethanolamine sclerotherapy (<i>n</i> = 56) for management of bleeding esophagogastric varices | Arrested acute bleeding in 66.7% of patients with gastric variceal bleeding. Recurrent bleeding in 8.6% in the combined therapy group <i>vs</i> 25% in the sclerosis group (<i>P</i> < 0.01). The mortality in the combined therapy group less than sclerosis group (3.5% and 8.8% respectively, <i>P</i> > 0.05) |
| Endoscopic variceal band ligation (EVBL) | | |
| Laine <i>et al</i> ^[65] | Meta-analysis of 7 RCTs/comparison of the effect of EVBL <i>vs</i> sclerotherapy in the treatment of patients with bleeding esophageal varices | EVBL (<i>vs</i> sclerotherapy) reduced the rebleeding rate (OR = 0.52, 95%CI: 0.37-0.74), the mortality rate (OR = 0.67, 95%CI: 0.46-0.98), and the rate of death due to bleeding (OR = 0.49, 95%CI: 0.24-0.996) |
| García-Pagán <i>et al</i> ^[63] | Meta-analysis of 10 RCTs comparing sclerotherapy with EVBL | Non-significant benefit of EVBL in achieving initial haemostasis <i>vs</i> sclerotherapy (pooled relative risk of 0.53 with 95%CI: 0.28-1.01) |
| Radiological transjugular intrahepatic portosystemic stent-shunts (TIPSS) | | |
| Monescillo <i>et al</i> ^[11] | RCT of patients (116) divided into low risk/high risk of rebleeding based on hepatic venous pressure gradient (HVPG) | Early TIPSS placement in patients with HVPG > 20 within 24 h of admission reduced in-patient and 1 yr mortality |
| García-Pagán <i>et al</i> ^[44] | RCT/role of early TIPSS in patients with oesophageal variceal haemorrhage (<i>n</i> = 32) within 72 h of admission <i>vs</i> continuation of vasoactive Tx and B-blocker/EVBL (<i>n</i> = 31) thereafter | Rebleeding or failure to control bleeding in 14 patients in the pharmacotherapy-EVBL group <i>vs</i> 1 patient in the early-TIPSS group (<i>P</i> = 0.001) |
| García-Pagán <i>et al</i> ^[114] | Post-RCT surveillance study/retrospective review of patients admitted for acute variceal bleeding and high risk of treatment failure treated with early-TIPSS (<i>n</i> = 45) or drugs/endoscopic therapy (ET) (<i>n</i> = 30) | Early-TIPSS group had a much lower incidence of failure to control bleeding/rebleeding than drug + ET (3 <i>vs</i> 15, <i>P</i> < 0.001). 1-yr actuarial survival was 86% <i>vs</i> 70% respectively (<i>P</i> = 0.056) |
| Yang <i>et al</i> ^[127] | Meta-analysis of 6 studies of covered stents <i>vs</i> bare metal stents | Use of polytetrafluoroethylene-covered stent-grafts associated with improved shunt patency without increasing the risk of hepatic encephalopathy and with a trend towards better survival |

achieve haemostasis with pharmacological or endoscopic techniques^[115], and achieved 100% immediate haemostasis rates in such a rescue setting. There was a stent migration in 25% of patients in this initial study and 10% of patients died within 5 d. Three other studies have further been published^[116-118], with a combined total of 57 patients. Successful stent placement ranged from 90%-100% and control of bleeding ranging between 70%-100%. Stent migration rates varied from 0%-18% with a total of 4 patients rebleeding. Such stents may be a promising option in refractory oesophageal haemorrhage as bridge therapy to definite treatment such as TIPSS. However, randomized controlled trials with comparison

to other interventions or even as an adjunct to current standard of care are necessary before they can be considered standard of care. Their mechanism of action would make them comparable to balloon tamponade and there is currently a study group in Barcelona exploring this (NCT01242280). Another United Kingdom study entitled "Effective haemostasis using self-expandable covered mesh-metal oesophageal stents *vs* standard endoscopic therapy in the emergency treatment of oesophageal variceal hemorrhage: A multicenter, open, prospective, randomized, controlled study-ISRCTN 98310189" is under way and recruiting. Preliminary data was recently presented in the use of stents compared to balloon tam-

ponade in variceal bleeding refractory to endoscopic and medical therapy. Escorsell *et al.*^[119] reported on 28 patients (15 Sengstaken and 13 metal stents) with the intention to treat analysis showing more frequent success of therapy in the stent arm (46% *vs* 3%, $P = 0.005$). There was a trend towards better control of bleeding ($P = 0.1$) and less transfusion requirements ($P = 0.08$) in the stent arm. Survival rates were comparable ($P = 0.4$). The authors concluded that oesophageal stents were indeed more effective than balloon tamponade for temporary control of variceal haemorrhage in treatment failures. The stents however do not have a role in gastric variceal bleeding in their current form.

Another new area of interest that has been the development of haemostatic powders/sprays. TC-325 (Hemospray, Cook TechnologyTM) is a granular non absorbable mineral powder used in the management of arterial wounds. It achieves hemostasis by activating platelets and increasing the concentration of clotting factors and also by forming a mechanical barrier over the wall of a bleeding vessel^[120] thus forming a mechanical plug at site of bleeding^[121]. It contains no proteins from animals or humans. The spray device kit contains an application catheter, a propellant CO₂ canister and also a chamber containing 20 g of powder. Its role has been studied in patients bleeding from peptic ulcers^[122].

In a pilot study by Ibrahim *et al.*^[123], the use of one such powder TC-325 (was studied in 2 tertiary care referral centres with primary haemostasis rates and rebleeding rates measured). Nine patients with confirmed variceal bleeding had treatment within 12 h of admission, with 21 g of haemostatic powder applied *via* a catheter in the accessory channel of the endoscope from the cardia up to 15 cm above the gastro-oesophageal junction. There was no rebleeding within 24 h and no mortality at 15 d. Although a small pilot study, further larger trials needed to secure its position in variceal haemorrhage. In another case series, its role in the management of portal hypertensive bleeding was studied in 4 patients - 3 with portal hypertensive gastropathies and 1 portal colopathy^[124]. All patients had cessation of bleeding with Hemospray and reduced transfusion requirements thereafter however in 1 patient a complication of viscus perforation was encountered and the patient died shortly after endoscopy - however it was unclear if perforation was secondary to instrumentation during the procedure or the spray itself. Its use has also been studied in small case reports in the management of bleeding gastric varices^[125]. This remains a promising area requiring further large trials securing its position in the management of variceal haemorrhage.

CONCLUSION

Variceal haemorrhage from oesophageal or gastric varices remains a life-threatening emergency requiring urgent specialist care. The development over the years of endoscopic access and therapies has transformed the management of variceal haemorrhage. This cou-

pled with improved medical management of variceal bleeding patients has resulted in improved mortality and rebleeding rates. However the delivery of optimal management of these patients in the “real-world” setting remains variable. With firm guidelines in place for the management of variceal haemorrhage and general management of upper gastrointestinal bleed patients, it is paramount that local centres aim to deliver such standards. Currently the gold standard management involves adequate and early resuscitation including airway support if required. The optimal circulating volume should allow good perfusion pressures however over transfusion recently has been contentious with further studies required in this area. Vasopressor and antibiotic treatments are now well established in variceal haemorrhage and should be instituted early in a presumed (or confirmed) variceal haemorrhage. Definitive endoscopic treatment is required, however the timing of endoscopy often depends on local units and ease of endoscopic services out-of-h. To develop optimal endoscopic services local and national auditing of services is required, but also training of competent endoscopists who can manage acute variceal haemorrhage optimally pre-, peri- and post-endoscopy.

In the management of oesophageal variceal haemorrhage, endoscopic band ligation should be the favored definitive treatment, with sclerotherapy reserved potentially for those whom EVBL cannot be performed (Figure 1). In gastric varices the optimal treatment remains to be ascertained between N-butyl-2-cyanoacrylate or thrombin, and a randomised controlled trial in this area would be helpful in the future. Much depends on the endoscopist familiarity with both injection methods, with thrombin being technically easier in our experience with potentially less complications. Other endoscopic therapies such as oesophageal stents and Hemospray are intriguing and may indeed have a role in patients who fail standard endoscopic treatments, however larger trials are also required for these agents.

If endoscopic therapy is difficult, or does not halt the bleeding in oesophageal variceal haemorrhage then a rescue TIPSS can be performed. The role of an “early” TIPSS in those who have had initial bleeding halted to prevent rebleeding and potentially improve mortality is something that requires further study and may potentially have significant implications for regional radiological centres offering TIPSS to other hospitals. Other interventional radiological procedures such as BRTO offer promise in refractory gastric variceal haemorrhage however their availability is dependent on the expertise of centre’s radiologists.

In summary, over the last few decades, much has been achieved in the management of variceal haemorrhage from an almost always life terminating event, to now, an event that can be adequately and aggressively managed, with the aim to completely reduce mortality from variceal bleeding. The next decade will be indeed an exciting time in the management of variceal haemorrhage.

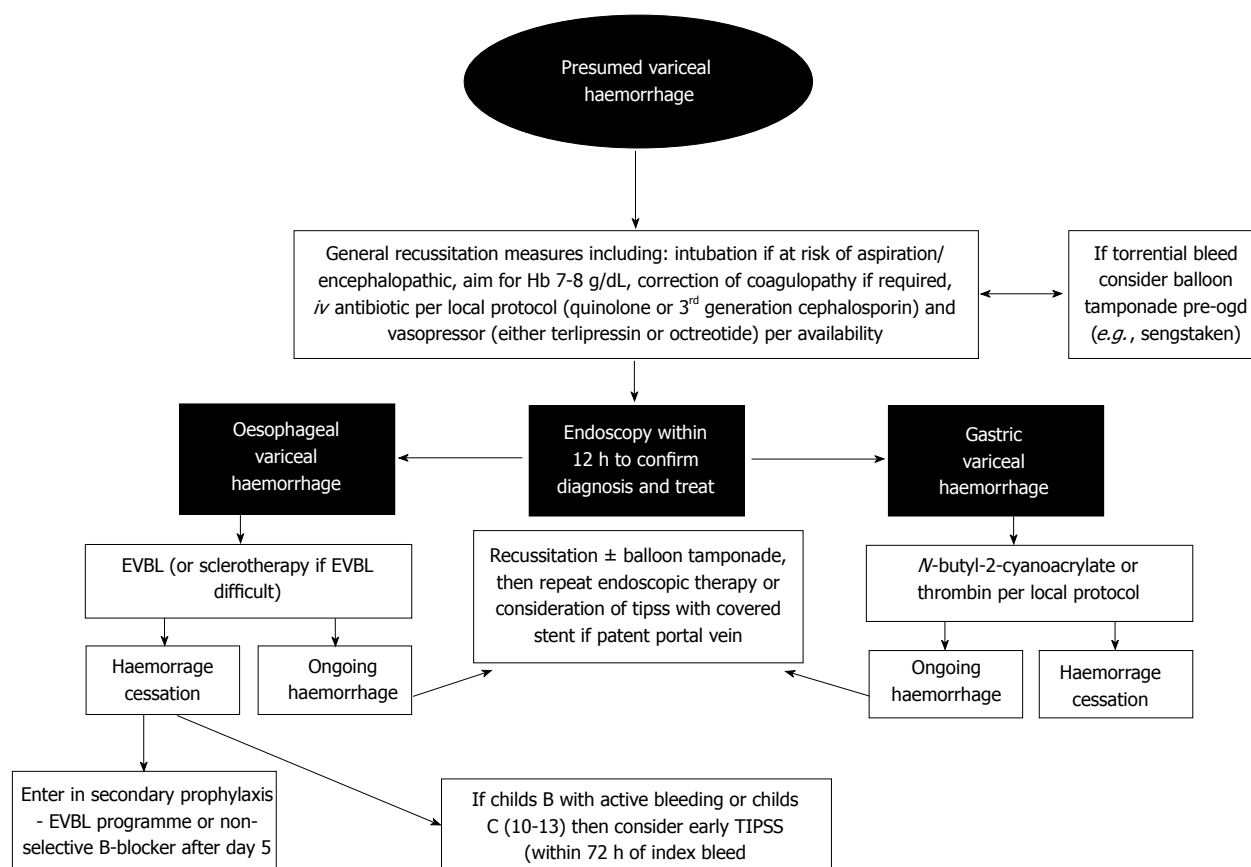


Figure 1 Summary in the management of acute variceal haemorrhage. EVBL: Endoscopic variceal band ligation; TIPSS: Transjugular intrahepatic portosystemic stent-shunts; Hb: Haemoglobin.

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Involvement of heat shock proteins in gluten-sensitive enteropathy

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Abstract

Gluten-sensitive enteropathy, also known as coeliac disease (CD), is an autoimmune disorder occurring in genetically susceptible individuals that damages the small intestine and interferes with the absorption of other nutrients. As it is triggered by dietary gluten and related prolamins present in wheat, rye and barley, the accepted treatment for CD is a strict gluten-free diet. However, a complete exclusion of gluten-containing cereals from the diet is often difficult, and new therapeutic strategies are urgently needed. A class of proteins that have already emerged as drug targets for other autoimmune diseases are the heat shock proteins (HSPs),

which are highly conserved stress-induced chaperones that protect cells against harmful extracellular factors. HSPs are expressed in several tissues, including the gastrointestinal tract, and their levels are significantly increased under stress circumstances. HSPs exert immunomodulatory effects, and also play a crucial role in the maintenance of epithelial cell structure and function, as they are responsible for adequate protein folding, influence the degradation of proteins and cell repair processes after damage, and modulate cell signalling, cell proliferation and apoptosis. The present review discusses the involvement of HSPs in the pathophysiology of CD. Furthermore, HSPs may represent a useful therapeutic target for the treatment of CD due to the cytoprotective, immunomodulatory, and anti-apoptotic effects in the intestinal mucosal barrier.

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Key words: Gluten-sensitive enteropathy; Coeliac disease; Heat shock proteins; Gluten-free diet; Intestinal barrier

Core tip: The only current effective therapy for the treatment of coeliac disease (CD) is a gluten-free diet. However, therapies targeting heat shock proteins (HSPs) for the treatment of various autoimmune disorders and cancers have been developed and have shown promising results. As CD is an autoimmune disorder, these new therapies may prove beneficial as an alternative treatment strategy. This review highlights and discusses recent data concerning the involvement of HSPs in the pathophysiology of CD.

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INTRODUCTION

Coeliac disease (CD), or gluten-sensitive enteropathy, is an autoimmune inflammatory disorder characterized by partial or total villous atrophy and crypt hyperplasia of the small intestine in genetically predisposed patients. Ninety-five percent of affected individuals carry one of two specific human leukocyte antigen (HLA) class II alleles, either DQ2 (HLA-DQA1*05-DQB1*02) or DQ8 (HLA-DQA1*03-DQB1*0302)^[1-4]. Since dietary gluten and related prolamins are present in different types of cereals (wheat, barley and rye), medicines, and various other products, including stamp and envelope adhesives, a lifelong exclusion of gluten presents a considerable challenge for patients with CD^[5,6]. Although the worldwide incidence of CD has continued to increase over the past decade, most cases remain undiagnosed^[7]. The increased incidence suggests that the disease manifestation is similar to that of other immune-mediated diseases, such as inflammatory bowel disease (IBD), allergies or asthma, and results from a combination of genetic predisposition and environmental factors. This hypothesis is supported by the fact that CD is often first detected following physical and emotional stress, such as from surgery, pregnancy, or viral infection^[8]. Heat shock proteins (HSPs) are known to exert immunomodulatory effects, and have thus been targeted for the treatment of autoimmune disorders. Recent evidence suggests that the expression of HSPs is altered in CD. This review presents and discusses the role of HSPs and various stress factors in the pathophysiology of CD.

EFFECT OF STRESS ON THE PATHOGENESIS OF CD

Stress represents an acute threat to an organism, which initiates and mediates the physiological adaptations necessary to maintain homeostasis and ensure survival^[9]. Stress can be caused by intrinsic factors, such as genes and endoplasmic reticulum stress, or extrinsic factors, such as heat, toxins, radiation, infection, mechanical force and metabolic disturbances. Stress factors affecting the gastrointestinal tract may induce inflammation and reduce its motility^[10], resulting in disrupted mucosal integrity and impaired epithelial barrier function^[11,12]. Such changes can lead to the development of CD in genetically predisposed individuals^[13].

In CD, the transport of incompletely digested wheat gluten peptides, such as gliadin, across a damaged epithelial layer into the lamina propria^[14] triggers oxidative stress and the release of pro-inflammatory cytokines^[15]. However, gluten can induce adaptive as well as innate immune responses, such as enhancing the production of interleukin (IL)-15 in epithelial cells, which also leads to cell damage through the activation of intraepithelial cytotoxic CD8+ T-cells^[16,17]. Activated transglutaminase 2 enzymes in the lamina propria^[18] deamidate neutral glutamine residues of gluten, thus creating epitopes with increased

immunostimulatory potential^[16]. These deamidated peptides are presented to CD4+ T-helper cells by the disease associated HLA-DQ2 and -DQ8 molecules from macrophages, dendritic cells (DCs) and B lymphocytes^[19], which promote the differentiation of B-cells producing anti-gliadin and anti-transglutaminase 2 antibodies^[20]. T-cells may also produce pro-inflammatory cytokines, such as tumour necrosis factor (TNF)- α and interferon (IFN)- γ , and activate intestinal fibroblasts leading to further damage of the epithelial cell layer, mucosal matrix degradation and tissue remodelling^[18]. Moreover, gliadin peptides can directly activate pattern recognition receptors such as Toll-like receptor (TLR) 2 and 4 on macrophages and DCs^[21], leading to a further upregulation of proinflammatory cytokines and chemokines^[22] (Figure 1). These inflammatory effects of stress lead to additional aggravation of the disease^[23].

DEFENSE AGAINST STRESS: ROLE OF HSPs

Stress results in the activation of various proteins such as proteolytic system components, RNA/DNA modifying enzymes, metabolic enzymes, regulatory, transport, detoxifying and membrane-modulating proteins, and molecular chaperones, or HSPs^[24]. HSPs were first discovered in *Drosophila melanogaster* in the early 1960s^[25], and have since been observed in all organisms after exposure to cellular stresses^[26], such as heat, UV light, cytotoxic agents^[27,28], and nutritional (*e.g.*, the absence of glucose and glutamine)^[29] and oxidative stress^[30]. HSPs are expressed in many tissues, including heart^[31], brain^[26], muscle^[32], lung^[33], kidney^[34], liver^[35], and intestinal and colonic epithelium^[36]. These highly conserved molecules are responsible for maintaining adequate protein folding^[37] and influencing the degradation of proteins^[38] and cell repair processes after damage^[39]. Furthermore, HSPs are involved in the modulation of immune responses^[40,41], autoimmunity^[27], cell signalling^[42], cell proliferation^[43], apoptosis^[44], and tumour cell differentiation and invasion^[45]. Based on their molecular weight they can be classified into six major families: small HSPs (molecular weight < 30 kDa), HSP60s, HSP70s, HSP90s, HSP100s^[24,46], and other non-ubiquitous HSPs^[47] (Table 1).

Oxidative stress and HSPs

Environmental and chemical agents inducing oxidative stress can enhance the generation of reactive oxygen species (ROS)^[48,49]. In CD, gluten itself can promote the generation of ROS by stimulating the expression of the inducible form of nitric oxide synthase (iNOS) and increasing nitric oxide levels^[50,51]. This process contributes to subsequent mucosal damage and villous atrophy of the small intestine^[52]. Interestingly, these same oxygen-free radicals, such as superoxide, also induce the expression of various HSPs which take part in the defence against oxidative stress^[53]. The inducible form of HSP70 (HSP70i) reduces iNOS expression by specifically binding to iNOS

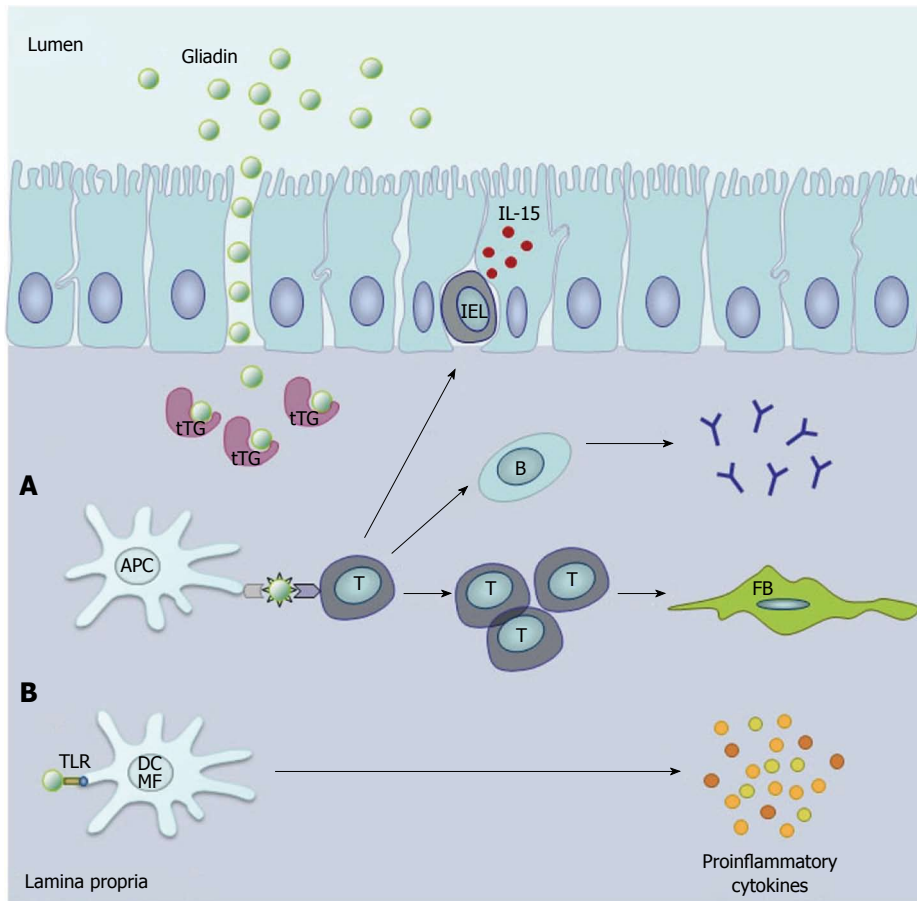


Figure 1 Key processes during the pathogenesis of coeliac disease. In the lamina propria. A: Gluten-derived gliadin peptides deamidated by tissue transglutaminase (tTG) are presented to T-cells by antigen presenting cells (APC). This process leads to the activation of anti-gliadin and anti-tTG antibody producing B-cells and other T-cells promoting the activation of intestinal fibroblasts (FB). Furthermore, gliadin enhances the production of IL-15, which activates intraepithelial T lymphocytes (IEL); B: Gliadin peptides can directly activate Toll-like receptor (TLR) 2 and 4 on macrophages (MF) and dendritic cells (DC), resulting in increased production of proinflammatory cytokines (Reproduced with permission from Sziksz *et al*^[1]).

| Table 1 Classification of heat shock proteins ^[46,113] | | | |
|---|------------------|--|--|
| Family | Subunit MW (kDa) | Family members | Cellular localization |
| HSP100 | 80-110 | HSP100, HSP104 | Cytoplasm, nucleus, mitochondria, plasma membrane |
| HSP90 | 82-96 | HSP90 α , HSP90 β | Cytoplasm, nucleus, mitochondria, endoplasmic reticulum |
| HSP70 | 67-76 | HSP70, HSP72, HSP73, HSP80 | Cytoplasm, nucleus, mitochondria, endoplasmic reticulum, lysosomes, extracellular compartments |
| HSP60 | 58-65 | HSP60, HSP65 | Mitochondria |
| Small HSPs | 8-40 | α B-crystallin, HSP25, HSP27, ubiquitin | Cytoplasm, nucleus |
| Others (not ubiquitous) | Various | HSP33 | Various |

MW: Molecular weight; HSP: Heat shock protein.

and its transcription factor Krueppel-like factor 6^[54]; moreover, its upregulation was shown to inhibit nuclear factor (NF)-B activation, thereby providing cellular pro-

tection against stress^[55]. In addition, glutamine-induced HSP72 was shown *in vivo* to protect against endotoxin-induced shock injury^[56], and HSP90 has been shown to exert antioxidative and anti-apoptotic effects against chemical-induced hypoxic injury^[57]. HSP60 contributes to the protection of small intestine by enhancing the cytoprotective function of intestinal epithelial cells against H₂O₂-induced injury^[58]. Finally, HSP32, also known as heme oxygenase-1, degrades heme into vasoactive carbon monoxide, free iron and biliverdin, and is also a potent antioxidant^[59].

Inflammation and HSPs

HSPs can act as “danger signals” for the immune system at sites of tissue injury^[60]. HSPs were shown to contribute to antigen presentation and the proliferation and activation of macrophages and DCs^[61], and natural killer cells^[62]. HSP70 and HSP90 bind to TLRs on the surface of DCs and macrophages^[63] resulting in enhanced expression of pro-inflammatory cytokines^[64,65], and HSP60 stimulates the release of TNF- α , IL-12, and IL-1 β , *via* TLR 4 signalling^[66]. However, HSP60 can also activate anti-inflammatory processes through TLR 2 signalling,

upregulating the suppressive function of regulatory T-cells and shifting the cytokine secretion balance toward a Th2 phenotype^[67,68], suggesting that the immunomodulatory effect can be cell and receptor type specific.

Altered expression of HSPs has been associated with intestinal inflammation. An increased epithelial expression of HSP70, HSP60 and HSP10 was observed in the colonic mucosa of patients with IBD^[69,70]. This upregulation may be protective, as Tanaka *et al*^[71] demonstrated that transgenic mice overexpressing HSP70 showed reduced apoptosis and suppressed expression of pro-inflammatory cytokines after dextran sulfate sodium-induced colitis. HSP47, a collagen-specific molecular chaperone, was also found in mesenchymal and submucosal cells in a murine model of colitis^[72].

Apoptosis and HSPs

Apoptosis is essential for the maintenance of intestinal epithelial function, as it regulates the normal turnover of enterocytes^[73]. The increased apoptosis of enterocytes in CD contributes to villous atrophy, which is mediated either by the direct toxicity of gliadin domains or by the gliadin-dependent activation of intraepithelial and lamina propria lymphocytes^[74]. Gliadin-induced apoptosis can be blocked by Fas cascade inhibitors^[75], although the activation of the Fas system can also contribute to cell survival in the gut by inducing the expression of HSP72 and HSP72-driven chemokines^[76]. HSP70 can also promote cell survival by inhibiting the mitochondrial translocation of Bax and subsequent release of cytochrome c and activation of caspase-9 and -3^[77,78], an intrinsic apoptotic pathway that is initiated by intracellular stress signals^[79]. Furthermore, HSP70 is a natural inhibitor of c-Jun N-terminal kinase^[80] and is also a modulator of the calcium signalling that play major roles in the regulation of apoptosis^[80-83]. Furthermore, HSP60 has been identified as a novel mitochondrial permeability transition regulator. HSP60 is a component of a mitochondrial multi-chaperone complex that includes HSP90 and its related molecule TNF receptor-associated protein 1, which associates with and antagonizes the pro-apoptotic, mitochondrial permeability transition pore modulator, cyclophilin D, thereby contributing to the preservation of organelle integrity and prevention of cell death^[84,85].

Intestinal epithelial integrity and HSPs

The intestinal mucosa forms a barrier that is essential for defending the intestine against the harmful effects of different stressors. Oxidative stress, inflammation and increased apoptosis all lead to mucosal damage and increased permeability^[86]. The integrity of the epithelial barrier is determined by an apical junctional complex composed of tight and adherent junctions^[87]. During heat stress, HSPs play a pivotal role in the preservation of the intestinal barrier by promoting the upregulation of the tight junction protein occludin^[88,89]. HSP70s protect intestinal epithelial cells by preserving the integrity of the actin cytoskeleton and cell-cell contact, and

HSP72 directly binds and stabilizes other tight junction-associated proteins on colonic epithelial cells, such as zonula occludens^[90]. Other HSPs, including members of the HSP110 subfamily, have also been shown to bind to junctional proteins^[91]. Tissue integrity is also influenced by matrix metalloproteinases (MMPs)^[92], which have been observed as increased in intestinal tissues of patients with CD^[93]. Extracellular HSP90 α was shown to activate MMP-2, which was enhanced by HSP70 and HSP40, leading to increased cell migration^[94]. HSP60 may also induce MMP production in macrophages^[95].

HSPs AND CD

HSPs are differentially expressed throughout the gastrointestinal tract, with gastric and colonic epithelial cells showing high expression of HSP25 and HSP72, likely the result of continuously low acidic pH, mechanical stress and/or bacterial fermentation^[96]. In contrast, the expression of HSPs in the small intestine is normally negligible^[97], but the expression of HSP25 and HSP70 is markedly increased under stress^[88]. The predominant localization of HSPs in intestinal epithelial cells suggests their primary role is in maintaining the integrity of the enterocyte layer, as demonstrated by Kojima *et al*^[98] who showed that *Bacteriodes fragilis* treatment of young adult mouse colonocyte cells increased the expression of HSPs mediated by lipopolysaccharide and other bacteria-derived factors. Using horseradish peroxidase to evaluate human intestinal epithelial permeability, Yang *et al*^[99] found that heat stress increased transport across an epithelial monolayer, which was inhibited by pretreatment with HSP70. Asea^[65] and Cario *et al*^[100] provided further supporting evidence by showing that HSP70 can behave as a ligand for TLR 2 and TLR 4^[101], the activation of which can contribute to the maintenance of intestinal barrier function by preserving the integrity of tight junction proteins, such as zonula occludens 1, under stressful conditions.

The role of HSPs in the pathophysiology of CD is not well understood, owing in part to the lack of experimental models. However, our lab has shown increased mRNA and protein expression of HSP72 in the duodenal mucosa of children newly diagnosed with CD^[102]. The most abundant expression of HSP72 was in villous enterocytes of the epithelium and immune cells of the lamina propria. Clinical symptoms were reduced with a gluten-free diet (GFD), which also reduced the level of intestinal HSP72, though levels were still higher than in control individuals. In contrast, Brottveit *et al*^[103] reported that suspension of a GFD for three days did not alter the mRNA expression of HSP70 or HSP27 in the mucosa of adult CD patients. This apparent discrepancy may be due to the difference in patient age, or in the experimental setting, for example, comparing the effect of dietary gluten elimination in newly diagnosed CD patients *vs* the return of dietary gluten in patients maintained on a long-term GFD. Ilanen *et al*^[104] found elevated expression of mitochondrial HSP65 in 80% of jejunal biopsies

Table 2 Involvement of heat shock proteins in coeliac disease

| Samples | Investigation | Localization/major findings | Ref. |
|--|---|---|-------|
| Duodenal biopsies from 16 children with newly diagnosed CD, 9 maintained on GFD, 10 controls | HSP72 mRNA expression, protein level and localization | HSP72 mRNA and protein are increased in CD, and decreased by GFD. HSP72 was localized in villous enterocytes of the epithelium and lamina propria immune cells | [102] |
| Duodenal biopsy specimens from 30 HLA-DQ2 (+) NCGS and 15 CD patients maintained on GFD | HSP27 or HSP70 mRNA expression, before and after challenge with gluten-containing bread daily for 3 d | mRNA expression of HSP27 and HSP70 in the duodenal mucosa was not different in any of the groups | [103] |
| Jejunal biopsies from 78 children with clinical suspicion of CD | Epithelial HSP65 expression | Increased mitochondrial HSP65 expression in the jejunal mucosa in 80% (16/20) of children with CD and in 24% (14/58) of non-CD patients. Strong correlation between HSP65, $\gamma\delta$ + T-cells and serum IgA endomysial autoantibodies. HSP65 is a potential mucosal integrity modulator | [104] |
| Duodenal biopsies from 12 patients with CD and 10 controls | Small HSP α B-crystallin expression and distribution | Increased α B-crystallin in CD, localized in the supra-nuclear region of enterocytes in the duodenal mucosa | [105] |
| Blood samples from 128 patients with CD and 94 healthy individuals | <i>HSPA1A</i> gene (HSP70-1) polymorphism | Altered frequency of an intermediate <i>HSPA1A</i> allele in CD (64.5%) <i>vs</i> normal (37.2%). HSP70-1 gene is part of a high-risk haplotype for CD | [106] |
| Blood samples from 19 families with CD patients and 95 healthy individuals | HLA-linked <i>HSPA1B</i> gene (HSP70-2) polymorphism | Altered <i>HSPA1B</i> allele frequencies in CD <i>vs</i> normal and non-affected MHC haplotypes | [107] |

CD: Coeliac disease; GFD: Gluten-free diet; HSP: Heat shock protein; NCGS: Non-coeliac gluten sensitivity; Ig: Immunoglobulin; HLA: Human leukocyte antigen; MHC: Major histocompatibility complex.

from children diagnosed with CD compared to 24% of specimens from children with a normal biopsy. The levels of HSP65 correlated with the number of + T-cells and serum IgA endomysial autoantibodies, suggesting that HSP65 may be an indicator of disease activity. Yeboah *et al*^[105] examined the duodenal mucosa of CD patients and found a close correlation between the distribution of the small HSP α B-crystallin and the degree of villous atrophy, indicating its involvement in the modulation of mucosal integrity.

Single nucleotide polymorphisms in the 5' regulatory region of the gene encoding HSP70-1 (*HSPA1A*) have been linked with CD. Ramos-Arroyo *et al*^[106] found a significantly higher frequency of an *HSPA1A* allele showing an intermediate electrophoretic mobility in patients with CD. Individuals expressing CD-associated HLA alleles that were homozygous for this intermediate *HSPA1A* allele were 12-fold more likely to develop CD, indicating that *HSPA1A* polymorphisms are an additional predisposing factor for CD as a component of a high-risk haplotype. Partanen *et al*^[107] found significantly deviated gene frequencies of the *HSPA1B* (HSP70-2) gene cluster in 19 families of patients with CD compared to that of a normal population, indicating that a polymorphism of the HLA-linked *HSPA1B* gene may be involved in the pathophysiology of CD. The main scientific findings indicating involvement of HSPs in CD are summarized in Table 2.

HSPs AND THERAPEUTIC TREATMENTS

Although promising results have been found using HSP-based vaccines for the treatment of cancer patients^[108], relatively little is known about the therapeutic potential of HSPs in the treatment of gastrointestinal diseases. There is evidence to suggest, however, that targeting of HSPs would be beneficial. The anti-ulcer drug geranyl-

geranylacetone (GGA) that reduced colitis in a mouse model was found to induce the intestinal expression of HSP70 and to suppress myeloperoxidase activity and reduce TNF- α and IFN- γ levels^[109]. Furthermore, it was demonstrated that the upregulation of HSPs by GGA is protective against intestinal damage from non-steroidal anti-inflammatory drugs such as indomethacin^[110]. Indeed, overexpression of HSP70 in mice decreased the number of indomethacin-induced apoptotic cells and the level of proinflammatory cytokines and chemokines (IL-1 β , IL-6) in the small intestine, suggesting that HSP70 is protective and can reduce the extent of small intestinal lesions^[36]. A strong correlation between the expression of HSPs and the advantageous effects of probiotics in IBD has also been suggested^[111], and probiotics containing eight different naturally occurring strains of "beneficial" bacteria may induce the expression of HSP25 and HSP72 in colonic epithelial cells^[88]. Moreover, probiotic *Lactobacillus GG* induces the expression of HSP72 in intestinal epithelial cells, contributing to the beneficial clinical effects through preservation of cytoskeletal integrity^[112]. These data suggest that HSP-inducers are promising drugs to treat gastrointestinal diseases, including CD, or ameliorate their symptoms.

CONCLUSION

HSPs are a class of highly conserved, stress-induced chaperones that are responsible for proper protein folding and regulating protein degradation, cell repair, immune responses, cell signalling, cell proliferation, apoptosis, and tumour cell differentiation. The increased expression of various HSPs observed in CD suggests that their antioxidant and anti-apoptotic features are protective. Furthermore, HSPs may be involved in the pathophysiology of CD through their immunomodula-

tory effects, serving as “danger signals” for the immune system at sites of tissue injury.

Intrinsic apoptotic pathways initiated by intracellular stress signals can be blocked by HSPs, which likely contributes to the maintenance of intestinal homeostasis. HSPs suppress the expression of iNOS and reduce the level of nitric oxide, thereby providing cellular protection against stress. HSPs are also involved in tissue repair and remodelling by regulating the production of matrix metalloproteinases in the intestine, which are increased in patients with CD. In conclusion, HSPs appear to influence the key features of CD through their contribution to the maintenance of mucosal barrier integrity, inhibition of apoptosis, and regulation of inflammatory processes. Therefore, therapies targeting the expression of HSPs in the intestinal mucosa should be pursued for the treatment of inflammatory gastrointestinal diseases.

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Treatment of refractory diabetic gastroparesis: Western medicine and traditional Chinese medicine therapies

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Abstract

Refractory diabetic gastroparesis (DGP), a disorder that occurs in both type 1 and type 2 diabetics, is associated with severe symptoms, such as nausea and vomiting, and results in an economic burden on the health care system. In this article, the basic characteristics of refractory DGP are reviewed, followed by a discussion of therapeutic modalities, which encompasses the definitions and clinical manifestations, pathogenesis, diagnosis, and therapeutic efficacy evaluation of refractory DGP. The diagnostic standards assumed in this study are those set forth in the published literature due to the absence of recognized diagnosis criteria that have been assessed by an international organization. The therapeutic modalities for refractory DGP are as follows: drug therapy, nutritional support, gastric

electrical stimulation, pyloric botulinum toxin injection, endoscopic or surgical therapy, and traditional Chinese treatment. The therapeutic modalities may be used alone or in combination. The use of traditional Chinese treatments is prevalent in China. The effectiveness of these therapies appears to be supported by preliminary evidence and clinical experience, although the mechanisms that underlie these effects will require further research. The purpose of this article is to explore the potential of combined Western and traditional Chinese medicine treatment methods for improved patient outcomes in refractory DGP.

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Key words: Refractory diabetic gastroparesis; Nutrition; Gastric electrical stimulation; Botulinum toxin; Traditional Chinese treatment; Surgery

Core tip: In this review, we summarize the latest therapeutic approaches for refractory diabetic gastroparesis (DGP), a condition that may be difficult to treat. For most patients with refractory DGP, invasive and expensive treatment options are frequently applied. They cause mental suffering and are expensive, and not all patients are likely to benefit from these therapies. Therefore, as with many chronic conditions, patients may seek complementary and alternative therapies. In clinical practice, traditional Chinese medicine (TCM) appears efficacious and offers a less invasive treatment option. Moreover, preliminary evidence in the literature has also suggested the efficacy of TCM.

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OUTLINE OF REFRACTORY DIABETIC GASTROPARESIS

Definition and clinical manifestations

First described in 1958, diabetic gastroparesis (DGP) is a well-established complication of diabetes mellitus (DM). As a chronic motility disorder of the stomach that is associated with both type 1 and type 2 DM, DGP is characterized by delayed gastric emptying (GE) of a solid meal with no evidence of mechanical obstruction. The most common disabling symptoms of DGP include nausea, vomiting, postprandial fullness, early satiety, and bloating^[1-3]. In some patients with DGP, symptoms such as severe, persistent nausea and vomiting may be very difficult to treat, which together define refractory DGP. This condition is often unresponsive to medical therapy, and because patients are unable to maintain nutrition *via* the oral route, frequent emergency department visits and/or hospitalization may be necessary^[2,4,5]. The overall prevalence of refractory DGP has not been determined; however, severe symptoms necessitating multiple hospitalizations are estimated to occur in 2% to 5% of patients with gastroparesis (GP)^[6].

Refractory DGP contributes to a range of morbidity issues, including weight loss, malnutrition due to inadequate caloric and fluid intake, electrolyte disturbances and dehydration, poor glycemic control, recurrent episodes of diabetic ketoacidosis, and increased healthcare utilization^[1,5-7]. These effects on health may also decrease work performance and disrupt normal life, which may themselves be disabling consequences. Notably, refractory DGP is increasingly recognized for its significant economic burden on the health care system^[8-10].

Pathogenesis

The pathogenesis of DGP has been related to autonomic neuropathy of the vagal innervation of the stomach in up to 40% of cases^[11,12]. Many of the metabolic and hormonal disturbances caused by DM may also lead to impaired gastric tonicity and antral contractions^[13].

Diagnosis

To date, there has been no accepted definition for the diagnosis of refractory DGP; most diagnostic parameters are drawn from the published literature and are based on reported clinical experiences. The key diagnostic criteria are as follows: (1) documented diagnosis of DGP for more than one year; (2) refractoriness or intolerance to antiemetics and prokinetics after more than 6 mo on a full regimen of standard medical therapy; (3) more than 7 emetic episodes per week or chronic daily nausea; and (4) delayed GE (> 60% retention at 2 h and/or > 10% at 4 h on the basis of a standardized scintigraphic method for GE of a solid meal)^[14-16]. Based on published literature, spleen-kidney yang deficiency syndrome is thought to be common for refractory DGP in traditional Chinese medicine^[17-20].

Efficacy evaluation

The efficacy of treatment for refractory DGP demands closer evaluation, as the subjective symptoms reported by patients, rather than sole reliance on results of GE tests, play an important role in the evaluation of treatment efficacy. The efficacy evaluation associated with an objective indicator may not be ideal, as some reports have indicated a poor correlation between improvement in GP symptoms and GE test results^[21,22]. Additionally, frequent vomiting and generalized weakness may also interfere with the performance of this complicated objective test, as it is very difficult to complete GE studies in patients with severe nausea and vomiting. The subjective symptoms are the only measures that directly reflect the patient's experience of symptom severity, functioning, and well-being^[23].

Quantification of the severity and characteristics of GP symptoms has been facilitated by the introduction of validated questionnaires. The Gastroparesis Cardinal Symptom Index (GCSI) has been the most widely used validated questionnaire and best reflects the subjective symptoms reported by patients, with a high correlation between these symptoms and GE. The GCSI is an assessment of symptom severity over a two-week period that incorporates nine symptoms assessed by three subscales (postprandial fullness/early satiety, nausea/vomiting, and bloating) and represents a subset of the more comprehensive Patient Assessment of Gastrointestinal Symptoms^[24-26]. Furthermore, weekly vomiting frequency and severity, the SF-36 Health Status Survey questionnaire, nutritional status, and weight of patients are also commonly measured parameters for study enrollment and efficacy evaluation in research trials.

TREATMENT OVERVIEW

Management

Strategies for the treatment of refractory DGP in clinical practice are based on controlling symptoms (particularly nausea and vomiting), improving delayed GE, and providing dietary and hydration measures to stabilize weight and support nutrition^[2]. First among the general principles that apply to refractory DGP treatment is the establishment of an accurate diagnosis of GP. Second, the metabolic status should be evaluated and glycemic control should be optimized, as hyperglycemia has been demonstrated to delay GE and to weaken the effects of prokinetic drugs on GE. In patients with DM, glycemic control includes dietary measures, which generally include restricted meal volumes, increased meal frequency, small-particle foods, and supplementary nutrition. The avoidance of excess fat and dietary fiber is necessary, as these dietary elements may aggravate delayed GE. With respect to drug therapy, standard drugs should be administered before patients are categorized as drug-refractory. For patients with medically refractory gastroparesis, gastric electrical stimulation (GES) is a minimally invasive surgical method that should be considered for severe symptoms, especially nausea and vomiting. Pyloric botulinum toxin

(BTX) injection has also been used recently for refractory DGP, as reported in several small case studies. More aggressive treatments include hospitalization for the intensive management of high glucose levels with insulin administration, intravenous hydration, and intravenous administration of antiemetic and prokinetic agents^[1,2,26-28]. Enteral or parenteral nutritional support and/or surgical intervention may also be necessary. Gastrectomy is performed as a last option and under conditions of strict patient selection. The different therapeutic modalities may be used alone or in combination, according to the needs of the individual patients. The use of traditional Chinese medicine (TCM) in the treatment of refractory nausea and vomiting is extensive. Preliminary evidence that has indicated the effectiveness of TCM appears to support the efficacy of TCM utilized in clinical practice. Additional research on the mechanism(s) of action of TCM, involving large-scale, randomized controlled trials, is warranted to strengthen the evidence base for future studies. The purpose of this article is to present the optimal, combined benefits of Western medicine and traditional Chinese treatments for refractory DGP for improved patient outcomes.

Drug therapy

The standard drug therapy for DGP, essentially unchanged over the past decade, mainly relies on the use of prokinetic agents, such as erythromycin, metoclopramide, and domperidone, which are the cornerstones of refractory DGP therapy. Tegaserod, cisapride, bethanechol, and other drugs are also commonly used; however, the use of cisapride is markedly restricted or may be discontinued due to side effects^[5]. Prokinetic agents have been demonstrated as the mainstay of treatment in patients with DGP, although the benefits may be short-lived and some diabetic patients may respond poorly^[1,2,26,27]. A meta-analysis involving 514 patients from 36 clinical trials revealed that erythromycin was the most potent stimulant of GE^[29], while a double-blind multicenter trial reported metoclopramide and domperidone as being equally effective in reducing symptoms of nausea and vomiting in patients with DGP^[30]. The antiemetic medications used included phenothiazine, a serotonin 5-HT₃ receptor antagonist, an antihistamine, and low-dose tricyclic antidepressants. The D₂ receptor antagonists metoclopramide and domperidone also have some anti-emetic effects and are usually administered to patients with nausea and/or vomiting. Although the use of antiemetic agents may delay stomach emptying, some patients who experience insufficient relief from prokinetic drug therapy or who develop unacceptable toxicity to prokinetics may respond positively to antiemetics. In patients with refractory DGP, prokinetic agents and antiemetic medications are often used in combination to reduce symptoms. Patients with DM should also be counseled regarding the need to achieve tighter glycemic control. More aggressive glucose monitoring that allows for the frequent dosing of short-acting insulin preparations to prevent post-prandial

hyperglycemia is likely to be effective, as the prevention of hyperglycemic spikes and widely fluctuating glycemia may be more important than the maintenance of a given steady-state blood glucose level^[31]. In summary, the monitoring of 2-h postprandial blood glucose levels may be useful, as the potential advantages of optimal glucose control are increased antral contractility, correction of gastric dysrhythmias, and accelerated GE^[32,33].

Recently, a proof-of-concept study^[34] demonstrated that a ghrelin receptor agonist (TZP-101) significantly improved GE and may be well tolerated in DM patients with moderate-to-severe chronic GP. Wo *et al.*^[35] conducted a multicenter, randomized, double-blind, placebo-controlled study of TZP-101 in DGP patients with severe nausea/vomiting. They reported a reduction in the GCSI Nausea/Vomiting subscale score of 3.82 ± 0.76 ($P = 0.011$) at the end of treatment compared with baseline in the 80 µg/kg group, a result that reached statistical significance. The investigators demonstrated that TZP-101 was able to reduce the frequency and severity of nausea and vomiting, as well as the overall symptoms, to a substantial and significant degree in a subset of the most refractory DGP patients.

Nausea and vomiting are often the most debilitating symptoms for patients with refractory DGP. Aprepitant, a neurokinin-1 receptor antagonist, has been approved in the US for nausea and vomiting associated with surgery and cancer chemotherapy. Chong *et al.*^[36] reported a case of refractory nausea in a patient with DGP who was successfully treated with aprepitant. This treatment was continued for 4 mo, and the patient showed a rapid and significant response to the agent, with improvements in both nausea and vomiting, despite an absence of significant effects of aprepitant on accelerated GE. The 5-HT₂ receptor antagonist mirtazapine^[37] has also been shown to be effective in a single report involving refractory DGP. The severe recurrent nausea and vomiting that persisted for over 7 mo in this case improved dramatically within a few days of once-daily mirtazapine administration. However, controlled studies may be warranted to further evaluate the benefit of these medications in improving symptoms in patients with DGP.

Other symptoms, such as early satiety, have been related to the functional dyspepsia caused by defects in fundic accommodation. The use of nitrates, buspirone, sumatriptan, and selective serotonin reuptake inhibitors may relax fundic muscles in this condition, but no relevant studies have indicated their efficacy in the management of early satiety in patients with GP^[5]. The pathogenesis of abdominal pain, which can be disabling in patients with GP, is postulated to be due to sensory rather than motor dysfunction^[38]. Thus, treatments that aim to reduce sensory afferent dysfunction may be more effective. However, agents commonly used in clinical practice, including tricyclic antidepressants, gabapentin, paroxetine, and opiates, have been generally unsatisfactory in treating this pain. In a case report^[39], a 50-year-old woman who had suffered from type 1 DM for 20 years was described

as having chronic abdominal pain and malnutrition due to severe DGP and was successfully treated with a celiac plexus block (CPB). This patient was able to successfully avoid any narcotic use, with successful analgesia achieved and maintained for 10 wk following CPB.

Nutritional support

Nutritional support is often overlooked in patients with DGP, and few randomized controlled trials have evaluated the effects of nutritional intervention on outcomes^[33]. For patients with refractory DGP who are unable to meet caloric and fluid needs *via* oral dietary modifications, enteral alimentation or total parenteral nutrition (TPN) should be considered.

Enteral alimentation is the preferred route for nutritional and hydration supplementation in patients with recurrent vomiting and unpredictable oral intake. Clinically common intubations for enteral alimentation are as follows: nasogastric tube, nasoduodenal/nasojejunal tube, gastrostomy tubes, PEG-J or Jet-PEG, jejunostomy, and dual gastrostomy and jejunostomy. The criteria for initiation of enteral nutrition supplementation include unintentional loss of more than 5%-10% of the usual body weight during a period of 3-6 mo and/or repeated hospitalizations for refractory symptoms^[40]. Enteral feedings may maintain nutrition, relieve symptoms, improve glycemic control, and reduce emergency department visits or hospitalizations in patients with normal small bowel function^[1]; however, a relatively high incidence of both major and minor complications, including infection, tube migration, and dislodgement^[41,42], have been reported.

Jejunostomy tube (J-tube) placement is the most common route for enteral alimentation and is a relatively inexpensive, safe, and physiological option for refractory DGP^[28]. The feeding J-tube that bypasses the paralyzed stomach is usually placed by laparotomy or laparoscopy^[43]. J-tubes enable jejunal nutrient and medication delivery and avoid gastric penetration that may interfere with proper electrode placement for gastric electrical stimulation. A disadvantage of the J-tube is the inability to vent the stomach, and the most common complications are tube obstruction and skin infection^[28,40]. Enteral feedings can be initiated 24 h after J-tube placement. They are initiated with diluted infusions and are gradually advanced to iso-osmolar preparations at relatively low infusion rates (*e.g.*, 20 mL/h) that are then increased to the target infusion rate that supports nutrition and hydration, typically at least 60 mL/h over 12-15 h/d^[28,40]. With regard to the important "rule of J-tube feeding," oral caloric intake should not occur when the J-tube is running, because calories within the small bowel will inhibit gastric emptying and induce further nausea and/or vomiting^[28].

Fontana *et al*^[43] investigated the long-term results and complications of J-tube placement. A retrospective analysis was performed on 26 patients with a mean follow-up of 47 mo (1-130 mo). The results indicated that 83% of the patients demonstrated improved overall health status, which was the only indicator that reached statistical sig-

nificance. Symptoms of nausea/vomiting, hospitalization rate, and nutritional status were also slightly improved. There were 23 major and 42 minor complications identified in this study, as well as one death that was directly caused by the J-tube placement, although a large proportion of the morbidity and mortality rates was clearly attributed to the malignant nature of DM and other chronic illness-related factors. Jacober *et al*^[44] reported the benefits of J-tube feeding in 4 diabetic patients with refractory GP. They demonstrated that the J-tube may be effective in providing nutrition, fluids, and medications in cases of either normal or abnormal small intestinal motor function. Technological improvements may influence the placement of J-tubes and their efficacy^[28,43]. Ginsberg *et al*^[45] used an endoscopically placed clip that assisted with tube placement and may have possibly prevented later accidental dislodgement. In summary, in cases of patients who fail medical therapy, the placement of a J-tube may present an available option with acceptable morbidity and mortality rates. Prospective, randomized controlled trials of J-tube use are required to provide more specific evidence for the efficacy of this therapy.

In patients with severe vomiting and fullness, the placement of a gastrostomy tube (G-tube) for intermittent decompression and the venting of secretions may help to provide some symptom relief, but it is a poor choice for feeding due to delayed GE. The G-tube can be placed endoscopically, surgically, or by fluoroscopy guidance. Hejazi *et al*^[28] indicated that G-tube decompression is advocated in cases of intestinal pseudo-obstruction rather than in the usual setting of gastroparesis given that the infusion of liquid meals into the stomach *via* a G-tube is unsafe due to the likelihood of symptom exacerbation and the risk of pulmonary aspiration caused by delayed GE. Data from Revicki *et al*^[46] revealed that 6 of 8 patients were able to return to work or school with a venting gastrostomy. Kim *et al*^[47] performed a non-randomized study of 8 patients, the results of which included relief of nausea and some gastric decompression in the treatment of refractory idiopathic GP.

Clinical data on other types of tubes have been limited until now. The nasogastric tube is usually inserted for acute management by gastric decompression. Short-term nasojejunal feeding is often used to help determine whether a patient may tolerate chronic small bowel feedings *via* permanent enteral access^[1,2,28,40].

Enteral alimentation is more acceptable than TPN in most patients. TPN introduces the risk of catheter-related infections, which is an important concern in diabetic patients; moreover, TPN is more expensive than enteral alimentation. However, in cases of severe malnutrition in which an adequate nutritional and hydration state cannot be maintained; in patients with small-bowel dysmotility, including chronic intestinal pseudo-obstruction, who are unable to tolerate J-tube feedings; and when surgery is planned, patients may benefit from the short-term use of TPN. TPN may maintain short-term normalization of GE and provide supplemental caloric support combined

with tight blood glucose control. In general, each liter of TPN requires the addition of 30-40 units of regular insulin, depending on the patient's previous insulin requirements and the TPN contents (1 unit of regular insulin per 5 g of carbohydrate or 15 g of protein)^[2].

GES

GES is a rapidly evolving treatment for patients with refractory symptoms related to DGP^[48]. GES involves the surgical implantation of unipolar electrodes into the muscular layer of the gastric antrum, as well as a pulse generator in the abdominal wall to deliver low-energy, 0.1-s pulse trains at a high frequency of 12 cycles/min of electrical energy, which is higher than the normal slow-wave gastric activity of 3 cycles/min^[49-51]. The early use of GES was reported by Lin *et al*^[52] and McCallum *et al*^[53] in 1998. The findings from two randomized trials that demonstrated good results prompted the US Food and Drug Administration to approve the usage of high-frequency, low-energy GES (Enterra Therapy System, Medtronic, Minneapolis, United States) with a Humanitarian Device Exemption in March 2000. The mechanism of action of GES is postulated to be the modulation of function of the autonomic nervous system and enteric nervous system, which affects the secretion of gastrointestinal hormones, reduces gastric sensitivity to distention, changes antral motor function, and enhances fundic relaxation^[48]. A direct central nervous system effect is also involved, whereby energy stimulates a central nausea and vomiting center in the brain^[49]. Recent studies have demonstrated that GES is an effective and safe treatment for patients with refractory DGP^[16,51,54-57].

Lin *et al*^[57] reported a GES study involving the largest sample size and the longest follow-up period to date. In this retrospective assessment, 221 patients (142 with DGP) were treated with Enterra (Medtronic); 188 patients underwent follow-up visits, and data were collected for up to 10 years (mean, 56 mo; range 12-131 mo). This therapy was reported to reduce the need for hospitalizations, limit the need for prokinetic and antiemetic medications, improve nutritional status, and decrease total symptom scores (TSSs). Two-hour gastric retention decreased from a median of 70% at baseline to 66%, and 4-h retention decreased from 37% to 30% at the time of last follow-up. The limited data showed a decrease in mean HbA1c levels. In this long-term study, the complications and overall safety of GES therapy were well documented; specifically, 24 patients (11%) underwent device removal, mainly due to infection, lack of efficacy, and small-bowel obstruction. This report also discussed the relationship between symptom improvement and stimulation energy, concluding that energy parameters and increasing voltage do not explain the likelihood of symptom reduction.

McCallum *et al*^[55] also investigated outcomes in 55 patients who kept the device on or off over one year in a prospective, placebo-controlled study. The median reduction in weekly vomiting frequency (WVF) during the initial 6 wk was 57% ($P < 0.001$) compared with the baseline

values. The WVF and TSS (frequency and severity) were not significantly different between patients who kept the device turned on or off during the crossover period. At 12 mo, the WVF decreased significantly compared with baseline, with a median reduction of 67.8% ($P < 0.001$), while the frequency and severity of the TSS were also reduced from baseline to 12 mo ($P < 0.001$). Patients also had significant improvements in GE, quality of life, and number of hospitalization days. The stimulation during the initial 6 wk significantly decreased DGP symptoms prior to the double-blind, randomized phase. An explanation for this finding may be the possibility of sustained memory or carryover effects from the 6 "active" weeks, which emphasizes the neuroplasticity of the mechanisms controlling nausea and vomiting by the central nervous system. Stimulation was administered for 1.5 mo before the randomized controlled trial (RCT) (6 mo) in the study by McCallum *et al*^[54], and the results from the RCT period may have been affected by the previous stimulation.

McKenna *et al*^[49] reported short-term results in 19 patients who had the device activated for a mean of 38 wk. Within 6 wk, the WVF had significantly decreased in 75% of patients with DGP, the TSS improved after 6 wk of stimulation and remained improved throughout the 12-mo follow-up, and nuclear medicine GE studies normalized in 80% of patients with DGP. However, there was no significant difference in health and quality of life as measured by the SF-36, most likely as a result of the relatively small sample size. This study concluded that GE correlated poorly with actual symptomatic outcomes, while Hou indicated that GES not only significantly improved symptoms but also improved 2- and 4-h GE in GP patients^[56]. Therefore, further studies are needed to resolve these results.

The Worldwide Anti-Vomiting Electrical Stimulation Study^[16] was a randomized, controlled, double-blind trial. During the 2 × 1-mo crossover phase, the device was alternately turned on and off, followed by a non-controlled observational phase. Included in the study were 33 patients, 17 of whom were diabetics, and most were followed for 12 mo. Patients in this study experienced a significant decrease in vomiting frequency and symptom scores, and they reported feeling significantly better when the devices were turned on than when they were turned off. The mean quality of life scores were nonequivalent at baseline. However, these changes did not reach statistical significance during the RCT phase, perhaps due to the relatively brief stimulation time, which lasted for only 2 mo.

In conclusion, the majority of studies on GES have demonstrated that GES therapy is effective in decreasing nausea and vomiting scores, promoting GE^[54-56], reducing gastroparesis-related hospitalizations^[54,57], improving health-related quality of life^[57], and controlling hemoglobin A1C levels in diabetics^[54-56,57]. Kastenmeier *et al*^[58] found GES to be beneficial for eliminating reliance on supplemental nutrition, with 60% of patients able to eliminate all supplemental nutrition within a mean of 5

mo post procedure. A meta-analysis showed similar feeding success postoperatively, with the majority (78%) of patients no longer requiring enteral or parenteral nutrition.

GES treatment is expensive and invasive, and not all patients are likely to benefit from this therapy^[59]. According to the literature, three parameters were found to have an impact on its clinical efficacy: the etiology of GP, main symptoms, and use of narcotics. Patients with DGP did better than patients with idiopathic GP, while patients in whom nausea and vomiting were the main symptoms exhibited a more favorable response than patients in whom abdominal pain was the main symptom. Additionally, patients who were not taking narcotics had better outcomes than those who were using narcotics at the study onset^[51]. Furthermore, there was a tendency for non-responders to be female and to have suffered GP symptoms for a longer period of time prior to initiating therapy^[59].

Pyloric BTX injection

BTX has been previously used for the treatment of spasm of the gastrointestinal sphincters. More recently, pyloric BTX injection has been evaluated for use in patients who are refractory to standard management in several small case studies, open-label trials, and RCTs, although BTX does not have an FDA-labeled indication for this use. BTX injection therapy achieved the goal of improving GE by decreasing the release of excitatory transmitter substances and promoting muscle relaxation^[24,60].

A few uncontrolled, open-label studies (< 30 cases) have indicated that the injection of 80-200 units of BTX during endoscopy in a circumferential manner at four to five sites into the pylorus was effective in improving GE and decreasing symptoms in patients with DGP^[61-64]. Symptoms decreased by an average of 45% (range, 29%-58%), while GE improved by a mean of 42% (range, 33%-50%), with a decreased symptom duration lasting 1-4 mo after injection^[40]. In a report by Coleski *et al*^[65] on 179 patients (81 patients with DGP), the duration of the response ranged from 1-4 mo, and 51.4% of the patients experienced a symptomatic response to BTX. The study concluded that responses to pyloric BTX depended on dose and were maintained with repeated injections. Another study^[66] showed that higher doses of BTX (150-200 units) were more likely to yield reductions in nausea and vomiting compared with a dose of 100 units and that a longer evaluation time also appeared to correlate with an improved response.

However, a study by Coleski *et al*^[65] indicated that the response rate (35%) in patients with vomiting as a major symptom of GP was significantly lower than that in patients without vomiting (57%). BTX injection may decrease the severity of subjective symptoms, but these symptoms may not include nausea and vomiting, which may even recur and worsen.

Additionally, this therapy has not been demonstrated to be effective in RCTs^[67,68]. In a randomized, double-blind, placebo-controlled trial^[68], 32 patients were randomized to BTX A or placebo for a 1-mo follow-up

period. The results indicated that symptom improvement was achieved in 37.5% and 56.3% of the individuals in the botulinum and control groups, respectively. Although improvement in GE was observed in the botulinum group, it was not superior to that in the placebo group. The BTX group demonstrated improvement in GE; however, this improvement was not significantly different compared with that in the placebo group. Some limitations existed, *e.g.*, the insufficient sample size and the short follow-up period. In the future, this therapy will require more in-depth testing and verification. To date, no serious adverse events have been attributable to BTX. The major limiting factors are related to insurance coverage and the inconvenience of undergoing endoscopy^[2].

Surgical therapy

Surgical therapy is increasingly employed in the treatment of patients with refractory DGP. Surgical therapy is a reasonable option to consider in patients who suffer from medically refractory nausea and vomiting, fail to achieve benefits from medical therapy, and present with weight loss or aspiration, as well as in patients who experience difficulty in taking oral medications. Unfortunately, this therapy has historically been plagued by high morbidity rates without consistent symptom response rates^[68]. Thus, in cases of judicious patient selection with careful assessments of the risk for malnutrition caused by surgery and following surgery, as well as the risk of renal failure, among other concerns, surgery may be performed. Traditional surgical options include sub-total gastrectomy or esophagojejunostomy, surgical drainage procedures (pyloroplasty or pyloromyotomy), gastric stimulator implantation, gastrostomy/jejunostomy tube insertion and total parenteral nutrition, pancreatic transplantation in diabetics. Generally, sub-total gastrectomy and completion gastrectomy comprise an effective therapy which can reduce symptoms, enhance delayed GE, and improve quality of life^[49,69-71].

Saridena *et al*^[69] reported on the outcomes in 9 patients with refractory GP (6 of whom were diabetic patients with a fully intact stomach) who underwent total gastrectomy with a mean follow-up of 3.5 years (range, 1-5 years), with six patients available for follow-up. In all patients, refractory nausea and vomiting were reduced by an average of 55%, and the frequency of hospitalization was significantly decreased. Their nutritional status was stabilized with the insertion of J-tubes, and there was a considerable improvement in the quality of life after total gastrectomy.

Watkins *et al*^[72] examined the long-term outcomes in patients with intractable vomiting due to DGP. Symptomatic relief in patients with a preoperative grade of Visick III-IV was evaluated objectively and reached 43% in 6 of 7 patients almost immediately after surgery. However, these results were accompanied by risks of subsequent renal failure and poor life expectancy.

Very limited data from uncontrolled studies have shown a small improvement of symptoms with pylo-

roplasty or pyloromyotomy^[73]. Surgical pyloroplasty resulted in some benefit in 30% of patients with GP^[74]. Hibbard *et al*^[75] reported excellent outcomes in patients who underwent minimally invasive pyloroplasty for refractory GP and proposed that a completely endoscopic pyloroplasty may be an even less invasive treatment option with advance of technology. A retrospective analysis reviewed the collective data of 28 patients, 26 of whom had undergone laparoscopic pyloroplasty and 2 of whom had undergone endoscopic pyloroplasty. The pre- and post-operative symptom severity scores (SSS), GES, and medication use were evaluated. The use of prokinetic agents was significantly reduced from 89% to 14%. The mean GES T-1/2 was decreased from 320 to 112 min ($P = 0.001$) and normalized in 71% of patients. Significant improvements in the SSS were observed at 1 mo and persisted for 3 mo. Symptoms were reported as improved at the 1-mo follow-up in 83% of patients.

Sarosiek *et al*^[76] initiated a controlled study in which GES was replaced with surgical pyloroplasty (PP) to improve GE and symptom control in patients with DM and other conditions. Forty-nine patients (17 diabetic, 9 idiopathic, and 23 post-vagotomy) underwent GES implantation, and 26 (53%) additionally received PP, with a mean follow-up of 7 mo. The TSS, 4-h GE, adverse events, and hospitalizations were observed at baseline and at the last follow-up. The results indicated that the TSS of patients in both the GES plus PP and GES groups significantly improved compared with their baseline scores; however, the TSS was not significantly different between the two groups. GE was improved by 64% at 4 h ($P < 0.001$) in patients with GES and PP, compared with 7% after GES therapy alone. No adverse events accompanied the addition of PP to GES. The authors concluded that in drug-refractory GP, the addition of PP to GES substantially accelerated GE, as well as that PP added to GES may sustain improved long-term symptom control, particularly in the post-vagotomy setting.

TCM

TCM and acupuncture are common modalities in complementary and alternative therapies, both of which have a long history of use in the treatment of refractory nausea and vomiting and also help to alleviate abdominal distension^[2,5,40]. Some clinical guidelines and reviews have recommended acupuncture as a treatment method for DGP^[2,5,40]. Many patients with incurable diseases, such as refractory DGP, particularly in China, are advised to visit a traditional Chinese physician^[2].

Professor Tong conducted a study^[77] based on a nearly six-year clinical practice. The method of retrospective analysis was applied to collect data on 47 patients with recurrent vomiting who were followed for three months. Xiaoban Xiatang combined with Suye Huanglian Tang was administered most frequently in this study. The severity of overall symptoms, particularly nausea/vomiting, and the postprandial fullness/early satiety and bloating subscale of the GCSI were evaluated before and after

treatment. The results indicated that symptom relief after treatment was significantly improved compared with baseline ($P < 0.05$) and that the efficacy increased with time. Fasting blood glucose levels and HbA1c also improved with treatment.

Generally, Xiaoban Xiatang combined with Suye Huanglian Tang contributes to improving symptoms of severe nausea and vomiting rapidly^[78-80]. Xiaoban Xiatang is composed of Rhizoma Pinelliae (Ban Xia) and ginger (Sheng Jiang). Ginger is a traditional Chinese antiemetic agent that exhibits weak 5-HT₃ receptor antagonist properties and gastric slow-wave anti-dysrhythmic effects in humans^[81,82]. This prescription may effectively alleviate nausea and vomiting *via* inhibition of NK1 receptor activity, antagonized motilin activity, and release of intestinal serotonin (5H-T)^[79,80,83,84]. Suye Huanglian Tang is composed of Rhizoma Coptidis (Huang Lian) and Perilla Leaf (Su Ye), which has been used to treat intractable vomiting, mainly based on clinical experience^[85-87] and without current pharmacological confirmation. Abdominal distention is also an important symptom in DGP. The traditional Chinese formula Zhizhu Tang is commonly used to treat postprandial fullness/early satiety and bloating by improving gastrointestinal motility. Zhizhu Tang is composed of Atractylodes macrocephala and Citrus aurantium. Liu *et al*^[88] reported that Tangweian Jianji, which is mainly composed of Zhizhu Tang, may reduce symptoms of diabetic gastrointestinal disorder *via* a pathway that involves changes in the morphometric and biomechanical remodeling of the esophageal and intestinal wall.

Professor Tong proposed the thought of a “combination of symptom, syndrome and disease” as a treatment guide for refractory DGP^[20]. In patients with recurrent nausea and vomiting that cause great distress, the main aim of treatment is to relieve symptoms. Because the main symptoms are viewed as the key factors in syndrome differentiation, the principal prescription should be related to the main symptoms^[20,84]. Xiaoban Xiatang combined with Suye Huanglian Tang is commonly employed, and the decoction must be taken in small doses at short intervals. When nausea and vomiting were mostly alleviated, Professor Tong performed “syndrome differentiation”. Based on its pathogenesis, “spleen-kidney yang deficiency syndrome” is believed to be common in refractory DGP, and the therapeutic method invokes warm yang to dissipate cold and an increase in qi to fortify the spleen. The Fuzi Lizhong Decoction is established as a fundamental prescription that may improve immune system function^[18-20,78,89]. The characteristics of “diseases” and the glycemic level also need to be considered.

Among the existing complementary and alternative therapies, acupuncture is the most-studied method for the treatment of nausea and vomiting of diverse etiologies. The antiemetic effects of acupoint PC6 stimulation have been well established based on a systematic review^[90]. The mechanism of action may be associated with effects on autonomic nerve function related to gastric

motility and changes in gastric hormone levels^[91,92]. Kim *et al.*^[93] presented a case report of a patient with refractory DGP who suffered from frequent nausea, vomiting, and lack of appetite. The patient was treated with 16 sessions of acupuncture for 8 wk as an adjunct to conventional drug treatment, which remained the same during the treatment course. After the treatment period, the GCSI score was reduced from 2.4 to 0.6 and the GE time, as measured by solid meal gastric scintigraphy, showed a significant reduction from 135 to 93 min. At a 4-mo follow-up visit after treatment, the patient reported complete reduction of subjective symptoms. According to a single-blinded, randomized pilot study^[92], acupuncture therapy may also reduce postprandial fullness as well as early satiety and bloating subscale scores at the end of treatment, as measured by the GCSI. Because most TCM treatments for severe gastroparesis in China are reported as case reports^[94,95], prospective RCTs are warranted to obtain a higher level of evidence.

In conclusion, TCM methods are often applied for the treatment of nausea and vomiting and to help alleviate abdominal distension. TCM methods, which are non-invasive and inexpensive, appear to be effective and promising based on clinical practice and some preliminary evidence. With regard to Western therapy, the process of diagnosis and treatment is standardized, and the effectiveness of Western methods has been demonstrated in large-scale clinical trials. The purpose of this review is to propose a combination of Western and TCM methods and to explore the respective advantages of these therapies.

CONCLUSION

The treatment of refractory DGP includes drug therapy, nutritional support, GES, pyloric BTX injection, endoscopic or surgical therapy, and complementary and alternative therapies in selected patients. The different therapeutic modalities may be applied alone or in combination according to the needs of the individual patient. In the past 10 years, experience with GES has represented a major advance in this field, offering new hope for patients with severe symptoms and for those who are refractory to standard medical therapy^[28]. While economically acceptable, effective and safe treatment modalities are limited, TCM and acupuncture therapies appear to be promising based on clinical practice and preliminary evidence, despite a low level of evidence. Surgical therapy with feeding tubes and/or gastric resection surgery is reserved for patients who do not respond to gastric stimulation therapy to augment nutrition and aid in medication delivery. The goals of therapy include the relief of symptoms, normalization of nutrition and hydration status, optimization of glycemic control in diabetic patients, and improvement in GE, when appropriate. Future therapies will be guided by our increased understanding of the pathophysiology of DGP, as well as by studies of non-invasive approaches that may be widely employed to treat refractory DGP.

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miR-132 inhibits colorectal cancer invasion and metastasis via directly targeting ZEB2

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Abstract

AIM: To investigate the biological role and underlying mechanism of miR-132 in colorectal cancer (CRC) progression and invasion.

METHODS: Quantitative RT-PCR analysis was used to examine the expression levels of miR-132 in five CRC cell lines (SW480, SW620, HCT116, HT29 and LoVo) and a normal colonic cell line NCM460, as well as in tumor tissues with or without metastases. The Kaplan-Meier method was used to analyze the prognostic significance of miR-132 in CRC patients. The biological effects of miR-132 were assessed in CRC cell lines using the transwell assay. Quantitative RT-PCR and western blot analyses were employed to evaluate the expression of miR-132 targets. The regulation of ZEB2 by miR-132 was confirmed using the luciferase activity assay.

RESULTS: miR-132 was significantly down-regulated in the CRC cell lines compared with the normal colonic cell line ($P < 0.05$), as well as in the CRC tissues with

distant metastases compared with the tissues without metastases (10.52 ± 4.69 vs 23.11 ± 7.84) ($P < 0.001$). Down-regulation of miR-132 was associated with tumor size ($P = 0.016$), distant metastasis ($P = 0.002$), and TNM stage ($P = 0.020$) in CRC patients. Kaplan-Meier survival curve analysis indicated that patients with low expression of miR-132 tended to have worse disease-free survival than patients with high expression of miR-132 ($P < 0.001$). Moreover, ectopic expression of miR-132 markedly inhibited cell invasion ($P < 0.05$) and the epithelial-mesenchymal transition (EMT) in CRC cell lines. Further investigation revealed ZEB2, an EMT regulator, was a downstream target of miR-132.

CONCLUSION: Our study indicated that miR-132 plays an important role in the invasion and metastasis of CRC.

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Key words: MicroRNA; miR-132; Colorectal cancer; Invasion; Metastasis; Epithelial-mesenchymal transition; Prognosis

Core tip: In this study, we reported the clinical significance and biological effects of miR-132 in colorectal cancer (CRC). We found that miR-132 was significantly down-regulated in tumor cell lines and CRC tissues with distant metastases. Down-regulation of miR-132 was associated with aggressive tumor phenotypes and adverse prognosis in CRC patients. Moreover, we showed that ectopic expression of miR-132 significantly inhibited cell invasion and the epithelial-mesenchymal transition (EMT), whereas knockdown of miR-132 promoted cell invasion and EMT in CRC cells. Further investigation revealed ZEB2, an EMT regulator, was a downstream target of miR-132.

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Xiao GC, Tong SL. miR-132 inhibits colorectal cancer invasion and metastasis *via* directly targeting ZEB2. *World J Gastroenterol* 2014; 20(21): 6515-6522 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i21/6515.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6515>

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in western countries^[1]. Recent development in the therapeutic strategies has helped to cure many patients with early-stage disease. However, the prognosis of patients with advanced disease and metastasis is still poor. Therefore, further investigation into the underlying molecular mechanisms of CRC progression to identify biomarkers to distinguish CRC patients with or without a high risk of metastasis is of great importance.

Tumor invasion and metastasis are parts of a complicated process in which the tumor grows, then detaches from the primary site and metastasizes to a distant organ. Aberrations of protein-coding genes have been widely accepted to play critical roles in the pathology of CRC, including both oncogenes and tumor suppressive genes^[2]. Recently, a series of studies have revealed that microRNAs (miRNAs) regulate various genes that play a pivotal role in the process of tumor progression and metastasis.

miRNAs are a class of short (approximately 18-22 nucleotides in length), endogenous, non-coding RNAs that can negatively regulate the expressions of various protein-coding genes by base pairing with their 3' untranslated region (3'-UTR)^[3-5], leading to their post-transcriptional translation inhibition or mRNA degradation^[6]. miRNAs have been found to be involved in the regulation of multiple pathological processes that contribute to tumorigenesis and metastasis, such as tumor cell proliferation, differentiation, apoptosis, and invasion^[7-9]. In CRC, several reports have indicated the role of miRNAs in regulating tumor invasion and metastasis. For example, miR-93 suppresses the proliferation and colony formation of human colon cancer stem cells by regulating HDAC8 and TLE4^[10]. Additionally, miR-21 is a potential biomarker in colon and rectal cancer^[11]. Previously, Zhang *et al.*^[12] reported that down-regulation of miR-132 by promoter methylation promotes pancreatic cancer development; Formosa *et al.*^[13] found that miR-132 is silenced by promoter CpG island methylation, which contributes to prostate cancer progression and metastasis. However, the role of miR-132 in CRC progression and metastasis remains unclear.

In the present study, we first examined the expression level of miR-132 in CRC tissues and cell lines. The results showed that miR-132 was significantly down-regulated in CRC tissues with metastasis compared with tissues without metastasis; the level of miR-132 was lower in CRC cell lines than in the NCM460 cell line (a normal colonic cell line). Ectopic expression of miR-132 markedly inhibited the invasiveness and epithelial-mesenchymal transi-

tion (EMT) of CRC cells. Further study indicated that ZEB2 (an EMT regulator) was a direct downstream target of miR-132. Collectively, these results demonstrated that miR-132 inhibited cell invasion and EMT in CRC cells through targeting ZEB2, providing a valuable target for cancer therapy.

MATERIALS AND METHODS

Human tissue specimens and cell lines

CRC tissues with distant metastases ($n = 32$) and CRC tissues without distant metastases ($n = 30$) were obtained from 62 CRC patients who underwent initial surgery at Renmin Hospital of Wuhan University between June 2005 and January 2008. All the patients had a histological diagnosis of CRC. Following resection, the specimens were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. All the subjects involved in this study provided written informed consent. This project was approved by the ethics committee of Wuhan University.

The human HT-29, HCT116, SW480, SW620 and LoVo CRC cell lines, the 293T embryonic kidney cell line, and the NCM460 normal colonic epithelial cell line were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum in a humidified 37°C incubator supplemented with 5% CO_2 .

RNA isolation and quantitative real-time PCR analysis

Total RNA from the tissues and cells was extracted using Trizol reagent (Invitrogen, Carlsbad, California, United States). RNA quality and concentration were determined using the Nanodrop 2000 system (Thermo Fisher Scientific, Wilmington, United States). For analysis of the miR-132 level, a TaqMan MicroRNA Assay Kit (Applied Biosystems) was used, and U6 snRNA was used as the reference. For measurement of the ZEB2 mRNA level, a SYBR Premix Ex TaqTM kit (Takara) was used, and β -actin expression was used as endogenous control. Real-time PCR was performed using the Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, United States). Data were analyzed using the $2^{-\Delta\Delta\text{Ct}}$ method.

Western blot analysis

Western blot analysis was performed as previously described^[14]. Briefly, total cellular protein was isolated, and the protein concentration was determined using the Bradford DC protein assay (Bio-Rad, CA, United States). A total of 40 μg of protein was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were then incubated with the following primary antibodies: ZEB2 (1:1000; CST, United States), E-cadherin (1:1000; CST, United States), α -catenin (1:1000; CST, United States), vimentin (1:1000; CST, United States), fibronectin (1:1000; CST, United States) and GAPDH (1:2000; Santa Cruz Biotechnology, United States). Proteins were visualized using the ECL procedure

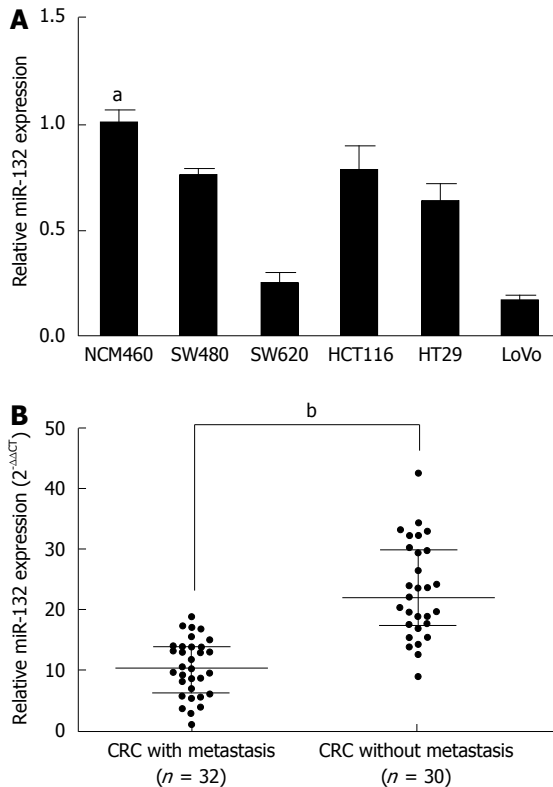


Figure 1 miR-132 is significantly down-regulated in colorectal cancer cell lines and tissues with distant metastases. A: Relative expression level of miR-132 in colorectal cancer (CRC) cell lines and the normal colonic cell NCM460 ($^*P < 0.05$); B: Relative expression level of miR-132 in CRC tissues with distant metastases ($n = 32$) and CRC tissues without metastases ($n = 30$) ($^*P < 0.01$).

(Amersham Biosciences, United States).

Oligonucleotide transfection

The hsa-miR-132 mimic, negative control (NC) oligonucleotides, has-miR-132 inhibitor and scramble oligonucleotides were purchased from Ribobio (Guangzhou, China). The cells were plated in a six-well plate the day before transfection. LoVo cells were transfected with the has-miR-132 mimic or NC (50 nmol/L), and HCT116 cells were infected with the has-miR-132 inhibitor or scramble oligonucleotides (100 nmol/L), using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, the cells were collected, and *in vitro* assays were performed.

Cell invasion assay

The cell invasive potential was evaluated using specialized transwell chambers (8- μ m pore; BD Biosciences). The cells (5×10^4 cells suspended in 500 μ L of serum-free medium) were added to the upper chamber of the inserts, which were coated with a Matrigel mix; fetal bovine serum (500 μ L) was added to the bottom chamber as a chemoattractant. Twenty-four hours later, the non-invading cells on the upper surface were removed, and the cells that invaded to the bottom side of the membrane were fixed with methanol, stained with 0.1% crystal violet, air dried and subjected to digital image acquisition. The

number of invasive cells was evaluated in five independent fields under a microscope. The mean of triplicate assays for each experimental condition was analyzed.

Vector construction and dual-luciferase reporter assay

For the luciferase assays, the potential miR-132 binding site in the ZEB2 3'-UTR was predicted using TargetScan (www.targetscan.org) and miRanda (www.microRNA.org). The 3'-UTR of the ZEB2 mRNA and a mutant ZEB2 mRNA were synthesized and cloned into the *Xba*I site of a pGL3 basic vector (Promega, United States) downstream from the luciferase stop codon and were designated as pGL3-wt-ZEB2 and pGL3-mt-ZEB2, respectively. Then, 293T cells (1×10^5 cells/well) were cultured in 24-well plates and co-transfected with the pGL3-Control (0.4 mg), pGL3-wt-ZEB2 (0.4 mg) or pGL3-mt-ZEB2 (0.4 mg) plasmid, the pRL-TK luciferase reporters (25 ng/well) and pcDNA-miR-132 (20 nmol/L) or pcDNA-miR-NC (20 nmol/L) using Lipofectamine 2000 (Invitrogen, United States). Forty-eight hours later, the cells were harvested, and luciferase activities were measured using a Dual-Luciferase Reporter Assay kit (Promega, United States).

Statistical analysis

The data were expressed as the mean \pm SD. Statistical significance was analyzed using Student's *t*-test (two-tailed). All statistical analyses were performed using SPSS 13.0 or the GraphPad Prism 5.0 software package. The Kaplan-Meier method and log-rank test were performed to analyze the prognostic significance. $P < 0.05$ was considered to be statistically significant in all tests.

RESULTS

MiR-132 was significantly down-regulated in CRC cell lines and CRC tissues with metastases

First, we analyzed the expression of miR-132 in five CRC cell lines and the normal colonic cell line NCM460. MiR-132 was significantly down-regulated in CRC cell lines compared with the normal colonic cell line NCM460 (all $P < 0.05$) (Figure 1A). Among the six CRC cell lines, LoVo possessed the lowest miR-132 level, and HCT116 exhibited the highest miR-132 level (Figure 1A). To explore the expression of miR-132 in CRC tissues with different metastatic characteristics, the miR-132 level was measured in tissues with distant metastases ($n = 32$) and tissues without distant metastases ($n = 30$). Interestingly, miR-132 was markedly lower in CRC tissues with distant metastases than that in CRC tissues without distant metastases (10.52 ± 4.69 vs 23.11 ± 7.84) ($P < 0.001$; Figure 1B). To evaluate the clinical value of miR-132 in CRC patients, we divided the patients into two groups according to the median value (15.40) of the miR-132 level. The correlation between miR-132 and clinicopathological characteristics was then analyzed (Table 1). Low expression of miR-132 was significantly associated with a larger tumor size ($P = 0.016$), distant metastasis ($P = 0.002$) and

Table 1 Correlations between miR-132 expression and clinicopathological characteristics in colorectal cancer patients *n* (%)

| Characteristics | <i>n</i> | miR-132 | | <i>P</i> value |
|--------------------|----------|---------|---------|----------------|
| | | Low | High | |
| Age | | | | 0.307 |
| < 60 yr | 34 | 19 (55) | 15 (45) | |
| ≥ 60 yr | 28 | 12 (42) | 16 (58) | |
| Gender | | | | 0.437 |
| Male | 37 | 20 (54) | 17 (46) | |
| Female | 25 | 11 (44) | 14 (56) | |
| CEA Level | | | | 0.127 |
| 0-5 ng/mL | 32 | 13 (41) | 19 (59) | |
| > 5 ng/mL | 30 | 18 (60) | 12 (40) | |
| CA199 Level | | | | 0.082 |
| 0-35 μ/mL | 46 | 20 (43) | 26 (57) | |
| > 35 μ/mL | 16 | 11 (69) | 5 (31) | |
| Tumor site | | | | 0.796 |
| Colon | 37 | 18 (49) | 19 (51) | |
| Rectum | 25 | 13 (52) | 12 (48) | |
| Tumor size (cm) | | | | 0.016 |
| ≤ 5 | 41 | 16 (39) | 25 (61) | |
| > 5 | 21 | 15 (71) | 6 (29) | |
| Differentiation | | | | 0.776 |
| Well/moderate | 45 | 23 (51) | 22 (49) | |
| Poor | 17 | 8 (47) | 9 (53) | |
| Distant metastasis | | | | 0.002 |
| Yes | 32 | 10 (31) | 22 (69) | |
| No | 30 | 21 (70) | 9 (30) | |
| TNM stage | | | | 0.020 |
| I / II | 11 | 2 (18) | 9 (72) | |
| III / IV | 51 | 29 (56) | 22 (44) | |

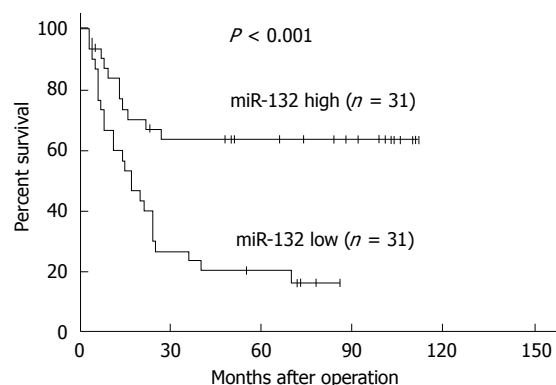
TNM stage ($P = 0.020$). However, age, gender, CEA level, CA199 level, tumor site and differentiation showed no association with miR-132 expression (Table 1). Kaplan-Meier analysis showed patients with low miR-132 expression had significantly worse disease-free survival than patients with high miR-132 expression ($P < 0.001$, Figure 2).

Ectopic expression/knockdown of miR-132 inhibited/promoted the invasion of CRC cells

Because miR-132 was significantly down-regulated in CRC tissues with distant metastases, we hypothesized that miR-132 could inhibit cell invasion. LoVo cells with low miR-132 expression were transfected with the miR-132 mimic to overexpress miR-132, whereas HCT116 cells with high miR-132 expression were transfected with the miR-132 inhibitor to knockdown endogenous miR-132. The efficiency of transfection was confirmed by real-time PCR (data not shown). As expected, ectopic expression of miR-132 reduced LoVo cell invasion by about 40% ($P < 0.05$; Figure 3A). By contrast, knockdown of miR-132 significantly increased the invasiveness of the HCT116 cells ($P < 0.05$; Figure 3B).

Overexpression of miR-132 reversed EMT in CRC cells

EMT is known to be important for tumor metastasis. Thus, we investigated the role of miR-132 in EMT in CRC cells. As shown in Figure 4A, ectopic expression of miR-132 induced a morphological change from the

**Figure 2** miR-132 predicts disease-free survival in colorectal cancer patients. The Kaplan-Meier curve of disease-free survival in patients with high miR-132 expression ($n = 31$) and low miR-132 expression ($n = 31$) ($P < 0.01$).

spindle-shaped mesenchymal form to a cobblestone-shaped epithelial-like form in LoVo cells. By contrast, knockdown of miR-132 in HCT116 cells led to more spindle-shaped mesenchymal characteristics (Figure 4B). Moreover, the expression of a set of EMT-related protein markers was also altered along with the morphological changes. Overexpression of miR-132 increased the protein levels of epithelial markers (E-cadherin and α -catenin) but decreased the expression of mesenchymal markers (vimentin and fibronectin) in LoVo cells (Figure 4C). By contrast, knockdown of miR-132 resulted in a decreased level of epithelial markers (E-cadherin and α -catenin) but increased levels of mesenchymal markers (vimentin and fibronectin) in HCT116 cells (Figure 4D).

ZEB2 was identified as a direct target of miR-132

To investigate the underlying mechanism of miR-132 in CRC, we first used TargetScan (www.targetscan.org) and miRanda (www.microRNA.org) to search for potential targets of miR-132. Because miR-132 could suppress CRC invasion, we focused on genes that could promote CRC metastasis. We found that the 3'-UTR of ZEB2 contains the complementary site for the seed region of miR-132. Additionally, ectopic expression of miR-132 could significantly reduce the ZEB2 mRNA and protein levels in LoVo and SW620 cells (Figure 5A and B). Furthermore, a luciferase activity assay demonstrated that miR-132 could significantly decrease the luciferase activity of the wild-type ZEB2 3'-UTR by 40%, a finding that was not observed for the mutant ZEB2 3'-UTR (Figure 5C).

DISCUSSION

Metastasis has been widely recognized as an adverse prognostic factor in CRC. miRNAs have been demonstrated as an important regulator of tumor progression and metastasis^[15]. In CRC, many miRNAs have been identified that are able to regulate known genes that are involved in the pathology of tumorigenesis and metastasis. For example, miR-21, miR-31 and miR-192 could induce CRC cell resistance to 5-fluorouracil (5-FU), a

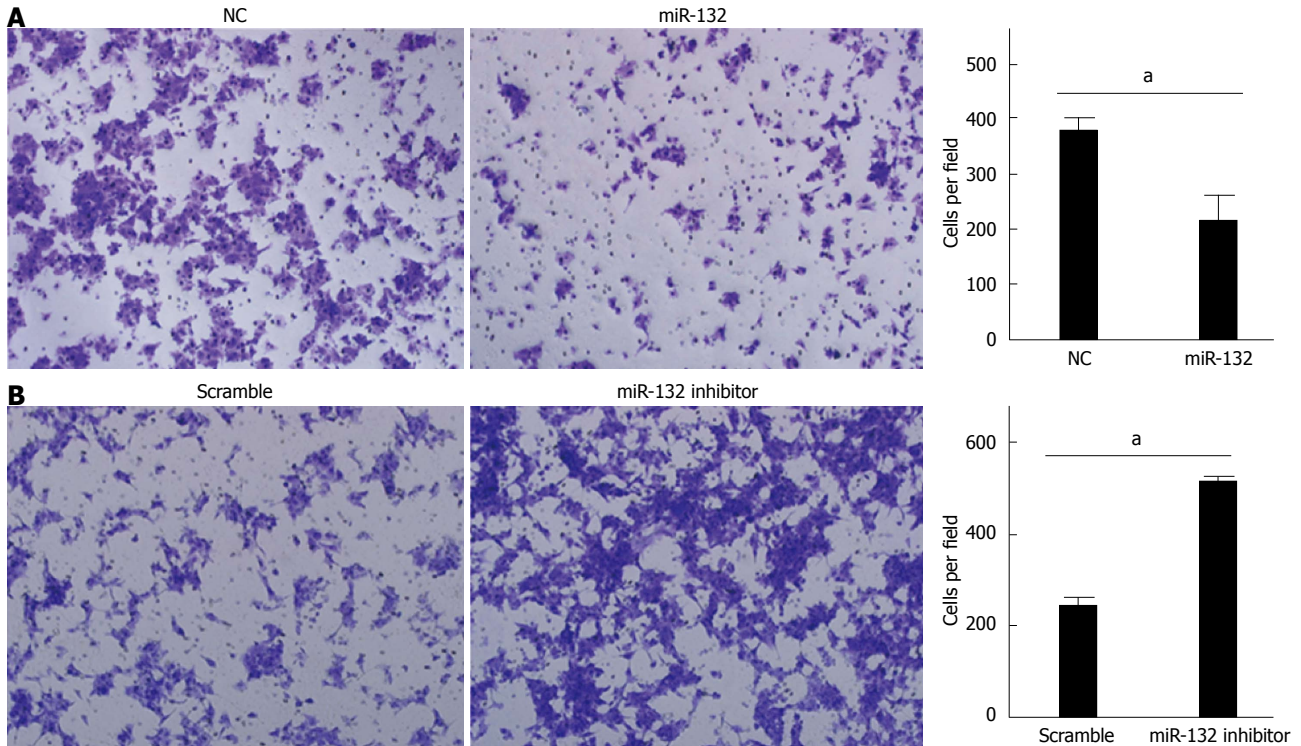


Figure 3 miR-132 inhibits colorectal cancer cell invasion. A: Ectopic expression of miR-132 inhibits cell invasion in LoVo cells ($^aP < 0.05$); B: Knockdown of miR-132 promotes cell invasion in HCT116 cells ($^aP < 0.05$).

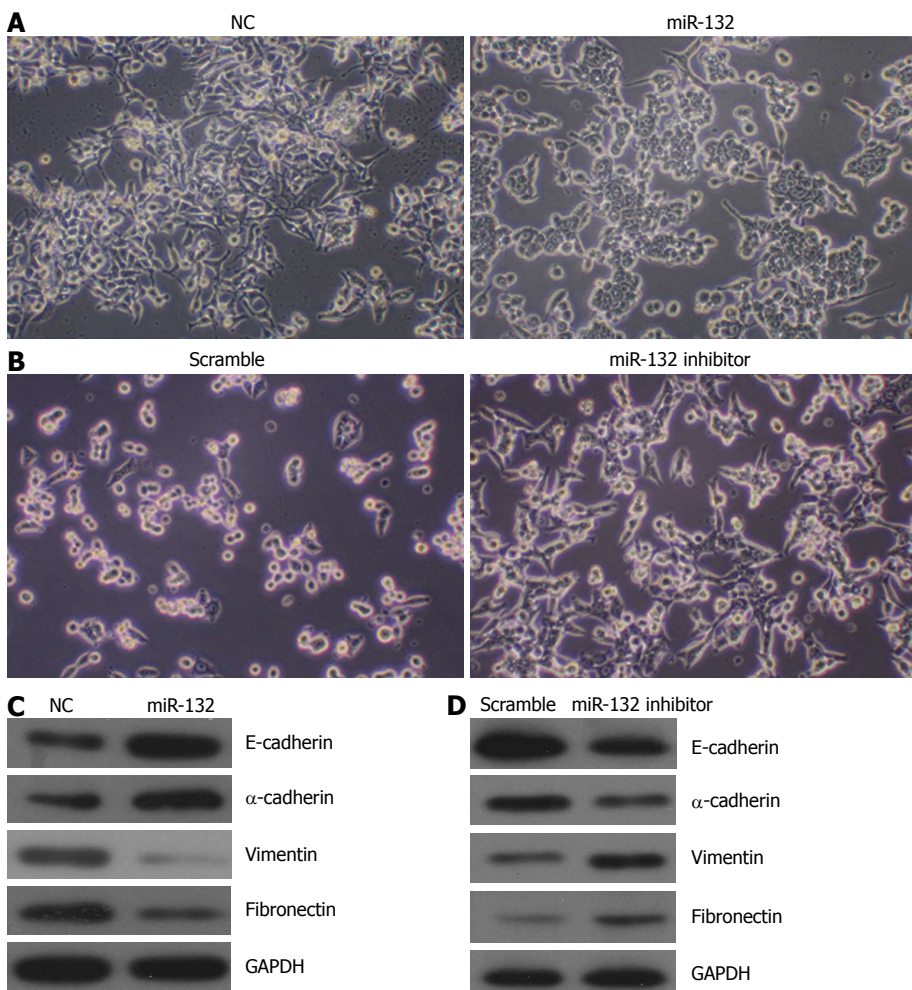


Figure 4 miR-132 regulates epithelial-mesenchymal transition in colorectal cancer cells. A: Ectopic expression of miR-132 leads to a cobblestone-shaped epithelial-like form in LoVo cells; B: Knockdown of miR-132 leads to spindle-shaped mesenchymal characteristics in HCT116 cells; C, D: Overexpression of miR-132 increased the protein levels of epithelial markers (E-cadherin and α -catenin) but decreased the expression of mesenchymal markers (vimentin and fibronectin) in LoVo cells, whereas knockdown of miR-132 resulted in decreased levels of epithelial markers (E-cadherin and α -catenin) and increased levels of mesenchymal markers (vimentin and fibronectin) in HCT116 cells.

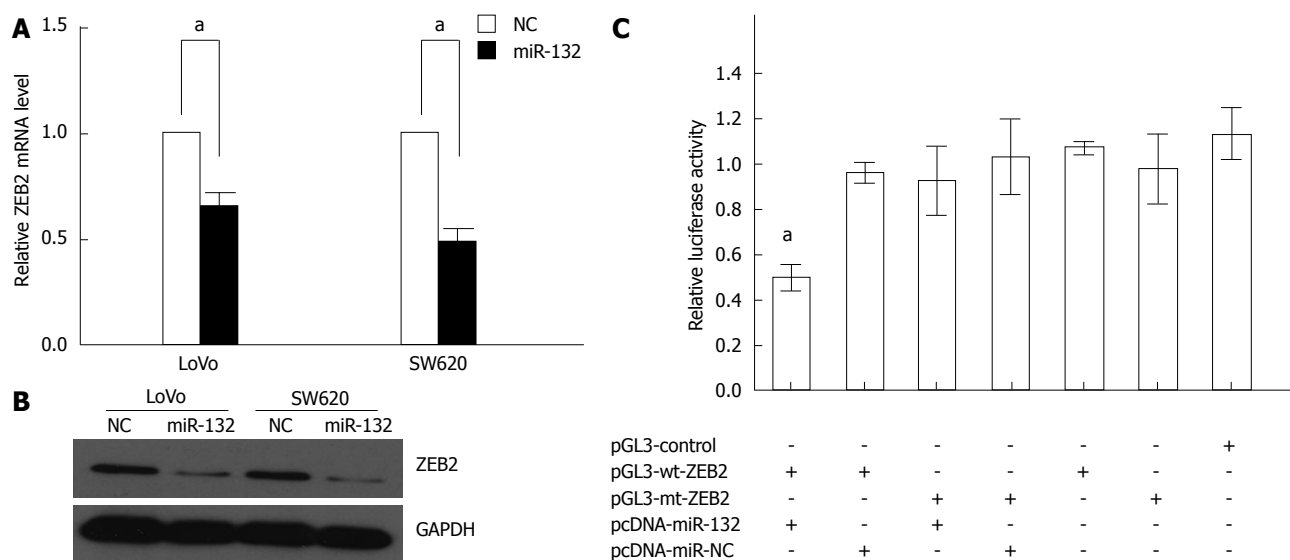


Figure 5 ZEB2 is a direct target of miR-132 in colorectal cancer cells. A: Ectopic expression of miR-132 reduces the ZEB2 mRNA levels in LoVo and SW620 cells ($P < 0.05$); B: Ectopic expression of miR-132 decreases the ZEB2 protein levels in LoVo and SW620 cells; C: Co-transfection of pcDNA-miR-132 and pGL3-wt-ZEB2 reduced the luciferase activity in 293T cells ($P < 0.05$).

finding that might shed light on chemotherapy for CRC patients^[16-18]; miR-28 inhibits CRC tumor growth and metastasis by CCND1 and NM23-H1^[19]. miR-132 has been found to be a tumor suppressor in a series of cancers, such as prostate cancer, hepatocellular carcinoma and ductal carcinoma *in situ* (DCIS) of the breast^[13,20,21]. Recently, a report indicated that miR-132 could regulate apoptosis in non-small cell lung cancer independent of acetylcholinesterase^[22]. In the present study, we showed that miR-132 was significantly down-regulated in CRC tissues with distant metastases.

Moreover, miR-132 was associated with tumor size, distant metastasis and TNM stage and could predict survival in CRC patients. These results indicated that miR-132 down-regulation might be a common occurrence in CRC, and miR-132 could be used as a biomarker to predict clinical outcome and metastasis in CRC patients.

EMT is a process in which adherent epithelial cells shed their epithelial traits and acquire mesenchymal properties, including fibroblastoid morphology and increased potential for motility, and, in the case of cancer cells, increased invasion, metastasis and resistance to chemotherapy^[23-25]. Although the signaling pathways of EMT are complicated, the hallmark of EMT in tumors is the down-regulation of E-cadherin, which is also considered to be a suppressor of invasion and metastasis. A series of transcription factors have been found to regulate EMT programs in cancer metastasis, including direct transcriptional repressors of E-cadherin-Snail (SNAI1), Slug (SNAI2), ZEB2 (SIP1), ZEB1, FOXC2 and Twist^[25]. In our study, we found that ectopic expression of miR-132 inhibited CRC cell invasion and EMT. By contrast, knockdown of miR-132 promoted cell motility and EMT progression. To the best of our knowledge, this is the first study to report the role of miR-132 in CRC metastasis.

MiRNAs are known to function by regulating target genes. Previous reports have found many target genes for miR-132 in cancer. For instance, Lagos *et al.*^[26] reported that miR-132 regulated antiviral innate immunity through suppression of the p300 transcriptional co-activator. Alvarez-Saavedra *et al.*^[27] showed that genes involved in chromatin remodeling (Mecp2, Ep300, Jarid1a) and translational control (Btg2, Paip2a) were direct targets of miR-132 in the mouse suprachiasmatic nucleus. In our study, we indicated that ZEB2, which is a transcriptional repressor of E-cadherin and plays an important role in EMT, was a direct target of miR-132 in CRC cells. These results showed that one miRNA might target different genes depending on the tumor types and cellular environment. However, further study is needed to investigate the underlying mechanisms of miR-132 in CRC.

In conclusion, we found that miR-132 was significantly down-regulated in CRC with distant metastasis. Moreover, miR-132 could predict disease-free survival and distant metastasis in CRC patients. Further investigation identified that the EMT regulator ZEB2 was a direct target of miR-132. Taken together, these data implicate that miR-132 might be used as a prognostic indicator and therapeutic target in CRC patients.

COMMENTS

Background

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in western countries. Although recent developments in therapeutic strategies have helped cure many patients with early stage disease, the prognosis of patients with advanced disease and metastasis remains poor. Further investigation into the underlying molecular mechanisms of CRC progression to identify biomarkers to distinguish CRC patients with or without a high risk of metastasis is of great importance.

Research frontiers

MiRNAs have been found to be involved in the regulation of multiple pathologi-

cal processes that contribute to tumorigenesis and metastasis, such as tumor cell proliferation, differentiation, apoptosis, and invasion. In CRC, studies have indicated that miRNAs play important roles in regulating tumor invasion and metastasis. Previously, Zhang *et al* reported that down-regulation of miR-132 by promoter methylation promotes pancreatic cancer development. Formosa *et al* found that miR-132 is silenced by promoter CpG island methylation, a process that contributes to prostate cancer progression and metastasis. However, the role of miR-132 in CRC progression and metastasis remains unclear and needs further exploration.

Innovations and breakthroughs

The authors found that miR-132 was significantly down-regulated in CRC tissues with metastasis compared with tissues without metastasis; the level of miR-132 was lower in CRC cell lines than in NCM460 cells (a normal colonic cell line). Ectopic expression of miR-132 markedly inhibited the invasiveness and epithelial-mesenchymal transition (EMT) in CRC cells. Further study indicated that ZEB2 (an EMT regulator) was a direct downstream target of miR-132. Collectively, these results demonstrated that miR-132 inhibited cell invasion and EMT in CRC cells by targeting ZEB2, thus providing a valuable target for cancer therapy.

Applications

The findings in this study indicated that miR-132 was significantly down-regulated in CRC with distant metastases. Moreover, miR-132 could predict disease-free survival and distant metastasis in CRC patients. Further investigation identified that the EMT regulator ZEB2 was a direct target of miR-132. Taken together, these data implicate that miR-132 might be used as a prognostic indicator and therapeutic target in CRC patients.

Terminology

MiRNA: a small non-coding RNA molecule (approximately 22 nucleotides in length) found in plants, animals, and some viruses that functions in transcriptional and post-transcriptional regulation of gene expression; epithelial-mesenchymal transition: a process by which epithelial cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties to become mesenchymal stem cells.

Peer review

The authors report on the biological and clinical significance of miR-132 in colorectal cancer, adding some information on a possible target of that microRNA. The results of the study are interesting and innovative. The sample size of the study is sufficiently large. The methods used are updated and well described, and the statistics are appropriate.

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IGFBPrP1 induces liver fibrosis by inducing hepatic stellate cell activation and hepatocyte apoptosis *via* Smad2/3 signaling

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Author contributions: Liu LX designed the study; Zhang Y analyzed the data and wrote the paper; Zhang QQ, Guo XH and Zhang HY performed the experiments.

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Abstract

AIM: To investigate the role and mechanism of insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) in the development of liver fibrosis.

METHODS: An *in vitro* model using hepatic stellate cell (HSC)-T6 cells and an *in vivo* model of rat liver overexpressing IGFBPrP1 were established using an IGFBPrP1-expressing recombinant adenovirus. The expression of IGFBPrP1 was examined by immunofluorescence, and the expression of collagen I and fibronectin was mea-

sured by real-time reverse transcription-polymerase chain reaction and Western blot analysis. The expression of Smad2/3 and p-Smad2/3 was examined by Western blot and immunohistochemistry. A shSmad3-expressing recombinant adenovirus (AdshSmad3) was designed and used to knockdown the *Smad3* gene in HSC-T6 cells and rat liver fibrosis transfected with IGFBPrP1. The expression of collagen I, fibronectin, and α -smooth muscle actin (α -SMA) was determined by Western blot analysis and immunohistochemistry. Hepatocyte apoptosis was assessed using TUNEL assay.

RESULTS: IGFBPrP1 overexpression induced collagen deposition and up-regulated the expression of α -SMA and p-Smad2/3, and AdshSmad3 inhibited IGFBPrP1-stimulated p-Smad2/3 activation and the expression of α -SMA, collagen I and fibronectin in HSC-T6 cells. Similarly, increased hepatocyte apoptosis ($38.56\% \pm 3.42\%$ vs $0.24\% \pm 0.03\%$, $P < 0.05$), α -SMA positive stained cells ($29.84\% \pm 1.36\%$ vs $5.83\% \pm 1.47\%$, $P < 0.05$), and increased numbers of Smad3 ($35.88\% \pm 2.15\%$ vs $10.24\% \pm 1.31\%$, $P < 0.05$) and p-Smad2/3 positive cells ($28.87\% \pm 2.73\%$ vs $8.23\% \pm 0.98\%$, $P < 0.05$) were detected in the livers of IGFBPrP1-overexpressing rats compared with the control group. Moreover, AdshSmad3 reduced IGFBPrP1-stimulated Smad3 expression and attenuated α -SMA expression ($29.84\% \pm 1.36\%$ vs $8.23\% \pm 1.28\%$, $P < 0.05$), hepatocyte apoptosis ($38.56\% \pm 3.42\%$ vs $6.75\% \pm 0.52\%$, $P < 0.05$), and both collagen I and fibronectin deposition in the livers of AdIGFBPrP1-treated rats.

CONCLUSION: IGFBPrP1 induces liver fibrosis by mediating the activation of hepatic stellate cells and hepatocyte apoptosis in a Smad3-dependent mechanism.

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Key words: Insulin-like growth factor binding protein-related protein 1; Liver fibrosis; Hepatic stellate cells; Hepatocyte apoptosis; Smad pathway

Core tip: This study investigated the role and mechanism of insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) in liver fibrosis using an adenovirus vector carrying IGFBPrP1 or a small interfering RNA targeting Smad3. We found that overexpression of IGFBPrP1 induced liver fibrosis by mediating hepatocyte apoptosis and hepatic stellate cells activation. We also identified the important role of the IGFBPrP1-Smad pathway in the regulation of IGFBPrP1 action in the development of liver fibrosis, and this pathway is a potential therapeutic target for liver fibrosis.

Zhang Y, Zhang QQ, Guo XH, Zhang HY, Liu LX. IGFBPrP1 induces liver fibrosis by inducing hepatic stellate cell activation and hepatocyte apoptosis via Smad2/3 signaling. *World J Gastroenterol* 2014; 20(21): 6523-6533 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6523.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6523>

INTRODUCTION

Hepatic fibrosis is characterized by excessive production and deposition of extracellular matrix (ECM) components including collagen and fibronectin, and often results in hepatic cirrhosis and carcinoma^[1]. During the process of liver fibrosis, hepatic stellate cells (HSCs) transform into myofibroblasts and are responsible for progressive ECM accumulation^[2-4]. Several cytokines and growth factors have been shown to regulate HSC activation, proliferation and ECM production^[5]. In addition, hepatocyte apoptosis may also contribute to HSC activation and the development of liver fibrosis. To date, there are no reports that a single molecule leads to hepatocyte apoptosis and HSC activation.

Insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) has been shown to be a tumor suppressor by regulating cell proliferation, senescence and apoptosis. We previously reported that IGFBPrP1 was up-regulated in the liver of patients with hepatic cirrhosis and in mice with thioacetamide (TAA)-induced hepatic cirrhosis^[6]. Most importantly, we demonstrated that recombinant IGFBPrP1 was capable of triggering HSC activation^[7,8]. These findings suggest that IGFBPrP1 plays an important role in liver fibrosis. However, the mechanism has not been described.

Recent studies found that IGFBPrP1 stimulated glioma growth or fibroblast activation by binding activin A, a transforming growth factor (TGF)- β superfamily member, to regulate TGF- β signaling. TGF- β combines with transmembrane type I and type II serine/threonine kinase receptors (T β RI and T β RII) to form a complex, which will activate the downstream Smad pathway^[9,10] or non-Smad pathway^[11-14], such as the p44/p42 mitogen-activated protein kinase pathway and phosphoinositide 3-kinase-Akt-mTOR regulating ECM production. The TGF- β -Smad signaling pathway is one of the most important

pathways responsible for regulating ECM production and liver fibrosis^[15]. Since IGFBPrP1 can regulate the TGF- β pathway, it is not surprising that IGFBPrP1 may contribute to liver fibrosis *via* the Smad signaling pathway.

The aim of this study was to identify the role and mechanism of IGFBPrP1 in liver fibrosis using an adenovirus vector carrying IGFBPrP1 (AdIGFBPrP1) or a small interfering RNA targeting Smad3 (AdshSmad3). We found that overexpression of IGFBPrP1 induced liver fibrosis by mediating hepatocyte apoptosis and HSC activation. We also identified the important role of the IGFBPrP1-Smad pathway in the regulation of IGFBPrP1 action in the development of liver fibrosis, and this pathway is a potential therapeutic target for liver fibrosis.

MATERIALS AND METHODS

Preparation of IGFBPrP1 adenoviral constructs

The recombinant replication deficient adenovirus 5 expressing EGFP was constructed as previously described. The full-length cDNA of rat IGFBPrP1 was obtained from the cDNA library using the PCR method, then subcloned into the shuttle vector AdMax for preparation of replication-deficient adenovirus type 5 expressing IGFBPrP1 (AdIGFBPrP1) or no cDNA (cAd) at the GenePharma Company (Shanghai, China). Both AdIGFBPrP1 and cAd contained an EGFP marker, which was used to determine the transduction efficiency and to optimize viral infection in HSCs.

Preparation of ShSmad3-expressing adenoviral constructs

Four shRNAs targeting rat Smad3 mRNA (nt553-572, 906-925, 958-977, and 1054-1073) and a scrambled shRNA used as a negative control (shNC) were designed using software found on the Ambio website and synthesized by the GenePharma Company (Shanghai, China). The most effective shSmad3 (1054-1073) or shNC was then used to construct the adenoviral vectors containing shSmad3 (AdshSmad3) or shNC (AdshNC). Both AdshSmad3 and AdshNC contained an RFP marker, which was used to determine the transduction efficiency.

Cell culture and transfection

The HSC-T6 cell line was a gift from Scott L. Friedman of the Mount Sinai School of Medicine (NY, United States) and was cultured in RPMI 1640 medium (Gibco, United States) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 g/mL streptomycin. After 24 h, HSC-T6 cells were transiently infected with AdshSmad3 or AdshNC in the presence of cAd or AdIGFBPrP1 at a multiplicity of infection (MOI) of 25, 50 and 100. The transfection efficiency was expressed as a percentage of the number of EGFP or RFP positive cells to the total cells.

Rats and adenovirus administration

Male wild-type Sprague-Dawley rats weighing 125-150 g

were obtained from Shanxi Medical University Laboratory Animal Center (Shanxi, China). All procedures were approved by the Shanxi Medical University Animal Care and Use Committee. All rats were injected with 2×10^9 PFU of AdshNC or AdshSmad3 in the presence of PBS or 2×10^9 PFU of cAd or AdIGFBPrP1 administered *via* the tail vein. Ten rats were included in each experimental group. Rats were sacrificed 14 and 28 d after adenovirus administration. Blood and liver tissues were harvested.

Real-time RT-PCR analysis

Total RNA was extracted from the cells or tissues with Trizol reagent (Invitrogen Life Technology, CA, United States). cDNA was obtained using the Reverse Transcription reagent kit (Fermentas Life Sciences, CA, United States). Quantitative real-time PCR was performed using the SYBR Green PCR kit (Fermentas Life Sciences, CA, United States). The primer sequences were as follows: (1) IGFBPrP1 forward primer (5'-GCGAG-CAAGGTCCTTCC AT-3') and reverse primer (5'-CG-GTCACCAGGCAGGAGTT-3'); (2) Collagen I forward primer (5'-AGCCAGCAGATCGAGAACAT-3') and reverse primer (5'-TCT TGTCCCTGGGGTTCTTG-3'); (3) Smad3 forward primer (5'-GGGAGACATTCCAC-GCTTCA-3') and reverse primer (5'-TAAGCTCCACG-GCTGCATT-3'); (4) α -smooth muscle actin (α -SMA) forward primer (5'-TTCGTTACTACTGCTGAGCGT-GAGA-3') and reverse primer (5'-AAAGATGGCTG-GAAGAGGGTC-3'); (5) fibronectin forward primer (5'-CCAGGCACTGACTACAAAGAT-3') and reverse primer (5'-CATGATACCAGCAAGGACTT -3'); and (6) β -actin forward primer (5'-CTGGCACCACCTTC-TACA-3') and reverse primer (5'-AGCACA GCCTG-GATAGCAAC-3'). β -actin was used as an internal control. Experiments were performed at least 3 times with similar results. The mRNA results were expressed as number of folds relative to the control group.

Western blot analysis

Western blot was performed as previously described with antibodies to (1) IGFBPrP1 (1:300, Santa Cruz Biotechnology, United States); (2) α -SMA (1:500, Abcam, United Kingdom); (3) collagen I (1:300, Santa Cruz Biotechnology, United States); (4) fibronectin (1:300, Santa Cruz Biotechnology, United States); (5) TGF- β 1 (1:200, Santa Cruz Biotechnology, United States); (6) Smad2/3 (1:300, Santa Cruz Biotechnology, United States); and (7) p-Smad2/3 (1:500, Abcam, United Kingdom). Immunoreactive blots were visualized using the Super ECL detection kit (Amersham Pharmacia Biotech, NJ, United States) according to the manufacturer's instructions. Specific signals were scanned using scanning densitometry and quantified with Quantity One Image software.

Histological examination and immunohistological staining

All paraffin-embedded liver tissues were cut into 4 μ m thick sections, and stained with hematoxylin and eosin

or Sirius Red stain. Immunohistochemical staining was performed to examine the expression of α -SMA, Smad3 and p-Smad3. The results were analyzed with Image-Pro Plus 7.0 software and expressed as a percentage of the area occupied by the signal.

TUNEL assay

Hepatocyte apoptosis in liver sections was measured by TUNEL assay, which was performed according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Boehringer Mannheim, Indianapolis, IN, United States). The data were expressed as a percentage of the area of TUNEL-positive cells in 10 random fields.

Hydroxyproline assay

Hydroxyproline content in whole liver specimens was quantified colorimetrically, which evaluated the total amount of collagen in the liver. The Hydroxyproline Assay Kit was purchased from Nanjing Jiancheng Bio-engineering (Nanjing, China). In brief, liver specimens were hydrolyzed, lyophilized and the absorbance of each sample at 550 nm was assayed for hydroxyproline content using a spectrophotometer.

Statistical analysis

All data are expressed as mean \pm SD. Statistical significance was determined using the Student's *t* test as appropriate.

RESULTS

IGFBPrP1 overexpression induces ECM production in HSC-T6 cells

Having shown that rIGFBPrP1 induces HSC activation, we sought to determine whether endogenously expressed IGFBPrP1 exerts similar effects. We first established IGFBPrP1 overexpression using the adenovirus in HSC-T6 cells, a rat HSC cell line. As shown in Figure 1A and B, adenoviral gene transfer of IGFBPrP1 (AdIGFBPrP1) increased IGFBPrP1 mRNA and protein expression in HSC-T6 cells in a time-dependent manner compared to cAd (0.254 ± 0.072 , 0.689 ± 0.023 , 0.856 ± 0.034 *vs* 0.038 ± 0.062 , $P < 0.05$). IGFBPrP1 overexpression similarly increased collagen I expression after transfection (Figure 1A, 0.614 ± 0.021 , 0.986 ± 0.027 , 1.294 ± 0.062 *vs* 0.596 ± 0.014 , $P < 0.05$).

IGFBPrP1-induced expression of α -SMA and type I collagen is regulated via Smad2/3 pathway

IGFBPrP1 has been shown to activate the TGF- β pathway in osteosarcoma cells. The TGF- β -Smad signaling pathway plays an important role in liver fibrosis. Smad2 and Smad3 are the main downstream mediators of TGF- β signaling in regulating ECM production. To determine the role of the Smad signaling pathway in mediating ECM up-regulation in response to elevated IGFBPrP1 levels, we measured Smad2/3 activation in HSC-T6 cells treated with AdIGFBPrP1. As shown in Figure 2A and B, Western blot analysis revealed that

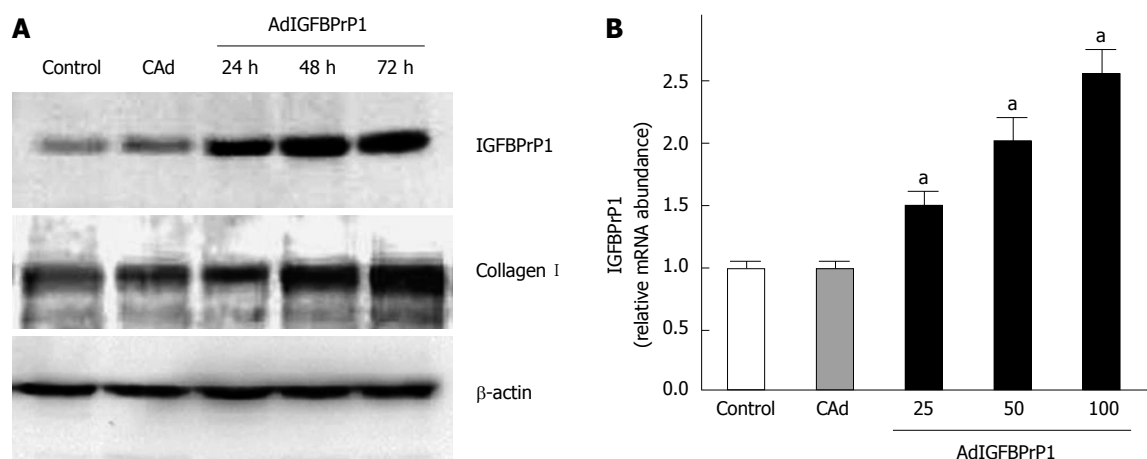


Figure 1 Insulin-like growth factor binding protein-related protein 1 overexpression induces extracellular matrix production in hepatic stellate cell-T6 cells. Hepatic stellate cell-T6 cells were infected with cDNA (cAd) or adenovirus vector carrying insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) (AdIGFBPrP1) for 24, 48 and 72 h at a multiplicity of infection of 100. Cell lysates were prepared from each culture. A: IGFBPrP1 and collagen I protein expression was detected by Western blot; B: IGFBPrP1 mRNA level was analyzed by real-time polymerase chain reaction. Data are expressed as mean \pm SD ($n = 4$ per group). ^a $P < 0.05$ vs the levels in the control group.

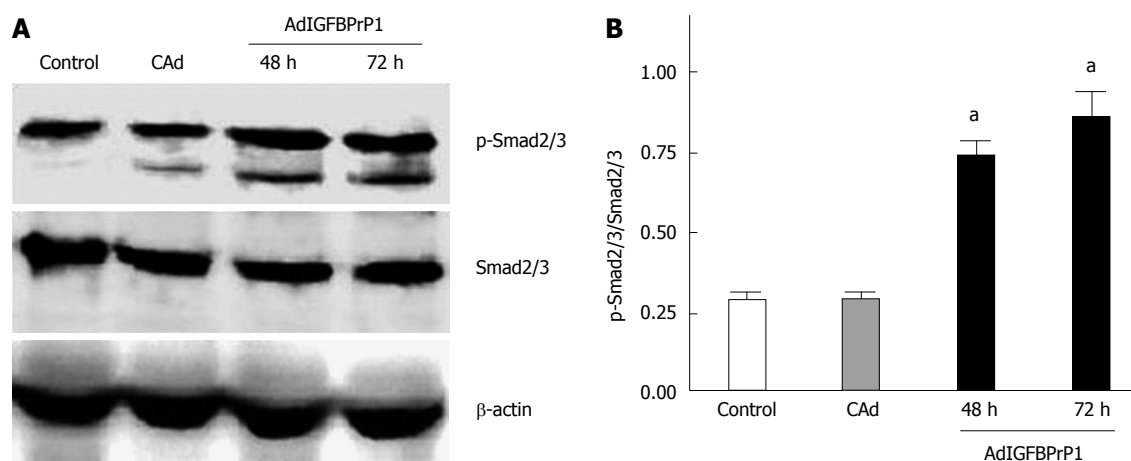


Figure 2 Insulin-like growth factor binding protein-related protein 1 overexpression stimulates Smad2/3 phosphorylation in hepatic stellate cell-T6 cells. A: Total and phosphorylated Smad2/3 protein expression in hepatic stellate cell-T6 (HSC-T6) cells was examined by Western blot 48 and 72 h after cDNA (cAd) or adenovirus vector carrying insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) (AdIGFBPrP1) transfection (multiplicity of infection = 100); B: P-Smad2/3 content normalized with total Smad3 content in cAd or AdIGFBPrP1-treated HSC-T6 cells. Data are expressed as mean \pm SD ($n = 4$ per group). ^a $P < 0.05$ vs the levels in the control group.

phosphorylation of Smad2/3 was 0.6-fold higher at 48 h and 1.5-fold higher at 72 h in HSC-T6 cells transduced with AdIGFBPrP1 than in the cAd group, suggesting that IGFBPrP1 overexpression activated the Smad2/3 pathway ($P < 0.05$).

To further investigate whether activation of the Smad2/3 pathway contributes to IGFBPrP1-stimulated ECM production, we designed an adenovirus harboring an shRNA targeting Smad3 (AdshSmad3) to knock down the expression of Smad3 gene. HSC-T6 cells were co-transfected with AdshSmad3 or negative control (AdshNC) and AdIGFBPrP1 at three different MOI (25, 50 and 100). As shown in Figure 3A, transfection efficiency in HSC-T6 cells was approximately $85.23\% \pm 10.2\%$ at an MOI of 100 ($P < 0.05$). As expected, real-time RT-PCR and Western blot results revealed that AdshSmad3 significantly reduced Smad3 protein by $68.45\% \pm 12.6\%$

($P < 0.05$) and mRNA by $76.45\% \pm 14.3\%$ ($P < 0.05$) at 72 h at an MOI of 100 (Figure 3B and C). Our results also showed that AdshSmad3 inhibited Smad3 protein by $68.6\% \pm 12.6\%$ and $58.6\% \pm 9.8\%$ at 72 and 96 h, respectively, in HSC-T6 cells compared with AdshNC at an MOI of 100 ($P < 0.05$, Figure 3D). In addition, AdshSmad3 inhibited Smad3 mRNA by $74.3\% \pm 11.2\%$ and $63.2\% \pm 10.4\%$ ($P < 0.05$, Figure 3E). Importantly, knockdown of Smad3 significantly abrogated IGFBPrP1-stimulated induction of α -SMA (0.196 ± 0.012 vs 0.723 ± 0.015 , $P < 0.05$), collagen I (0.482 ± 0.019 vs 1.268 ± 0.027 , $P < 0.05$) and fibronectin expression (0.334 ± 0.024 vs 1.146 ± 0.015 , $P < 0.05$) (Figure 3F). Similarly, up-regulation of α -SMA, collagen I and fibronectin mRNA in response to IGFBPrP1 overexpression was suppressed by AdshSmad3 ($P < 0.05$, Figure 3G). Taken together, these data indicated that IGFBPrP1-

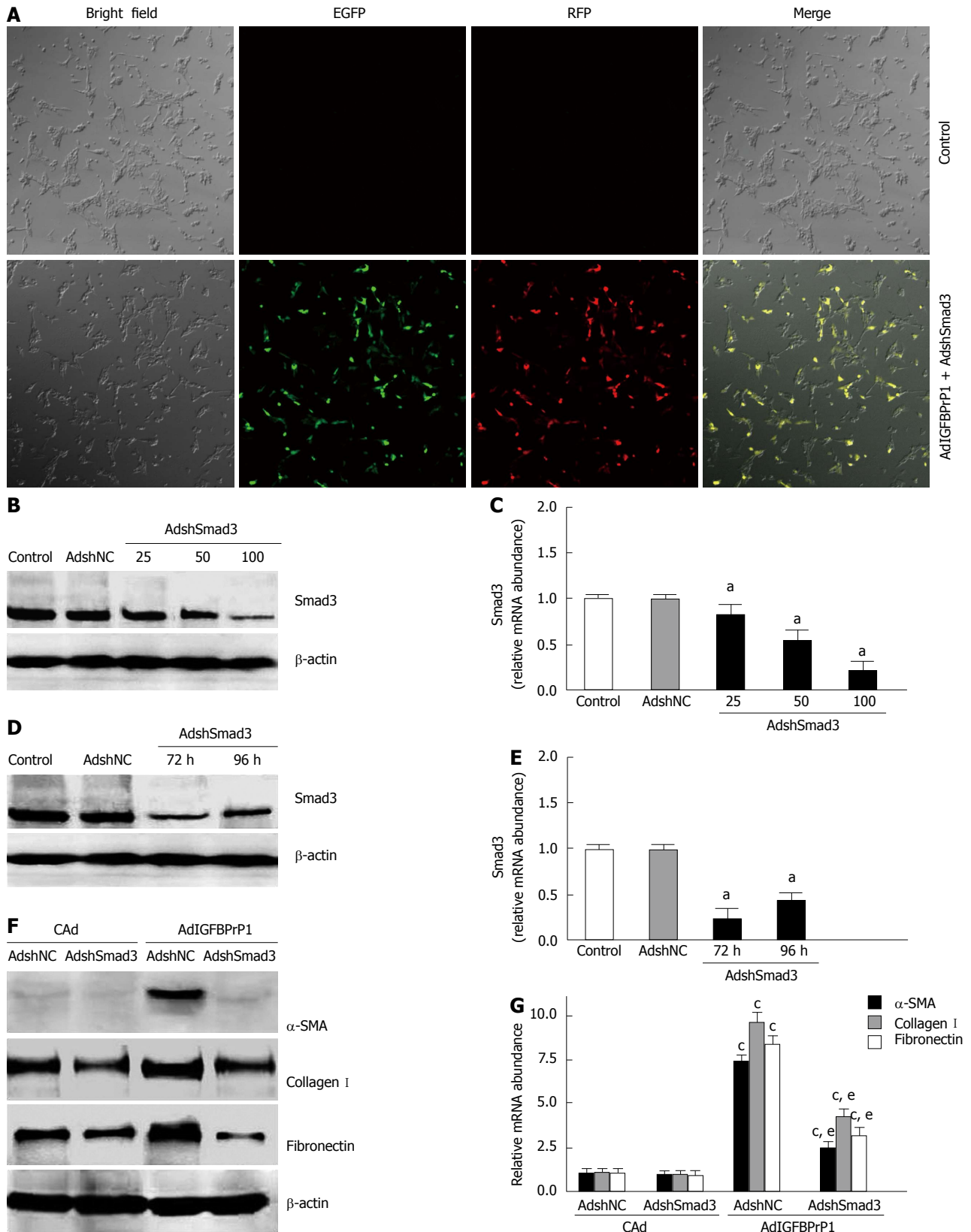


Figure 3 Insulin-like growth factor binding protein-related protein 1-induced extracellular matrix expression is mediated through the Smad pathway in hepatic stellate cell-T6 cells. Hepatic stellate cell-T6 (HSC-T6) cells were co-infected with adenovirus vectors containing shSmad3 (AdshSmad3) or shNC (AdshNC) and adenovirus vector carrying insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) (AdIGFBPrP1). A: Expression of enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) in HSC-T6 cells was visualized by confocal microscopy after treatment with AdshSmad3 (magnification $\times 200$); B, C: Smad3 protein (B) and mRNA (C) expression in HSC-T6 cells was detected by Western blot and real-time polymerase chain reaction (RT-PCR) after treatment with different multiplicity of infection (MOI) of AdshSmad3, respectively; D, E: Smad3 protein (D) and mRNA (E) expression was detected by Western blot and real-time RT-PCR 72 h or 96 h after AdshSmad3 treatment (MOI = 100), respectively; F, G: Protein (F) and mRNA (G) expression of α -smooth muscle actin (α -SMA) and extracellular matrix in HSC-T6 cells was analyzed by Western blot and real-time RT-PCR 72 h after AdshSmad3 treatment (MOI = 100), respectively. Data are expressed as mean \pm SD ($n = 4$ per group). ^a $P < 0.05$ vs the levels in the control group; ^c $P < 0.05$ vs the levels in cAd + AdshNC; ^e $P < 0.05$ vs the levels in AdIGFBPrP1 + AdshNC.

induced ECM expression in HSC-T6 cells was Smad dependent.

Smad2/3 expression in IGFBPrP1-induced rat liver fibrosis

Based on these *in vitro* data, we hypothesized that IGFBPrP1 leads to the development of liver fibrosis *via* the Smad pathway. Rats were injected with 2×10^9 PFU of cAd or AdIGFBPrP1 *via* the tail vein. Expression of IGFBPrP1 in AdIGFBPrP1 or cAd-treated rat livers was examined by immunohistochemistry. As shown in Figure 4A and B, IGFBPrP1 was expressed mainly in hepatocytes and sinusoidal cells 2 d after adenovirus injection. Hepatocyte steatosis, cellular infiltration and excessive collagen deposition were observed at 28 d in IGFBPrP1-treated rats compared with cAd-treated rats (Figure 4C and D). Collagen content, quantified by Sirius Red staining, was markedly increased at 28 d in the liver of AdIGFBPrP1-injected rats compared with cAd-injected rats (Figure 4E and F).

We then examined Smad2/3 expression. Immunohistochemistry revealed faint expression of Smad3 and phosphorylated Smad2/3 (p-Smad2/3) in the liver of normal rats. Moreover, Smad3 and p-Smad2/3 were strongly expressed in the IGFBPrP1-induced fibrotic liver (Figure 4G-J). The positive areas in IGFBPrP1-induced fibrotic liver were larger than those in the cAd group (Smad3, $35.88\% \pm 2.15\%$ *vs* $10.24\% \pm 1.31\%$, $P < 0.05$; p-Smad2/3, $28.87\% \pm 2.73\%$ *vs* $8.23\% \pm 0.98\%$, $P < 0.05$). Consistent with the immunohistochemistry staining results, Western blot results also showed that the expression of Smad2/3 and p-Smad2/3 protein was increased 14 and 28 d after IGFBPrP1 administration (Smad3, 1.342 ± 0.075 , 1.586 ± 0.116 *vs* 0.657 ± 0.032 , $P < 0.05$; p-Smad2/3, 0.682 ± 0.043 , 0.856 ± 0.064 *vs* 0.189 ± 0.007 , $P < 0.05$) (Figure 4K) and the ratio of p-Smad2/3 to total Smad2/3 was significantly up-regulated in the AdIGFBPrP1 group compared with the normal and cAd groups ($P < 0.05$) (Figure 4L).

AdshSmad3 attenuates fibrosis in IGFBPrP1-treated rat liver

To further elucidate the effect of the Smad2/3 pathway on IGFBPrP1-induced liver fibrosis, we injected SD rats with 2×10^9 PFU of AdshNC or AdshSmad3 in the presence of 2×10^9 PFU of AdIGFBPrP1 into the tail vein. The levels of serum ALT and AST increased in AdIGFBPrP1-treated rats compared with the control group and decreased in AdshSmad3-treated rats compared with AdIGFBPrP1-treated rats. As shown in Figure 5A-J, AdshSmad3 ameliorated liver fibrosis induced by IGFBPrP1 as demonstrated by both hematoxylin and eosin and Sirius red staining. Hydroxyproline content in the AdshSmad3 group was down-regulated by $48.5\% \pm 12.6\%$ as compared with the AdshNC group and IGFBPrP1 group ($P < 0.05$, Figure 5K). Moreover, the expression of Smad3, collagen I and fibronectin proteins was significantly up-regulated at 14 and 28 d in fibrotic livers

induced by IGFBPrP1 (Smad3, 1.128 ± 0.164 , 1.345 ± 0.156 *vs* 0.626 ± 0.021 , $P < 0.05$; collagen I, 0.832 ± 0.031 , 1.324 ± 0.076 *vs* 0.534 ± 0.018 , $P < 0.05$; fibronectin, 0.647 ± 0.037 , 1.225 ± 0.039 *vs* 0.324 ± 0.022 , $P < 0.05$) and was markedly down-regulated 14 and 28 d after AdshSmad3 treatment as demonstrated by Western blot analysis (Smad3, 0.594 ± 0.147 *vs* 1.128 ± 0.164 , 0.742 ± 0.189 *vs* 1.345 ± 0.156 , $P < 0.05$; collagen I, 0.626 ± 0.025 *vs* 0.832 ± 0.031 , 0.728 ± 0.014 *vs* 1.324 ± 0.076 , $P < 0.05$; fibronectin, 0.428 ± 0.018 *vs* 0.647 ± 0.037 , 0.532 ± 0.024 *vs* 1.225 ± 0.039 , $P < 0.05$, Figure 6A).

AdshSmad3 inhibits hepatocyte apoptosis and HSC activation in IGFBPrP1-treated rats

The TGF- β /Smad pathway is not only associated with HSC activation, but also participates in hepatocyte apoptosis. In light of the mechanism of the Smad pathway in the development of liver fibrosis induced by IGFBPrP1, hepatocyte apoptosis and HSC activation in rat livers were evaluated 28 d after co-infection with AdIGFBPrP1 and AdshSmad3. As shown in Figure 6B-D, no TUNEL-positive cells were identified in normal liver, whereas scattered TUNEL-positive cells were observed in AdIGFBPrP1-treated rat liver ($38.56\% \pm 3.42\%$ *vs* $0.24\% \pm 0.03\%$, $P < 0.05$). Interestingly, AdshSmad3 reduced AdIGFBPrP1-induced TUNEL-positive cells ($6.75\% \pm 0.52\%$ *vs* $38.56\% \pm 3.42\%$, $P < 0.05$). We then examined α -SMA expression, a marker of HSC activation, by immunohistochemistry. α -SMA-positive cells were more abundant in the liver of IGFBPrP1-treated rats compared with normal rats ($29.84\% \pm 1.36\%$ *vs* $5.83\% \pm 1.47\%$, $P < 0.05$). More importantly, AdshSmad3 reduced AdIGFBPrP1-induced α -SMA-positive cells ($8.23\% \pm 1.28\%$ *vs* $29.84\% \pm 1.36\%$, $P < 0.05$, Figure 6E-G).

DISCUSSION

Liver fibrosis is thought to be a reversible disease. HSCs have been recognized to play an important role in the development of liver fibrosis. Thus, many effective therapeutic approaches have intensified interest in regulating HSC activation and proliferation^[16-19]. Recently, the IGFBPrP1 gene was found to be significantly increased during HSC activation^[6]. Therefore, we examined the role of IGFBPrP1 in liver fibrosis. We found that anti-IGFBPrP1 antibody can attenuate TAA-induced hepatic fibrosis^[7]. Moreover, siRNA targeting IGFBPrP1 reduced HSC activation and ECM production stimulated by TGF- β . Most importantly, we previously reported that recombinant IGFBPrP1 induces HSC activation *in vitro*^[8]. However, the molecular mechanism underlying this process and the *in vivo* effect of IGFBPrP1 have not been elucidated. In this study, we demonstrated that overexpression of IGFBPrP1 induced liver fibrosis by stimulating hepatocyte apoptosis and HSC activation, and the underlying mechanism involved the Smad2/3 pathway.

IGFBPrP1, also known as Mac25 or IGFBP7, is a member of the IGFBP superfamily. It appears to be in-

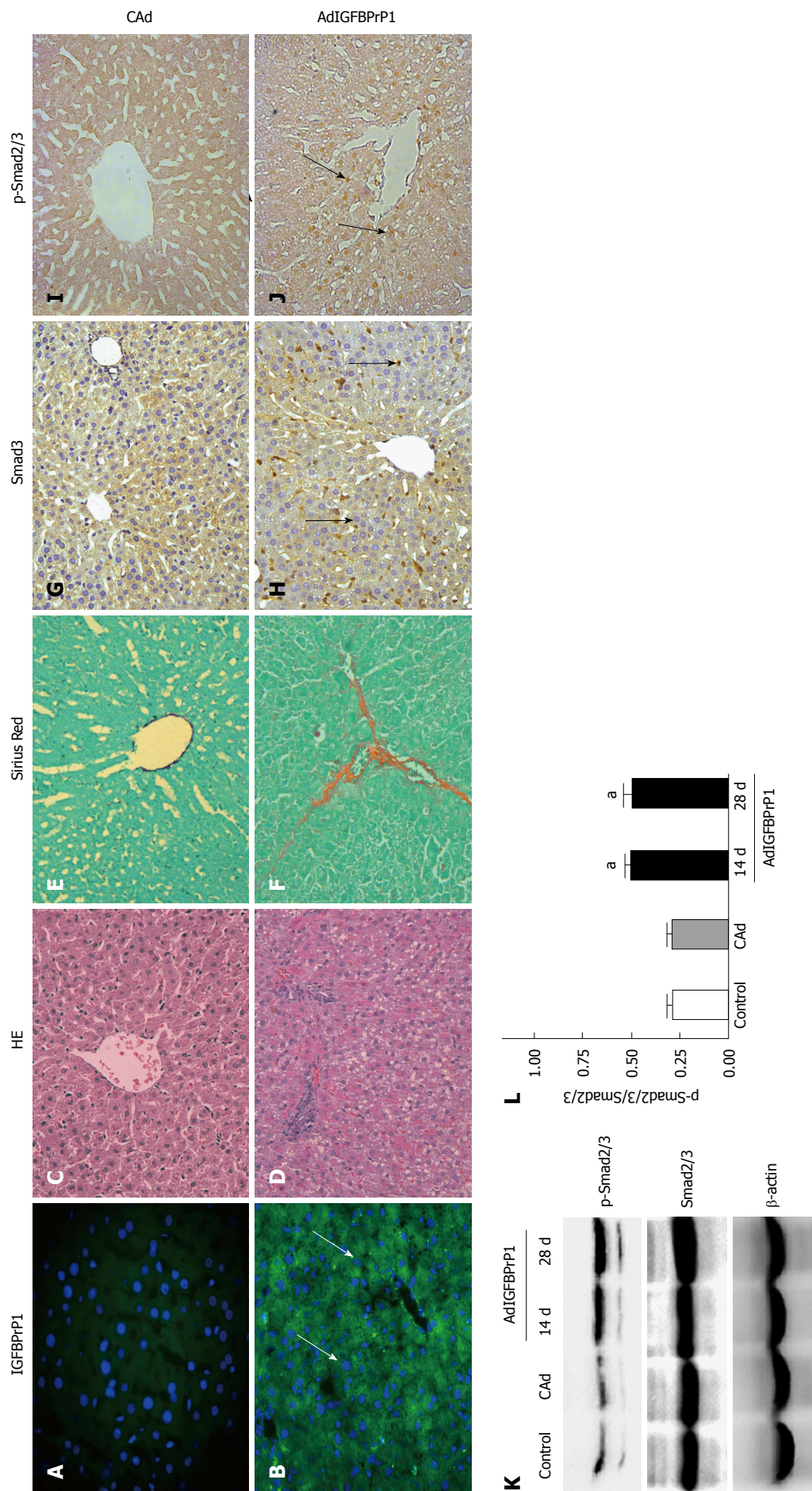


Figure 4 Smad2/3 expression in insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) (AdIGFBPrP1) and AdIGFBPrP1-treated (A) and AdIGFBPrP1-treated (B) rats was evaluated by immunofluorescence; Histopathology (C, D) and collagen content (E, F) in the livers of cAd-treated and AdIGFBPrP1-treated rats were demonstrated by HE and Sirius Red staining; expression of total Smad2/3 (G, H) and phosphorylated Smad2/3 (I, J) in hepatocytes and HSCs (arrows) in the livers of cAd-treated and AdIGFBPrP1-treated rats was examined by immunohistochemistry (magnification $\times 400$); K: Protein expression of total and phosphorylated Smad2/3 in the livers was examined by Western blot analysis; L: P-Smad2/3 content normalized with Smad2/3 content in the livers of the control, cAd or IGFBPrP1 group. Data are expressed as mean \pm SD ($n = 10$ per group). $^*P < 0.05$ vs the levels in the control group.

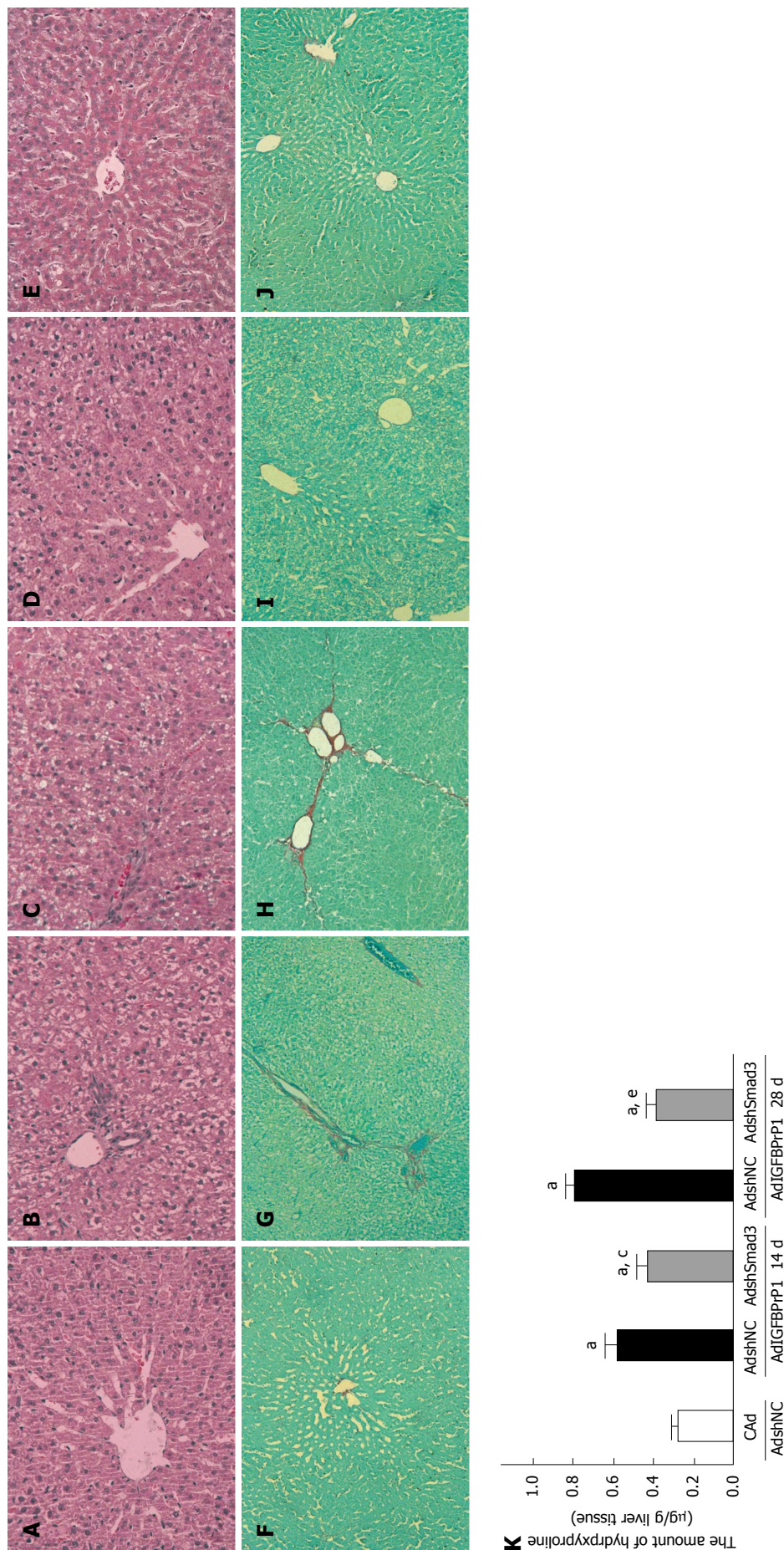


Figure 5 Adenoviral vectors containing shSmad3 ameliorated insulin-like growth factor binding protein-1-induced liver fibrosis. Rats were sacrificed 14 and 28 d after injection with adenovirus vectors containing shSmad3 (AdshSmad3) or shNC (AdshNC) in the presence of cDNA (cAd) or adenovirus vector carrying insulin-like growth factor binding protein-1 (IGFBPrP1) (AdIGFBPrP1) (AdIGFBPrP1). Histopathology (A-E) and collagen content (F-J) in the livers were demonstrated by HE and Sirius Red staining (A, F: cAd + AdshNC; B, G: AdIGFBPrP1 + AdshNC; C, H: AdIGFBPrP1 + AdshSmad3 14 d; D, I: AdIGFBPrP1 + AdshSmad3 28 d; E, J: AdIGFBPrP1 + AdshNC 28 d; magnification $\times 200$); K: The amount of collagen in rat livers was determined using the hydroxyproline assay. Data are expressed as mean \pm SD ($n = 10$ per group). $^{\#}P < 0.05$ vs the levels in the cAd + AdshNC group; $^{\circ}P < 0.05$ the levels in the AdIGFBPrP1 + AdshSmad3 14 d group vs those in the AdIGFBPrP1 + AdshNC 14 d group; $^{\circ}P < 0.05$ the levels in the AdIGFBPrP1 + AdshNC 28 d group.

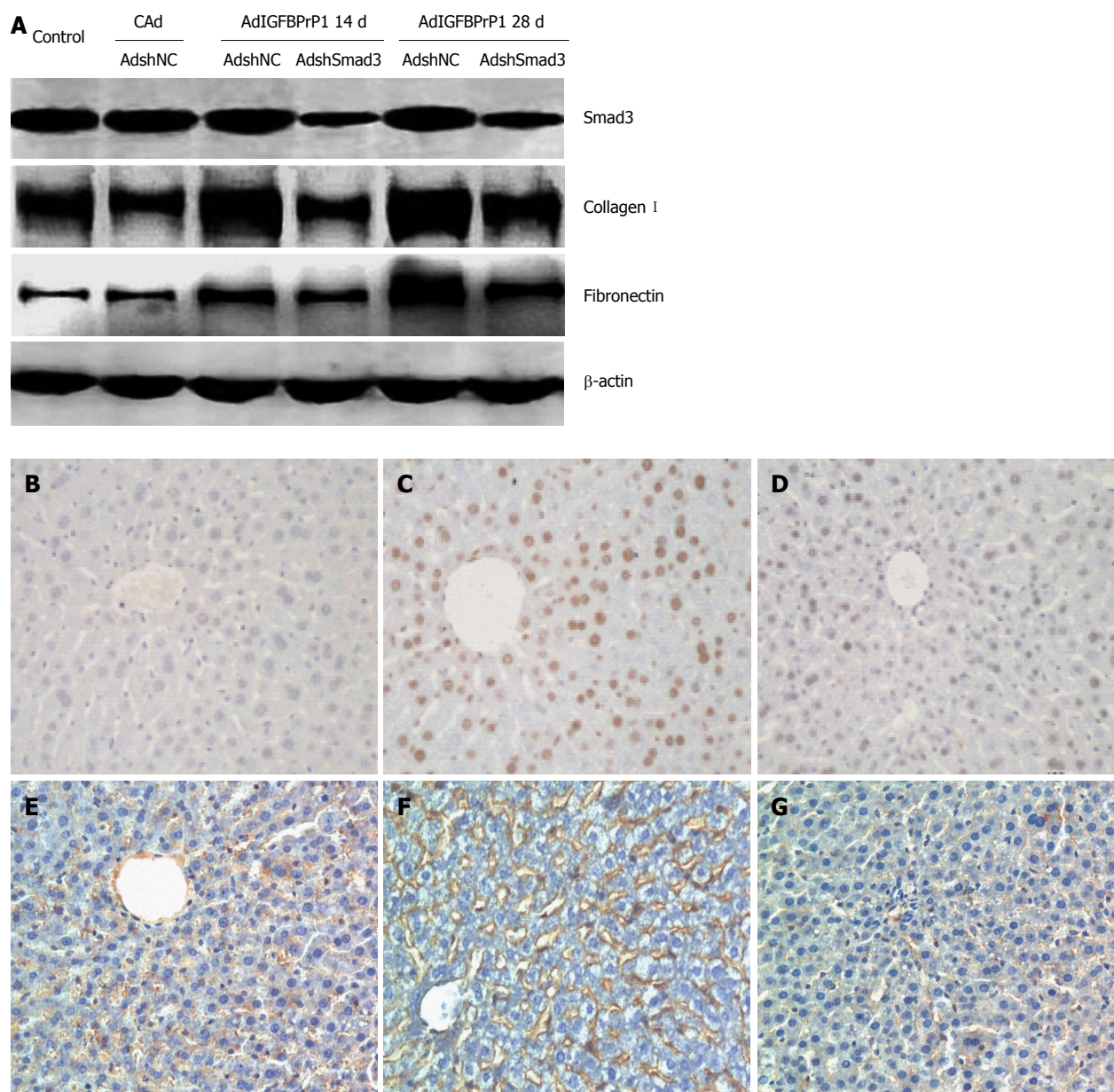


Figure 6 Adenoviral vector containing shSmad3 inhibits fibrogenic expression in insulin-like growth factor binding protein-related protein 1-treated rats. **A:** Expression of Smad3, collagen I and fibronectin in the livers were analyzed 28 d after treatment by Western blot; **B-D:** Hepatocyte apoptosis was examined 28 d after treatment by TUNEL assay; **B:** cDNA (cAd) + adenovirus vector containing shNC (AdshNC); **C:** Adenovirus vector carrying insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) (AdIGFBPrP1) + AdshNC; **D:** AdIGFBPrP1 + adenovirus vector containing shSmad3 (AdshSmad3); **E-G:** α-smooth muscle actin (α-SMA) expression was examined 28 d after treatment by immunohistochemistry; **E:** cAd + AdshNC; **F:** AdIGFBPrP1 + AdshNC; **G:** AdIGFBPrP1 + AdshSmad3. Data are expressed as mean ± SD ($n = 10$ per group).

involved in diverse biological functions, such as regulation of cell growth, stimulation of prostacyclin production, and binding of type IV collagen, IGFs and insulin^[20-23]. Interestingly, IGFBPrP1 demonstrated positive and negative roles in tumor progression by mediating fibroblast activation or epithelial cell senescence^[24,25]. However, the relationship between IGFBPrP1 and liver fibrosis has not been investigated. We established an *in vitro* and an *in vivo* model in which we transiently overexpressed IGFBPrP1 in HSC-T6 cells and in rat liver by adenoviral-mediated *IGFBPrP1* gene transfer, respectively, as the replication-deficient recombinant adenovirus has very high efficient delivery into target cells and was reported to be suitable

for liver fibrosis^[26,27]. With this approach, we showed that overexpression of IGFBPrP1 caused activation of HSCs and ECM production in HSC-T6 cells, which resulted in liver fibrosis, and AdIGFBPrP1-treated rats developed liver steatosis and fibrosis.

HSCs are known to have an important role in liver fibrosis, however, hepatocyte apoptosis is now emerging as a critical event in the progression of liver fibrosis^[28,29]. Engulfment of apoptotic bodies by HSCs stimulates the activation of HSCs and ECM production. Hepatocyte apoptosis may also be responsible for the generation of inflammatory mediators leading to liver inflammation and fibrosis. We observed increased hepatocyte apoptosis and

HSC activation in IGFBPrP1-treated fibrotic liver.

The TGF β -Smad signaling pathway is the main pathway regulating ECM production and liver fibrosis. Recent studies found that IGFBPrP1 stimulated glioma growth or fibroblast activation by binding activin A to regulate the TGF- β pathway. Activin A belongs to the TGF- β superfamily and activates the Smad pathway in systemic sclerosis^[30,31]. Therefore, it is not surprising that IGFBPrP1 may induce liver fibrosis *via* the activin A-Smads pathway. Kitamura *et al*^[15] reported that Smad expression increased in the nucleus of HSCs in liver fibrosis both *in vivo* and *in vitro*. We found that overexpression of IGFBPrP1 up-regulated p-Smad2/3 expression in cultured HSC-T6 cells and IGFBPrP1-induced liver fibrosis. Our results were consistent with stimulation by TGF- β ^[32], suggesting that IGFBPrP1 activated the Smad2/3 pathway in activated HSCs both *in vivo* and *in vitro*. Furthermore, our results also showed that strong p-Smad2/3 expression was observed in the nucleus of hepatocytes in IGFBPrP1-induced liver fibrosis. Taken together, our data suggest that the Smad pathway participated in IGFBPrP1-induced liver fibrosis.

Latella *et al*^[33] previously demonstrated that targeted disruption of Smad3 inhibits the development of TAA-induced hepatic fibrosis in mice. In order to further evaluate the effect of the Smad pathway on IGFBPrP1-induced liver fibrosis, we successfully used AdshSmad3 to knockdown the *Smad3* gene in AdIGFBPrP1-treated HSC-T6 cells and rat liver as demonstrated by real-time RT-PCR and Western blot analysis. Furthermore, AdshSmad3 attenuated AdIGFBPrP1-induced liver fibrosis and reduced the expression of α -SMA, collagen I and fibronectin both *in vivo* and *in vitro*. More importantly, AdshSmad3 attenuated AdIGFBPrP1-induced hepatocyte apoptosis. It was reported that the Smad2/3 pathway not only stimulated HSC activation, but also induced hepatocyte apoptosis^[34,35]. Taken together, these data demonstrated that IGFBPrP1 may contribute to liver fibrosis by inducing HSC activation and hepatocyte apoptosis in a Smad-dependent manner.

In summary, we have shown that adenovirus-mediated IGFBPrP1 overexpression induced HSC activation and ECM production *in vitro via* the Smad pathway. More importantly, overexpression of IGFBPrP1 induced hepatocyte apoptosis and HSC activation *in vivo* in a Smad-dependent manner. These data suggest a novel potential therapeutic target for liver fibrosis.

COMMENTS

Background

Hepatic stellate cells (HSCs) play an important role in the development of liver fibrosis. Insulin-like growth factor binding protein-related protein 1 (IGFBPrP1) is a novel protein, which is involved in the activation of HSCs and regulates cell proliferation, senescence and apoptosis.

Research frontiers

Significant IGFBPrP1 expression has been detected during fibrogenesis and our previous study demonstrated that rIGFBPrP1 activated HSCs and increased extracellular matrix production *in vitro*. In this study, the authors focused on the

in vivo effects and the mechanism of endogenous IGFBPrP1 expression by administration of a recombinant adenovirus expressing IGFBPrP1 or shSmad3.

Innovations and breakthroughs

Recent studies have highlighted the importance of IGFBP5 and IGFBPrP1 in the activation of HSCs. The authors provide the first evidence that overexpression of IGFBPrP1 induces liver fibrosis by inducing HSC activation and hepatocyte apoptosis in a Smad3-dependent manner.

Applications

By understanding the effect and mechanism of IGFBPrP1 in the development of liver fibrosis, IGFBPrP1 could be a novel potential therapeutic target for liver fibrosis.

Terminology

HSCs are responsible for extracellular matrix deposition during liver fibrosis. The activation of HSCs is regulated by many cytokines. Hepatocyte apoptosis may also contribute to HSC activation. IGFBPrP1 can induce hepatocyte apoptosis and HSC activation.

Peer review

The authors demonstrated that overexpression of IGFBPrP1 induced liver fibrosis by mediating hepatocyte apoptosis and HSC activation and identified the IGFBPrP1-mediated pathway involved in liver fibrosis development. The interesting conclusion of the study was that this pathway could be a potential therapeutic target for liver fibrosis.

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Changes in iron transporter divalent metal transporter 1 in proximal jejunum after gastric bypass

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transporter 1 (DMT1) shows in patients after Roux-en-Y gastric bypass (RYGB) surgery.

METHODS: Prospective and analytical study of DMT1 level at the brush border of proximal jejunum in patients having undergone RYGB surgery. The mucosa of proximal jejunum forming the gastrojejunal anastomosis was biopsied during surgery and after 6 mo later with an endoscopic biopsy. All the patients received precise instructions regarding feeding and nutritional supplementation. Both samples were processed at the same time by immunohistochemistry and western blot. Samples were analysed by a pathologist. For statistical analysis, the χ^2 and Wilcoxon tests were used.

RESULTS: Sixteen patients were recruited, 13 of whom completed the study. Twelve were women. Average age and body mass index (BMI) were 44.1 and 40.4, respectively. Both body weight and BMI decreased significantly during the study period, with an average percent excess weight loss (%EWL) of $60\% \pm 13.3\%$ and an average percent excess BMI loss (%EBMIL) of $79.6\% \pm 21.6\%$. Only two patients presented with mild anaemia 6 mo after surgery, but their ferritin levels stayed within normal ranges. Staining for DMT1 showed a significant increase in the cytoplasm of enterocytes located at the tips of the villi ($\chi^2 = 6.03$; $P = 0.049$). Nevertheless, the total quantity of DMT1 decreased significantly ($Z = 2.04$; $P = 0.04$). Associated with these results, we observed a significant increase in goblet cells in the villi 6 mo postoperatively ($Z = -2.47$; $P = 0.013$).

CONCLUSION: Six months after RYGB surgery, patients exhibit an increase in DMT1 expression in the enterocytes of the tips of the villi at the proximal jejunum.

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Abstract

AIM: To describe the variation that divalent metal

Key words: Roux-en-Y gastric bypass; Bariatric surgery; Divalent metal transporter 1; Anaemia; Iron

Core tip: Anaemia after Roux-en-Y gastric bypass surgery is one of the most common nutritional deficiencies. Different nutritional supplementation strategies have been developed to prevent this complication, but a subset of patients still develop it. This study brings readers the first report on the molecular changes that occur in the physiology of iron absorption in these patients.

Marambio A, Watkins G, Castro F, Riffo A, Zúñiga R, Jans J, Villanueva ME, Díaz G. Changes in iron transporter divalent metal transporter 1 in proximal jejunum after gastric bypass. *World J Gastroenterol* 2014; 20(21): 6534-6540 Available from: <http://www.wjgnet.com/1007-9327/full/v20/i21/6534.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6534>

INTRODUCTION

Nutritional deficiencies are common complications of bariatric surgery, especially in association with Roux-en-Y gastric bypass (RYGB). Of particular importance are iron deficiency and anaemia. It is accepted that such patients must receive vitamin supplements perpetually, and iron supplements depending on the presence or absence of factors that influence the development of anaemia, such as gender or age. In spite of this, a fraction of the patients will still develop iron deficiency and in some cases will require the administration of intravenous iron supplementation^[1].

The mechanisms that result in iron deficiency are mainly related to a lack of gastric acidity and the exclusion of the duodenum and part of the jejunum, the main sites of iron absorption^[2]. The iron transporter divalent metal transporter 1 (DMT1) has been detected in these regions. DMT1 is a transmembrane protein found on the apical membrane of the enterocyte that, by the proton-motive force, transports ferrous iron (Fe^{2+}) into the cell^[3].

DMT1 is mainly found in the duodenum, and its expression decreases along the digestive tract. In conditions of iron deficiency, the duodenum is capable of adapting by overexpressing DMT1, and in overload conditions by diminishing its expression^[4]. In humans there are no reports of the presence or absence of this phenomenon at the proximal jejunum. Moreover, there are no reports concerning over-expression in patients undergoing RYGB surgery, which is an iron deficiency model involving the described mechanism.

The aim of the current study is to describe the changes in DMT1 expression in a group of patients having undergone RYGB surgery. Because to our knowledge, no research has been carried out on this topic, the intention is to start a new line of research addressing the molecular aspects that regulate the occurrence of anaemia in such patients.

MATERIALS AND METHODS

For this prospective and analytical study, patients from the Surgery Department of the University of Chile Clinical Hospital were recruited. To be enrolled, patients had to be scheduled for RYGB surgery. All patients were informed of the nature of the study and authorised their inclusion by signing an informed consent. The study was approved by the ethics committee of our hospital. The design consisted of an initial assessment that included anamnesis and full physical examination. Age, weight, height, body mass index (BMI), and medical, surgical and family history were documented. All patients underwent a complete blood count and a serum ferritin test. Exclusion criteria were preoperative iron deficiency, previous use of iron supplements, poor tolerance to iron supplements, the presence of diseases that affect the red series or jejunum, habitual smoking (> 1 pack per week) and current pregnancy. In addition, patients who subsequently presented significant early or late postoperative complications (leak, haemorrhage, stenosis, *etc.*) were excluded from the final analysis.

All patients underwent RYGB surgery by a standard technique^[5]. During the surgery, a sample of jejunal mucosa was collected at the level of the gastrojejunal anastomosis (GJA) for histological analysis (jejunum approximately 25 cm distal to Treitz's angle). Once discharged, all the patients received precise indications concerning their feeding (similar guidelines for all patients having undergone RYGB surgery) and vitamin supplement intake. The latter consisted of Maltofer vitaminado[®] (multivitamin complex with minerals, trace elements and iron as iron III 60 mg from Andrómaco laboratory, Santiago, Chile), 1 tablet per day for 1 mo, which was then changed to Berocca Plus[®] (B complex, calcium and magnesium from Bayer laboratory, Santiago, Chile), 1 tablet per day permanently. In addition to the former, patients were prescribed Neurobionta[®] (B complex, 10000 U, from Merck laboratory, Santiago, Chile), 1 intramuscular ampoule per month for 3 mo, and then every 3 or 6 mo for women or men respectively.

Six months after surgery, patients underwent a complete blood count, a serum ferritin test and an upper digestive endoscopy with biopsy of the jejunal mucosa at the alimentary limb (approximately 10 cm distal to the GJA). The histological specimens were fixed in 4% formaldehyde (for haematoxylin-eosin staining and immunohistochemistry analysis) and stored in liquid nitrogen at -80°C (for western blot analysis).

Haematoxylin and eosin staining

Immediately upon collection, the tissue specimens were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 24 h. The specimens were subsequently dehydrated and embedded in paraffin in an automatic processor to create paraffin blocks in standard plastic cassettes. Sections 5 μm thick were obtained using a rotation microtome with disposable blades. These were mounted on xylan-coated glass slides and dried in an oven at 60°C .

for 24 h.

Sections for haematoxylin and eosin (H and E) staining were deparaffinised in xylene and hydrated in a sequence of ethanol in decreasing concentrations ending in distilled water. Nuclear contrast was obtained with Harris haematoxylin for 3 min. The sections were then washed under running water to turn the nuclei blue. Cytoplasmic staining with aqueous 0.5% eosin Y was applied for 30 s. Finally, sections were dehydrated with ethanol, clarified with xylene and mounted with permanent synthetic medium.

Immunohistochemistry

Immunohistochemistry (IHC) sections were processed in the same way as for H and E staining but then rehydrated and pretreated with 1 mmol/L ethylenediamine-tetraacetic acid (EDTA), pH 8.0 for 25 min at 96 °C in a steamer for antigen retrieval. The slides were washed in distilled water, followed by incubation with 3% aqueous hydrogen peroxide to block the endogenous peroxidase. The slides were then washed 3 times for 2 minutes with 0.01 mol/L Tris-buffered saline with 200 iL/L Tween 20 (TBST), pH 7.6, and blockage of unspecific reactivity was carried out with horse serum for 10 min at room temperature (RT).

Once the blockage was finished, sections were incubated with anti-DMT1 antibody (pan-DMT1 rabbit polyclonal antibody, which recognises the amino-terminal sequence MVLGPEQKMSDDSVSGDH present in all the isoforms of human DMT1 and which was prepared by the Immunology Service of the School of Sciences of the University of Chile) in 1:200 dilution in TBST, for 45 min at 37 °C in a humid chamber. In parallel, sections were incubated with horse serum under the same conditions, as a negative control. After incubation with the antibody, sections were washed with TBST and treated with the detection kit Vector PK 7200 (Vector Laboratories Inc. 30 Ingold Road, Burlingame, CA 94010 United States). Sections were incubated for 25 min at RT with biotinylated secondary antibody from the kit, followed by a wash with TBST and finally incubation with developing solution (1 mg/mL dimethylaminobenzidine in 0.01 mol/L saline phosphate buffer pH 7.6 with 0.003% hydrogen peroxide) for 2 min at RT. The reaction was interrupted with distilled water. Nuclear contrast was obtained with Harris haematoxylin for 1 min. Finally, the sections were dehydrated with ethanol, clarified with xylene and mounted with permanent synthetic medium.

Western blot

The western blot protocol consisted of (1) initial homogenisation of the specimens with a tissue lysis buffer solution in presence of enzymatic inhibitors (RIPA solution) in a glass-glass Wheaton® homogeniser; sonication of the sample for 7 min; further homogenisation to ensure its total distribution; and then centrifugation of the sample at 10000 rpm at 4 °C for 10 min and extraction of the supernatant that contained the protein of interest;

(2) quantification of the protein in the extract by Lowry's method; (3) electrophoretic separation of the proteins (according to their molecular weight), carried out in acrylamide/polyacrylamide gels (8%); (4) electrotransfer of the proteins to a nitrocellulose membrane; (5) incubation of membranes with a specific antibody against the protein of interest (anti-DMT1) and a secondary horseradish peroxidase-conjugated antibody, followed by immunodetection with the method of chemoluminescence and its subsequent detection with auto radiographic film; and (6) quantification of protein bands by appropriate software showing the results as total pixels per quantifiable band.

Histological analysis

The samples were analysed by a single pathologist (MV) who was blinded to the groups (preoperative *vs* postoperative). For morphological analysis of the villi and goblet cells, haematoxylin and eosin staining was used. For the analysis of DMT1 staining, the enterocyte was divided into cytoplasm and brush border. Qualitative analysis was performed to compare the intensity of the staining at 0 and 6 mo after surgery. The staining was classified into three levels: (+) mild, (++) moderate and (+++) intense.

Statistical analysis

All data are presented as the mean \pm SD. Stata 8.1 software (Stata Corp., Lakeway Drive, TX, United States) was used for statistical analysis. Because the data were normally distributed, we applied Wilcoxon's test and the χ^2 test, considering $P \leq 0.05$ statistically significant.

RESULTS

We recruited 16 patients who met the inclusion/exclusion criteria, but 3 patients were lost from the study because they failed to complete the 6-mo post-surgery follow-up control. There were no postoperative complications. Among the 13 patients who were analysed, the average age was 44.1 ± 12.7 years (20-66), and only one patient was a male. Four of the patients had type 2 diabetes mellitus, 6 had arterial hypertension, and 6 had dyslipidaemia. Twelve patients underwent non-resective laparoscopic RYGB, whereas the patient with the highest BMI (50.9) underwent an open technique with resection of the gastric remnant.

Both weight and BMI decreased significantly ($P = 0.001$ for both variables), for an average percent excess weight loss (%EWL) of $60\% \pm 13.3\%$ and an average percent excess BMI loss (%EBMIL) of $79.6\% \pm 21.6\%$. Two patients presented with mild anaemia six months after surgery (haemoglobin: 11.2 g/dL and 11.4 g/dL, respectively). Nevertheless, their ferritin levels stayed within normal ranges. When the group was analysed as a whole, there was not a significant decrease in the level of haemoglobin or ferritin. Table 1 shows the evolution of anthropometric and haematologic variables.

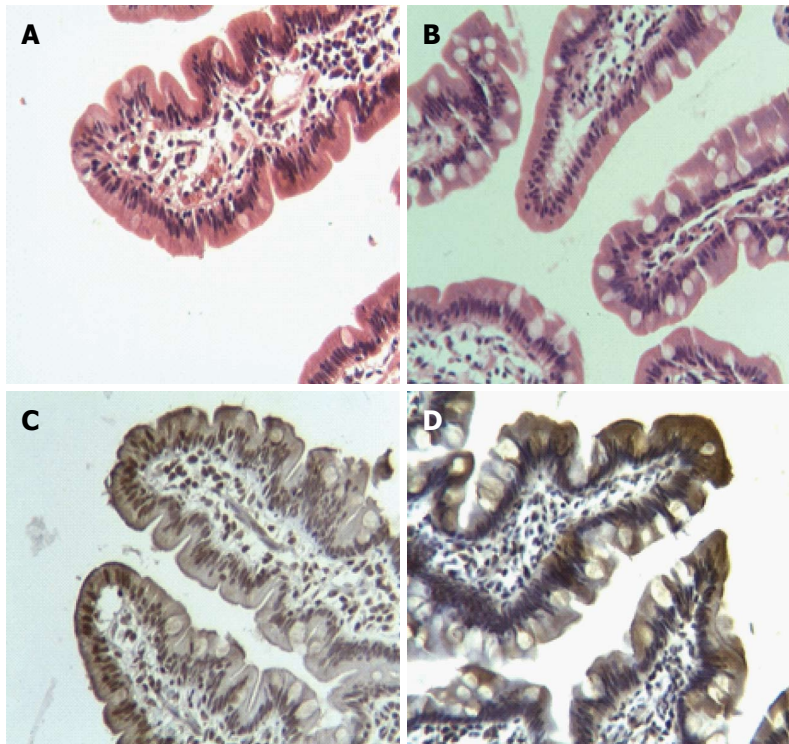


Figure 1 Morphological and immunohistochemical changes in the jejunal villus of patients subjected to Roux-en-Y gastric bypass. A and B are jejunal villus stained with haematoxylin-eosin at 0 and 6 mo after surgery, respectively. Note the preserved normal morphological pattern together with an increase of goblet cells. C and D show immunohistochemical staining for divalent metal transporter 1 during the same period, with an evident increase of such staining in the cytoplasm of epithelial cells at the apex of the villi.

Table 1 Progress of the anthropometric and haematologic variables

| Variable (averages) | Preoperative | 6 mo postop. | P ¹ |
|---------------------|--------------|--------------|----------------|
| Weight (kg) | 99.8 ± 17.7 | 71.7 ± 12.3 | 0.001 |
| BMI | 40.4 ± 5.7 | 29.1 ± 4 | 0.001 |
| Haematocrit (%) | 41.4 ± 2.7 | 39.7 ± 3.5 | 0.272 |
| Haemoglobin (g/dL) | 13.7 ± 1 | 13.1 ± 1.1 | 0.071 |
| Ferritin (ng/mL) | 85.2 ± 76 | 69.3 ± 58.7 | 0.208 |

¹Wilcoxon test. BMI: Body mass index.

During histological analysis, most of the villi and crypts showed preservation of the normal morphologic pattern 6 mo after surgery (Figure 1A, B). Villi showed an increase in the number of goblet cells per millimetre of epithelium ($P = 0.013$) (Figure 2). By IHC, DMT1 was present in the whole epithelium, mainly concentrated at level of the brush border.

At 6 mo there was a significant increase of DMT1 in the cytoplasm of epithelial cells ($\chi^2 = 6.03$; $P = 0.049$) at the apex of the villi (Figure 1C, D). At level of the brush border, the expression of DMT1 did not show significant variation (Table 2). In spite of these findings and having analysed the total amount of the receptor in the jejunal mucosa by western blot, we found a significant decrease of DMT1 ($P = 0.040$) 6 mo post-surgery (Figure 3).

DISCUSSION

The number of bariatric surgeries has progressively

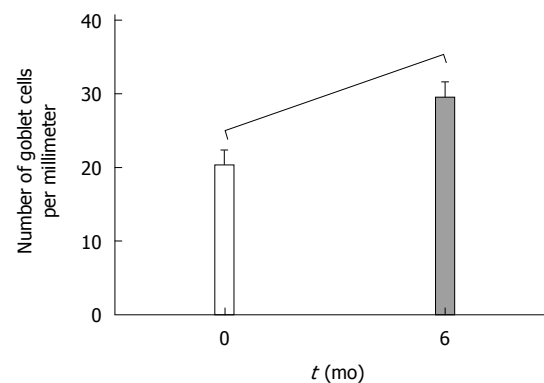


Figure 2 Change in the number of goblet cells in the proximal jejunum 6 mo after Roux-en-Y gastric bypass. Wilcoxon test: $Z = -2.47$; $P = 0.013$.

increased given the obesity epidemic that affects the population of many countries, especially in the West^[6]. RYGB is one of the most frequent bariatric surgeries due to its excellent results, mainly in relation to the resolution of comorbidities^[7] and its low rate of complications^[8]. Nevertheless, as the quantity of operated patients increases, so do the long-term complications, some of the most important of which are nutritional deficiencies. The most frequent nutritional deficiencies after bariatric surgery is iron deficiency and secondary anaemia^[9-11], although in many cases it follows the deficit of other minerals or vitamins, such as vitamin B12 and the folates. This is why there are established follow-up and nutritional supplementation protocols, usually with

Table 2 Change in staining intensity for divalent metal transporter 1 in jejunal mucosa from patients 6 mo after surgery

| Patients | Cytoplasm | | <i>P</i> ¹ 0.049 | Brush border | | <i>P</i> ¹ 0.218 |
|----------|-----------|------|--------------------------------|--------------|------|--------------------------------|
| | 0 mo | 6 mo | | 0 mo | 6 mo | |
| 1 | ++ | +++ | | ++ | ++ | |
| 2 | ++ | + | | ++ | ++ | |
| 3 | + | + | | ++ | ++ | |
| 4 | ++ | + | | ++ | ++ | |
| 5 | ++ | ++ | | ++ | ++ | |
| 6 | + | ++ | | + | ++ | |
| 7 | ++ | ++ | | ++ | ++ | |
| 8 | ++ | +++ | | ++ | ++ | |
| 9 | ++ | ++ | | ++ | ++ | |
| 10 | + | +++ | | ++ | +++ | |
| 11 | + | + | | + | ++ | |
| 12 | ++ | + | | ++ | ++ | |
| 13 | ++ | +++ | | ++ | ++ | |

¹χ² test. Staining intensity: (+) mild; (++) moderate; (+++) intense.

multivitamin and iron oral intake. However, a subset of patients still will still manifest the deficit.

No randomised and controlled studies have addressed the iron deficiency in such patients. There are only retrospective data and case series. From the latter, it is possible to estimate that between 30% and 50%^[11-15] of patients will show iron deficiency or anaemia, depending on the study. These might manifest months or even years after the surgery, but in most cases the disorder appears within the first 12 mo^[16]. Thus, these proportions might vary depending on the moment at which the measurement is carried out. Some patients have a higher tendency than others to present this complication because it is more common in women of fertile age (due to increased blood loss), adolescents and pregnant women (the latter due to higher iron requirements). This disorder is seen frequently before surgery, as a result of the eating and living habits of such patients^[17,18].

There are various reasons for the greater tendency towards iron deficiency and anaemia among bariatric surgery patients, mainly the lack of gastric acidity, the exclusion of the main absorption site (duodenum), decreased ingestion, postoperative bleeding and the presence of marginal ulcers^[2]. The longer the alimentary limb, the higher the incidence of this pathology^[11], because of the eventual increase of malabsorption. It must be taken into account that these patients are also at risk of presenting other mineral or vitamin deficiencies that might also cause anaemia, such as vitamins from the B complex, copper, vitamin C and protein deficiencies, although these are less frequent reasons.

The iron in our diet comes in two forms, either as haeme iron or as inorganic iron. The former is highly bioavailable and is mainly found in red meats. Its absorption mechanism is not completely clear, but haeme carrier protein 1 (HCP1) has been suggested as the iron transporter at the brush border^[19]. The non-haeme iron is widely distributed and is absorbed in its ferrous state (Fe²⁺) by DMT1, which has the ability to transport non-specific metals such as manganese, lead, cadmium, zinc

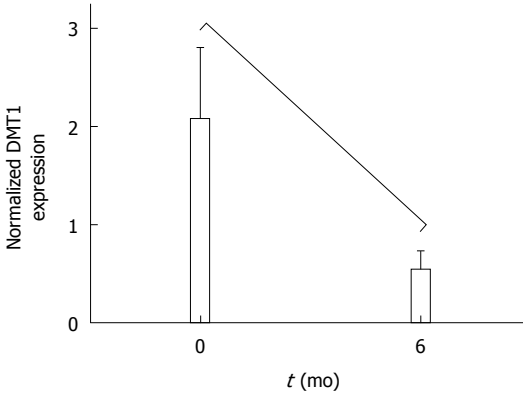


Figure 3 Change in expression of divalent metal transporter 1 in the proximal jejunum 6 mo after Roux-en-Y gastric bypass. Wilcoxon test: Z = 2.04; P = 0.04.

and copper^[3]. In its ferric form (Fe³⁺), iron is absorbed in parallel by mobilferrin^[20]. However, the presence of an apical ferric reductase (Dcytb) has been described at the duodenal mucosa, where it reduces Fe⁺³ to facilitate its absorption.

In conditions of iron deficiency, the duodenal mucosa is the mucosa that adapts the most. The amount of DMT1 mRNA in the duodenum increases in iron deficiency^[4,21-24], as does Dcytb^[4,23]. The molecular mechanisms for this adaptation are not well known, although it has been suggested that the apical transporters are regulated by local signals and the basolateral ones by systemic signals^[25]. We have demonstrated that in patients undergoing RYGB, DMT1 expression in the cytoplasm of enterocytes located in the apex of the villi of the proximal jejunum is increased 6 mo after surgery. Nevertheless, we were surprised that the total quantity of the receptor in the same area decreased. To reduce the possibility of error in the measurements and in the handling of samples, biopsies at 0 and 6 mo were kept under the same conditions and finally processed simultaneously under the same laboratory conditions and by the same staff. While the biopsies varied in size, western blots were performed by normalising protein levels to the tissue size, thereby eliminating the variable amount of tissue as a bias.

Therefore, a possible explanation for our protein expression findings are the cellular changes seen in the villi in such patients, such as the significant increase in the number of goblet cells that displace enterocytes which have greater absorptive capacity than goblet cells. This might be triggered by the physical/chemical stimulus of food, now undigested by the exclusion of the stomach and the duodenum, a mechanism that acts within these cells^[26,27]. This might be the beginning of a compensation mechanism between the enterocyte decrease and the increased DMT1 in the remaining enterocytes. This adaptation would allow our patients to maintain their iron reserves at normal levels despite all the side effects of the RYGB that affect iron absorption, which after 6 mo has already decreased^[28]. These changes might explain the high rate of iron metabolism disorders observed in

all these patients during a longer-term follow-up, keeping in mind that many other individual factors may influence the development of anaemia. Undoubtedly, further cellular research is needed, prospective in nature and for longer periods, in patients undergoing RYGB, including other molecules involved in iron metabolism, such as Dcytb, ferroportin, hepcidin and hephaestin. This would improve our understanding of the mechanisms that produce iron metabolism disorders in bariatric surgery patients.

In conclusion, 6 mo after RYGB surgery, patients showed increased expression of DMT1 in the cytoplasm of the enterocytes located in the apex of the villi of the proximal jejunum. This could be a compensation mechanism because it is associated with a decrease of the total quantity of the receptor in the mucosa, most likely following the cellular changes experienced by the intestinal villi in RYGB patients.

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COMMENTS

Background

Iron deficiency and anaemia are common complications affecting patients after Roux-en-Y gastric bypass (RYGB) surgery. Various mechanisms could explain this, especially the exclusion of the duodenum, the main site of iron absorption effected by the divalent metal transporter 1 (DMT1). In spite of various protocols of supplementation and nutritional management, iron deficiency is still present in a proportion of patients.

Research frontiers

DMT1 is a transmembrane protein found on the apical membrane of the enterocyte that, by the proton-motive force, transports ferrous iron (Fe^{2+}) into the cell. In conditions of iron deficiency, the duodenum is capable of adapting by over-expressing the DMT1 transporter, and in overload conditions by downregulating it. In this study, the authors demonstrate that 6 mo after RYGB surgery, patients exhibit an increase in DMT1 expression in the enterocytes of the tips of the villi at the proximal jejunum.

Innovations and breakthroughs

Several studies have highlighted the importance of nutritional deficiencies in patients subjected to RYGB surgery, in particular iron deficiency, which occurs in 30%-50% of patients. In this study, the authors demonstrate that the variations in the iron receptor DMT1 may help to explain why some patients develop anaemia despite being supplemented.

Applications

Understanding the changes experienced by these patients in terms of iron absorption mechanisms will help to create future strategies to prevent the development of anaemia.

Peer review

The authors examined the variation that DMT1 shows in patients after Roux-en-Y gastric bypass surgery. This study reveals that 6 mo after surgery, patients exhibit an increase in the expression of DMT1 in the cytoplasm of enterocytes of the tips of the villi at the proximal jejunum. This could be a compensation mechanism because it is associated with a decrease in the total quantity of the transporter in the mucosa, most likely as a result of the cellular changes present at the intestinal villi of such patients. The results are interesting and

represent the first report on the molecular changes that occur in the physiology of iron absorption in these patients.

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Effects of bile acids on cyclooxygenase-2 expression in a rat model of duodeno-esophageal anastomosis

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Abstract

AIM: To examine the expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) in rat esophageal lesions induced by reflux of duodenal contents.

METHODS: Thirty 8-week-old male Wistar rats were exposed to duodenal content esophageal reflux. All animals underwent an esophagoduodenal anastomosis (EDA) with total gastrectomy to elicit chronic esophagitis. In ten rats sham operations with only a midline laparotomy were performed (Control). The rats were sacrificed at the 40th week, their esophagi were taken for hematoxylin and eosin staining and for examination of expression of COX2, PGE2, and proliferating cell nuclear antigen (PCNA), and total bile acids in the esophageal lumen was measured.

RESULTS: After 40 wk of reflux, columnar dysplasia, squamous cell carcinoma and adenocarcinoma were observed. Total bile acids in the esophageal lumen were significantly increased in the EDA group compared with the sham operated rats. PCNA labelling index and esophageal tissue PGE2 levels were higher in dysplastic and cancer tissues than in control tissues. Overexpression of COX2 was observed in dysplastic and cancer tissues.

CONCLUSION: Reflux of duodenal contents induces COX2 expression and increases prostaglandin synthesis in dysplastic and cancer tissues. This result suggests a possible mechanism by which bile acids promote esophageal cancer.

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Key words: Bile acids; Cyclooxygenase-2; Prostaglandin E2; Esophageal cancer; Esophagoduodenal anastomosis

Core tip: It is known that reflux of duodenal contents (bile acids) can induce mucosal injury, stimulate cell proliferation, and promote tumorigenesis. We examined the expression of cyclooxygenase-2 (COX-2) and prostaglandin E2 in rat esophageal lesions induced by reflux of duodenal contents. All animals underwent an esophagoduodenal anastomosis with total gastrectomy to elicit chronic esophagitis. We demonstrated that reflux of duodenal contents induces COX-2 and increases prostaglandin synthesis in dysplastic and cancer tissues. This result suggests a possible mechanism by which bile acids promote esophageal cancer.

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INTRODUCTION

Reflux of duodenal contents appears to contribute to the development of esophagitis and Barrett's esophagus^[1,2]. This idea is supported by several observations. In patients with gastroesophageal reflux disease, the concentration of bile acids in the esophageal refluxate correlates with

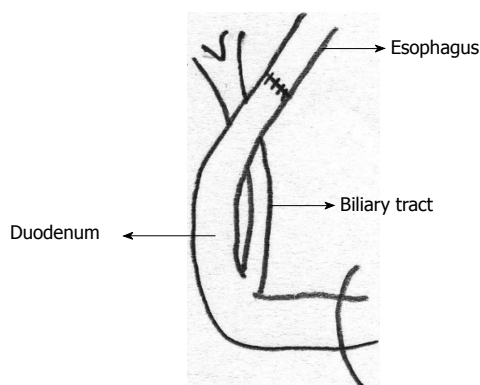


Figure 1 Esophagoduodenal anastomosis model: Esophagoduodenal anastomosis with total gastrectomy.

the degree of esophageal mucosal injury^[3]. In experimental animals, induction of a duodeno-esophageal anastomosis led to esophagitis, Barrett's esophagus and esophageal cancer^[4]. The precise mechanism by which duodenal reflux causes esophageal injury and predisposes to neoplasia is uncertain. However, there is considerable evidence to suggest that bile acids contribute to this mechanism. Bile acids can induce mucosal injury^[5], stimulate cell proliferation^[6] and promote tumorigenesis^[7].

Two isoforms of cyclooxygenase (COX), designated COX-1 and COX-2, catalyze the synthesis of prostaglandins (PGs) from arachidonic acid. COX-1 is a house-keeping gene that is expressed constitutively in most tissues. COX-2 is an immediate-early response gene that is induced by a variety of mitogenic and inflammatory stimuli^[8]. Elevated levels of COX-2 have been detected in both inflammatory^[9] and neoplastic conditions^[10]. For example, COX-2 is up-regulated in peptic ulcer disease, Barrett's esophagus and esophageal cancer. Taking this information together, it seems likely that COX-2 plays a role in the pathogenesis of duodenal reflux-related diseases of the esophagus.

In this study, we investigated the effects of bile acids and duodenal reflux on COX-2 expression in a rat model of duodeno-esophageal anastomosis.

MATERIALS AND METHODS

Eight-week-old male Wistar rats weighing approximately 300 g were used for the experiments. They were allowed to acclimatize for 2 wk prior to surgery. Solid food was withdrawn 1 d before and for 1 d after surgery. Esophagoduodenal anastomosis (EDA) was performed in 30 rats under general anesthesia (pentobarbital 50 mg/kg body weight, *i.p.*) through an upper midline incision. The gastroesophageal junction was ligated, and the distal esophagus was transected 2 mm above the ligature. Furthermore, the gastroduodenal junction was also ligated, and the proximal duodenum was transected 3 mm distal to the pylorus. A total gastrectomy was performed with the removal of the entire stomach, and end-to-end anastomosis of the esophagus and duodenum. The abdomi-

nal incision was closed in two layers (Figure 1). In 10 rats, sham operations with only a midline laparotomy (control group) were performed. Postoperatively, the rats were allowed to drink water after six hours and were fed the following day. This procedure was approved by the Animal Care and Facilities Committee, Kinki University.

All of the rats were killed as described previously^[11]. Special care was taken to separate the esophagus from the duodenum based on the suture line. For the animals killed at the 40th week, all of the esophagi were cut longitudinally and fixed in 10% buffered formalin. The formalin-fixed esophagus was Swiss-rolled, processed and embedded in paraffin. Five-micron sections were mounted on glass slides and used for pathological and immunohistochemical analyses.

Immunohistochemical analysis

COX-2: Localization of COX-2 protein was determined by immunohistochemical staining with a specific antibody. The DAKO EnVision system (Dako Cytomation Japan Co. Ltd., Kyoto, Japan) was used with autoclave acceleration. After blocking endogenous peroxidase, deparaffinized sections were covered with a protein block and serum-free medium (Dako) and were incubated overnight at 4 °C with a primary anti-COX2 monoclonal antibody (1:50; BD Transduction Laboratories, San Jose, CA). Sections were treated with a secondary biotinylated antibody (Dako), 3,3'-diaminobenzidine tetrahydrochloride was used as the chromogen, and the sections were counterstained with hematoxylin.

Proliferating cell nuclear antigen: Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was performed by the avidin-biotin complex method using a mouse anti-human PCNA monoclonal antibody and the appropriate Histostain Gold AEC kit. The PCNA labeling index has been widely used to assess cell proliferation. In this study, the index was defined as the number of squamous epithelial cells with a PCNA-positive nucleus (or nuclei)/100 squamous epithelial cells (%).

Measurement of PGE2 production

Each tissue sample frozen at -80 °C was weighed and homogenized using a Polytron homogenizer PT-MR2100 in 0.5 mL of homogenization buffer (0.1 mol/L phosphate, pH 7.4, containing 1 mmol/L ethylenediamine tetraacetic acid and 10 µmol/L indomethacin) containing 100 mg tissue. Then, 2 volumes of acetone were added to the samples and vigorously spun. The samples were incubated at room temperature for 5 min, and centrifuged at 1500 × *g* for 10 min to remove precipitated proteins. The supernatant was transferred into a clean tube, and dried to remove the acetone using a gentle stream of nitrogen. One mL of 1.0 mol/L acetate buffer (pH 7.4) was added to dissolve the samples, which were immediately affinity purified with an SPE cartridge (Cayman Chemical, Ann Arbor, MI). The samples were assayed using a PGE2 EIA kit (Cayman Chemical), according to the manufac-

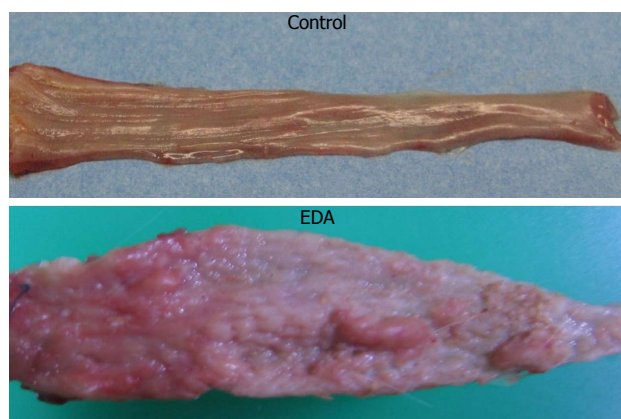


Figure 2 Macroscopic appearance of resected esophagi from esophagoduodenal anastomosis and control rats. EDA: Esophagoduodenal anastomosis.

turer's instructions. PGE2 levels were shown as pg/mg of tissue.

Measurement of bile acids in the esophageal lumen

After each rat was sacrificed, their esophagi were removed and lavaged with 0.5 mL of saline. The lavage was centrifuged at $1500 \times g$ at 4°C for 5 min. The supernatant was frozen and stored. Total bile acid concentration was measured with an ENZA BILE kit (Daiichi Chemical, Tokyo).

Statistical analysis

Data are expressed as mean \pm SD of each group. Student's *t* test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

General observations

A total of 37 of 40 (92.5%) rats completed the study. In the EDA group, 27 (90%) rats completed the study, and 3 rats died from complications, such as malnutrition and pneumonia. In the control group, 10 (100%) rats completed the study.

Macroscopic findings

The middle and lower esophagus of animals in the EDA group was wide and thickened. There was gross evidence of severe esophageal mucosal injury in the EDA group, which included epithelial thickening and extensive hyperplasia of the lower two thirds of the esophagus. Ulceration was frequently present in the area above the anastomosis (Figure 2).

There was a small polypoid tumor in the lower esophagus in the EDA group. The tumor was squamous cell carcinoma and adenocarcinoma. Most of the nodular lesions were also associated with carcinomas, and the others with esophagitis. In addition, there were grossly normal tissues in the control group.

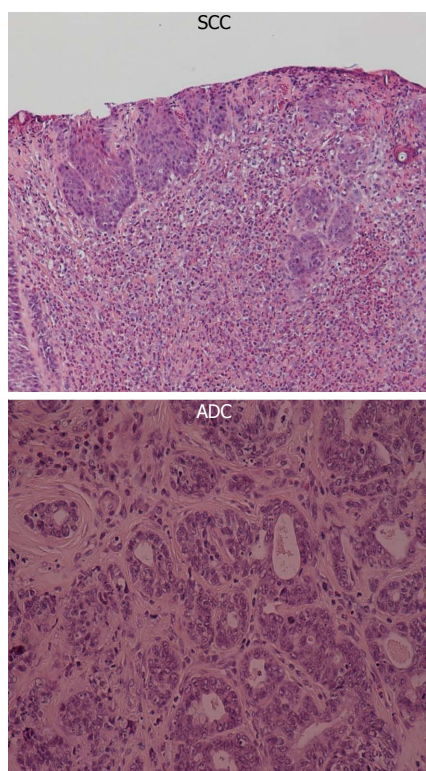


Figure 3 Microscopic findings in the distal portion of the esophagus from esophagoduodenal anastomosis rats. ADC: Adenocarcinoma; SCC: Squamous cell carcinoma.

Microscopic findings

The esophagi of the control rats did not reveal any pathological findings, but various squamous cell lesions were observed in the middle and lower esophagus in the EDA group (Figure 3).

All animals from the EDA group showed histological features of esophagitis, including marked hyperplastic changes with increased thickness of the squamous epithelium, hyperkeratosis and regenerative changes with papillomatosis, and basal cell hyperplasia. These features were not found in the control group. Columnar lined epithelium (CLE) and epithelial ulceration were frequently present adjacent to the anastomosis. CLE was observed in 40% of rats at the 40th week. Severe dysplasia in the lower esophagus occurred in 100%, squamous cell carcinoma (SCC) was observed in 40% and adenocarcinoma (ADC) was observed in 30% at the 40th week.

To assess the biological behavior of various squamous lesions, we performed immunohistochemical staining for PCNA because the proliferative index is often increased in dysplastic and cancerous tissues. The PCNA labeling index of dysplasia and cancer ($75\% \pm 5\%$) was higher than that of control ($30\% \pm 5\%$) ($P < 0.05$) (Figure 4).

Total bile acids in the esophageal lumen ($\mu\text{mol/L}$)

Total bile acids in the esophageal lumen were significantly increased in the EDA group (180 ± 50) compared with

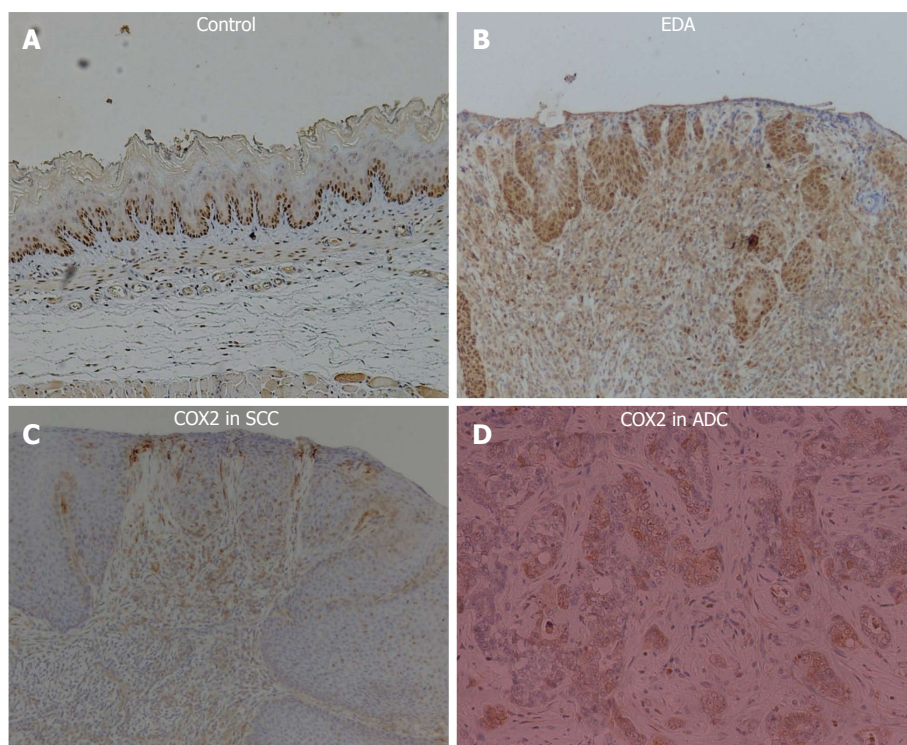


Figure 4 Immunohistochemical findings. A: For proliferating cell nuclear antigen in control; B: Esophagoduodenal anastomosis (EDA) rats; C: For cyclooxygenase-2 (COX2) in squamous cell carcinoma (SCC); D: Adenocarcinoma (ADC) in EDA rats.

the sham operated rats (35 ± 5) ($P < 0.05$).

Immunohistochemistry of COX-2

Every animal that suffered from reflux demonstrated COX-2 protein expression in the lower esophagus. COX-2 was abundantly expressed in both inflammatory and proliferative esophageal mucosa of rats exposed to chronic EDA. Some SCC and ADC epithelial cells strongly expressed the COX-2 protein (Figure 4).

Measurement of PGE2 production (pg/TPmg) in esophageal tissue

PGE2 synthetic activity was significantly increased in the EDA group (260 ± 50) compared with the sham operated group (25 ± 5) ($P < 0.01$).

DISCUSSION

Overexpression of COX-2 has been linked to a variety of inflammatory and neoplastic conditions. Hence, it is logical to postulate that endogenous inducers of COX-2 could predispose to inflammation and malignancy. Previously, Song *et al*^[12] reported that unconjugated bile acids induced COX-2 expression.

Recent evidence suggests that bile acids, major constituents of the duodenogastroesophageal refluxate, can also promote the development of Barrett's esophagus and esophageal cancer. Bile reflux is particularly common in individuals with gastroesophageal reflux disease who subsequently develop Barrett's esophagus^[13,14]. Barrett's esophagus also develops in patients who have undergone total

gastrectomy, a situation in which bile reflux is common. Development of Barrett's esophagus and subsequently esophageal cancer occurs in a rat model of esophago-duodenostomy. The present study demonstrates that it is duodenal contents, not gastric contents, that induce esophageal carcinogenesis through reflux. Because this carcinogenesis required no administration of carcinogens, and because spontaneous esophageal carcinoma is rare in animals, duodenal contents are most likely carcinogenic in the development of esophageal carcinoma.

The histological pattern of esophageal carcinoma induced in the present study was classified into 2 types; ADC and SCC.

ADC always occurred near the esophagoduodenostomy and always in the columnar lined epithelium. Human esophageal ADC mostly arises in the lower third of the esophagus, and when it does occur, it is usually associated with Barrett's esophagus. The majority of Barrett's esophagus cases result from chronic gastro-esophageal reflux. SCC was observed distant from the site of the anastomosis, and was surrounded by chronic squamous esophagitis with features of basal-cell hyperplasia and regenerative thickening.

It is widely accepted in humans that regurgitation of duodenal contents is closely linked to Barrett's esophagus and to the development of esophageal ADC; however, esophageal SCC is not reported to be related to reflux^[15], but is strongly associated with tobacco smoking and alcohol consumption. Gastroesophageal reflux does not appear to be an independent risk factor for esophageal SCC, but it may enhance the acknowledged risk factors

such as tobacco smoking and alcohol consumption. In contrast, results from several studies using rat duodenal content reflux models have shown the development of esophageal carcinomas including SCC^[16]. In this study, the incidence of pure ADC is lower than that of SCC. It is unclear what factors lead to the formation of carcinomas of specified histology.

The precise mechanisms by which duodenal reflux causes esophageal injury and predisposes to esophageal cancer are uncertain. There is considerable evidence, however, that bile acids contribute to this process. Total bile acids in the esophageal lumen were significantly increased in the EDA group compared with the control group. Bile acids induce AP-1-mediated gene transcription^[17-19] and enhance the activity of protein kinase C^[20]. Recent evidence has linked bile acid induced tumorigenesis to increased activity of COX-2. It is also unclear which bile acids in the refluxate contribute to COX-2 induction.

As discussed above, bile acids represent one of the important constituents of duodenal fluid that has been implicated in esophageal mucosal injury^[21]. Bile acids strongly induce COX-2 by either transcriptional or post-transcriptional mechanisms in multiple gastrointestinal tract cancers, including cancer of the colon, pancreas, stomach, liver, esophagus and bile duct^[22].

An animal model was used to determine whether duodenoesophageal reflux caused induction of COX-2. We observed markedly enhanced expression of COX-2 in dysplastic and cancerous mucosa obtained from rats in which an esophagoduodenal anastomosis had been created. In contrast, COX-2 was undetectable in esophageal and duodenal mucosa from the control rats. Esophageal tissue PGE2 levels were significantly increased in rats that developed dysplasia and cancer. This result suggests a possible mechanism by which bile acids promote esophageal cancer.

Bile acids increase cellular proliferation and the number of mitotic events in colonic mucosa^[23]. Enhanced DNA synthesis has been demonstrated in the epithelium of the large intestine of rats treated with bile acids^[24]. Reduced susceptibility to apoptosis occurs in animal and human models of colon cancer following bile acid treatment^[25]. Taken together, the data suggest that bile acids are important mediators of carcinogenesis.

In conclusion, our findings suggest that reflux of bile acids induced the development of esophageal cancers. COX-2 induced by bile acids might be responsible for tumor angiogenesis, an important process in the development of esophageal cancers.

COMMENTS

Background

It is known that reflux of duodenal contents (bile acids) can induce mucosal injury, stimulate cell proliferation, and promote tumorigenesis.

Innovations and breakthroughs

In this study, bile reflux of duodenal contents induces cyclooxygenase-2 (COX-2) expression and increases prostaglandin synthesis in dysplastic and cancer tis-

sues. This result suggests a possible mechanism by which bile acids promote esophageal cancer.

Terminology

Hematoxylin eosin, COX-2, PGE2, and proliferating cell nuclear antigen.

Peer review

This is an excellent experimental study which probably adds to the existing literature.

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Dual-priming oligonucleotide-based multiplex PCR using tissue samples in rapid urease test in the detection of *Helicobacter pylori* infection

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Abstract

AIM: To investigate whether tissue samples processed by the rapid urease test (RUT) kit are suitable for dual-priming oligonucleotide-based multiplex polymerase chain reaction (DPO-PCR) to detect *Helicobacter pylori* (*H. pylori*).

METHODS: A total of 54 patients with specific gastrointestinal symptom were enrolled in this study. During endoscopy, gastric biopsy specimens were taken for histology, RUT, and DPO-PCR. DPO-PCR was performed on gastric biopsy samples and tissue samples that were analyzed by RUT at 2 separate institutes. In detecting *H. pylori*, the concordance rate of the DPO-

PCR tests between the tissue samples that had been submitted to RUT and the gastric biopsy samples was investigated.

RESULTS: *H. pylori* co-occurred with 76.0% (19/25) of gastric ulcers, 64.3% (9/14) of duodenal ulcers, and 33.3% (4/12) of gastritis cases. *H. pylori* infection was found in 100% (3/3) of the patients with both gastric and duodenal ulcers. Overall, *H. pylori* was detected in 35 of 54 (64.8%) patients. The diagnostic sensitivities of histology, RUT, and DPO-PCR were 85.7% (30/35), 74.3% (26/35), and 97.1% (34/35), respectively ($P = 0.02$). The positive predictive value (PPV) of DPO-PCR was 94.4%, whereas the negative predictive value (NPV) was 94.7%. In the rapid urease test (CLOtest)-negative cases, the frequency of positive DPO-PCR and histologic results was 20.0% (7/35). The concordance rate of the DPO-PCR tests between the tissue samples from the RUT kit and the gastric biopsy samples was 94.4% (51/54). The rate of DPO-PCR and silver stain positivity in the RUT-negative cases was 20.0% (7/35).

CONCLUSION: In diagnosing *H. pylori* infection, DPO-PCR can be performed on tissue samples that have been processed by the RUT kit. Particularly, in patients with RUT-negative results, DPO-PCR on these tissue samples could be helpful in detecting of *H. pylori* infection.

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Key words: *Helicobacter pylori*; Diagnosis; Dual-priming oligonucleotide-based multiplex polymerase chain reaction

Core tip: The rapid urease test (CLOtest) alone is unreliable in diagnosing *Helicobacter pylori* (*H. pylori*) infec-

tion and does not provide information about resistance to clarithromycin. Therefore, we investigated whether tissue samples that have been analyzed by the CLOtest kit are suitable for dual-priming oligonucleotide-based multiplex PCR (DPO-PCR) to detect *H. pylori*. Our results demonstrated that the DPO-based multiplex PCR test using tissue samples processed by the CLO kit is appropriate for detecting *H. pylori* and clarithromycin resistance. Particularly, in patients with CLO-negative results, this method is helpful for diagnosing *H. pylori* infection. Moreover, it would be beneficial in economical aspects.

Chung WC, Jung SH, Oh JH, Kim TH, Cheung DY, Kim BW, Kim SS, Kim JI, Sin EY. Dual-priming oligonucleotide-based multiplex PCR using tissue samples in rapid urease test in the detection of *Helicobacter pylori* infection. *World J Gastroenterol* 2014; 20(21): 6547-6553 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6547.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6547>

INTRODUCTION

In patients with *Helicobacter pylori* (*H. pylori*) - related diseases, a reliable diagnosis of infection with this bacterium is crucial, but no single test can be considered the gold standard. The rapid urease test (RUT) is the most commonly used biopsy-based method to diagnose *H. pylori* infection because it is simple, rapid and accurate. However, it requires a high density of bacteria, and anything that reduces the bacterial load may produce false-negative tests^[1]. Moreover, various medications may affect the presence of urease in the gastric mucosa. Within 2 wk of taking a proton pump inhibitor, bismuth or antibiotic, most of the *H. pylori* organisms have disappeared, making the RUT negative. Moreover, *H. pylori* does not distribute evenly in the stomach^[2]. Several factors including gastric pH, inflammatory cells, atrophic gastritis, and intestinal metaplasia affect its distribution^[3,4]. If the biopsy sample is taken from an area of intestinal metaplasia, the RUT will fail^[2,3].

Molecular methods are widely used to diagnosis *H. pylori* infection, as are analyses of its virulence and resistance patterns^[4-7]. Polymerase chain reaction (PCR) is the most sensitive and specific method for detecting of *H. pylori* in gastric biopsy specimens. It has great sensitivity with a detection limit of 0.02 pg *H. pylori* DNA, which corresponds to only 10 organisms^[8]. However, in clinical practice, PCR is complicated and it is not always simple to achieve the desired result. It is a time-consuming and labor-intensive method. Recently, a commercial dual-priming oligonucleotide (DPO) primer has been developed to detect single-nucleotide polymorphisms (SNP) using a 1-step PCR assay^[9]. Detection is accurate and rapid using the specific primers. Moreover, DPO-based multiplex PCR (DPO-PCR) can provide information

about clarithromycin resistance because clarithromycin resistance is the main predictor of failure of eradication treatments; therefore, the detection of clarithromycin resistance is important.

Previously, tissue samples taken for rapid urease testing have also been analyzed by PCR, which can detect *H. pylori* DNA in gastric tissue samples obtained for the RUT kit^[10]. When *H. pylori* infection is not detected in cases of peptic ulcer bleeding or peptic ulcer disease with chronic atrophy, an additional biopsy specimen and endoscopic procedure should be performed. In addition, in case repeated eradication therapy fails and the patient is clinically suspected of having an infection with a clarithromycin resistant strain, an additional biopsy specimen is necessary. Unfortunately, taking extra biopsy specimens is burdensome for clinicians and patients. In this study, we aimed to evaluate DPO-PCR in diagnosing of *H. pylori* infection, and to determine whether the tissue samples that already been submitted to the RUT kit are suitable for the DPO-PCR test compared with the result of DPO-PCR performed on gastric biopsy samples, RUT, and histologic results.

MATERIALS AND METHODS

Study population

All patients with specific gastrointestinal symptoms were enrolled at a teaching hospital of the Catholic University of Medicine, St. Vincent's Hospital, from November 2011 to May 2012. Patients who were referred to the endoscopy unit were recruited for this prospective study.

Patients were eligible to enter the study if they were older than 18 year of age and had gastric *H. pylori* infection. None of the patients had a history of *H. pylori* eradication, had undergone previous gastric surgery or had taken antibiotics in the 2 mo preceding the study. Patients were also excluded if they had significant renal, hepatic, cardiovascular, metabolic or hematological disorders. Additionally, pregnant or lactating women were excluded from our investigation.

Sample size

An estimated sample size of 50 subjects per group would give an 80% power to detect a difference of 15% in the *H. pylori* detection rate compared to other tests (assumed to have an detection rate of 85%), with a 2-sided alpha = 0.05. Thus, with a 10% drop out rate we needed to recruit at least 55 patients for each group.

$$n = [Z_{\alpha/2}(P_0 Q_0)^{2/1} + Z_{\beta}(P_0 Q_0)^{2/1}]^2 / [(\epsilon - |\delta|)^2]$$

DPO-Based multiplex polymerase chain reaction PCR

Genomic DNA from gastric biopsy and tissue samples analyzed by RUT (CLOtest; Kimberly-Clark, Utah, United States) were extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, United States). DNAs was stored at -20 °C until it was required for analysis. A novel commercialized DPO-PCR (Seeplex® ClaR-*H. pylori* ACE Detection, Seoul, South Korea) was

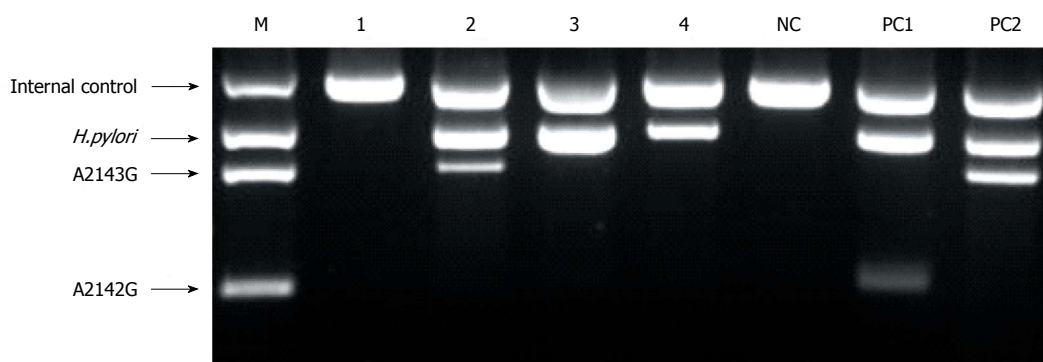


Figure 1 Representative example of the dual-priming oligonucleotide-based multiplex polymerase chain reaction. Lane M: Amplicon size marker (Seegene Inc., Korea); Lane 1: Negative; Lane 2: Mutant type of A2143G; Lane 3-4: Wild type; PC1: A2142G mutant positive control; PC2: A2143G mutant positive control; NC: Negative control.

performed according to the manufacturer's recommendations. This method uses 2 forward and 2 reverse DPO primers against the *23S rRNA* gene. A 4-primer combination mixture (HP-F, HP-R, A2142G-F, and A2143G-R) that amplifies 3 fragments (*i.e.*, a *H. pylori* common sequence, an A2142G mutant, and an A2143G mutant) is made for the multiplex PCR (Figure 1). The A2142G and A2143G mutations of the *23S rRNA* gene in *H. pylori* are associated with resistance to clarithromycin^[11,12]. DPO-PCR is a multiplex PCR that can be performed in any standard thermocycler. It is analyzed using a semi-automated system (*i.e.*, Screen tape[®]), which allows ultra rapid migration and analysis of the PCR products in small polyacrylamide gels. 8-Methoxysporalen was added during the mix preparation to intercalate between the double-stranded nucleic acids generated during amplification, thereby limiting carry-over contamination after UV irradiation and before the PCR product analysis. The kit also includes a primer pair for internal control.

Methods

During endoscopy, gastric biopsy specimens were taken from the greater curvature of the mid-antrum and corpus for histology, CLOtest and DPO-PCR. The diagnosis of *H. pylori* infection was made based on (1) histologic evidence of *H. pylori* in any 2 specimens taken from the antrum or corpus by silver stain; or (2) positive CLOtest and serological test results. If only the CLOtest was positive, serological test was performed additionally. All patients with peptic ulcer disease were prescribed proton pump inhibitor (PPI) therapy for 2-4 wk, and the remaining patients were treated according to their symptoms for 2 wk. If the silver stain and CLOtest were *H. pylori*-negative, we obtained an additional biopsy specimen under endoscopy 4-8 wk after initial examination. Patients did not take PPIs for at least 2 wk before re-endoscopy.

The specimens were fully immersed in the CLO reagent, and the test was interpreted at 1 and 9 h in the endoscopy room. If the CLOtest was positive, the

specimens were placed at -15 to -20 °C. If the CLOtest was negative, it was re-interpreted 24 h later in ambient air. DPO-PCR tests were performed on the gastric biopsy samples (Seegene Institute of Life Science, Seoul, South Korea) (Figure 1) and on tissue samples obtained from the CLOtest kit at 2 separate institutes (Research Institute of St. Vincent Hospital, Suwon, South Korea) (Figure 2).

Ethics statement

The study was approved by the institutional review board of the Catholic University of South Korea (VC11EISI0200). Each patient provided written informed consent to participate.

RESULTS

Basal characteristics of the enrolled patients

Gastric tissue samples were taken from 57 patients, but 3 patients were excluded because of inadequate DNA samples. A total of 54 patients (43 males, 11 females, mean age 58.7 ± 14.2 years) were enrolled in this study. Of these patients, 25 (46.3%) had gastric ulcers, while 14 (25.9%) had duodenal ulcers. Three (5.6%) patients had both gastric and duodenal ulcers, and 12 (22.2%) patients had chronic gastritis. *H. pylori* was detected in 76.0% (19/25) of the gastric ulcer patients, 64.3% (9/14) of the duodenal ulcer patients, and 33.3% (4/12) of the gastritis. *H. pylori* was detected in 100% (3/3) of the patients with both gastric and duodenal ulcers.

Diagnostic sensitivity

H. pylori was detected in 35 of 54 (64.8%) patients. The diagnostic sensitivities of histology, CLOtest, and DPO-PCR were 85.7% (30/35), 74.3% (26/35) and 97.1% (34/35), respectively ($P = 0.02$) (Table 1). The positive predictive value (PPV) is the proportion of patients with positive test results who are correctly diagnosed. The negative predictive value (NPV) is the proportion of patients with negative test results who are correctly diagnosed. The PPV of DPO-PCR was 94.4%, whereas

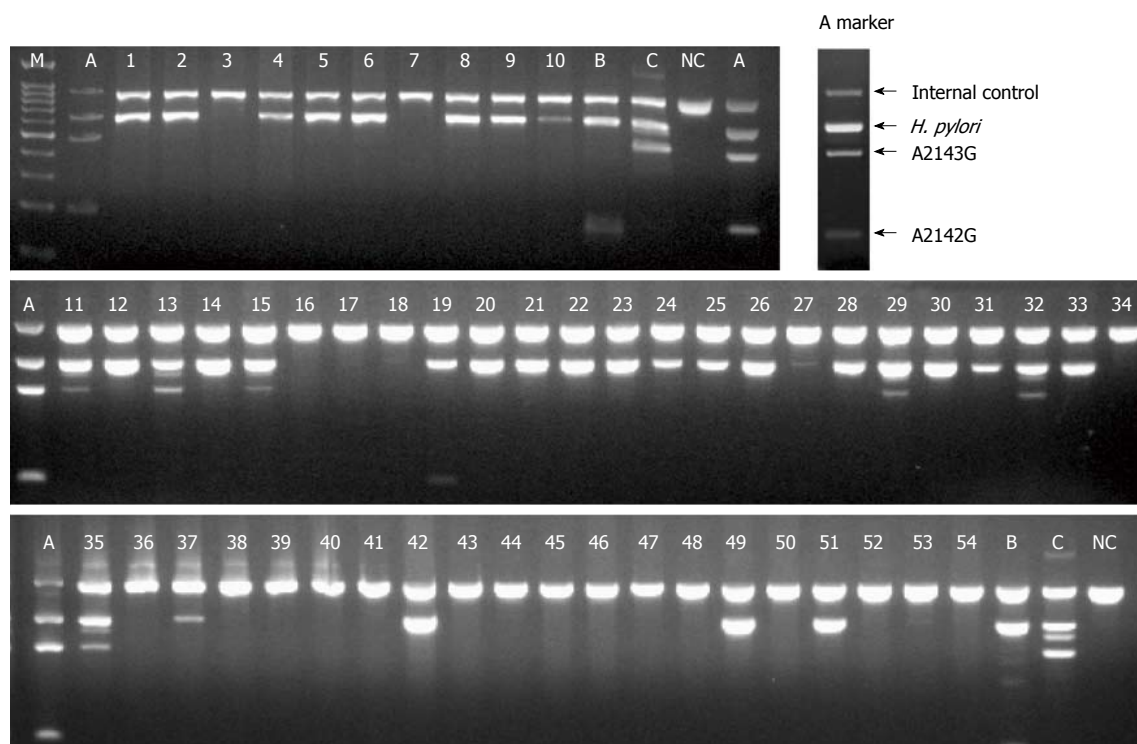


Figure 2 Detection of *Helicobacter pylori* and the A2143G/A2142G of the 23S rRNA gene on the basis of the dual-priming oligonucleotide-based multiplex polymerase chain reaction product in enrolled patients. M: 100 bp Marker; A: ClaR Marker; B: A2142G Positive Marker; C: A2143G Positive Marker; NC: Negative Marker; 1-54 : Samples.

Table 1 Diagnosis of *Helicobacter pylori* in the enrolled patients of this study *n* (%)

| Method of detection | Number of positivity | Number of <i>H.pylori</i> infection |
|--|----------------------|-------------------------------------|
| Silver stain - 1 st session | 30 | 30 (85.7) |
| 2 nd session | 2 | |
| CLOtest 1 st session | 26 | 26 (74.3) |
| 2 nd session | 3 | |
| DPO-PCR of gastric biopsy | 37 | 34 (97.1) |
| DPO-PCR using tissue sample of CLO kit | 34 | 33 (94.3) |

DPO-PCR: Dual-priming oligonucleotide-based multiplex polymerase chain reaction; *H. pylori*: *Helicobacter pylori*; CLOtest: The rapid urease test.

its NPV was 94.7%. In CLOtest-negative cases, DPO-PCR and histology were both positive in 20.0% of the patients (7/35).

Concordance rate of DPO-PCR tests

DPO-PCR was positive in 68.5% (37/54) of the gastric biopsy samples, whereas the CLOtest kit was positive in 61.1% (33/54). The concordance rate of DPO-PCR tests between gastric biopsy samples and tissue samples analyzed by the CLOtest kit was 94.4% (51/54). There were only 2 false-positives in the gastric biopsy samples. Despite repeated histologic examinations, negative results were observed. In 1 case, both of DPO-PCR tests were all negative and there was positive histologic examination.

Resistance to clarithromycin and eradication therapy

Among the 35 patients with *H. pylori* infection, 7 patients (20.0%) had 23S rRNA point mutations associated with clarithromycin resistance. The mutation subtypes included 6 patients with A2143G and 1 patient with A2142G.

A total of 28 patients with peptic ulcer disease were recommended to undergo eradication therapy of *H. pylori*, and follow-up was incomplete in 4 patients. Twenty-four patients completed the standard 7-d eradication therapy. In the absence of a 23S rRNA point mutation in *H. pylori*, the patients were treated with PPI-based triple therapy - twice daily with 1000 mg of amoxicillin, 500 mg of clarithromycin and 30 mg of lansoprazole. If a mutation was present, the patients took metronidazole containing triple therapy, which consisted of 1000 mg of amoxicillin and 30 mg of lansoprazole twice daily and 500 mg of metronidazole 3 times daily. Eradication was determined by the C¹³-urea breath test 6 wk after the eradication therapy. *H. pylori* eradication (intention to treat) was successful in 23/28 (90.3%) patients and the per-protocol analyses is showed a rate of 95.8% (23/ 24).

DISCUSSION

Despite the highly sensitive and specific nature of PCR, it can provide false-positive or false-negative results. To reduce the risk of false-positive results in PCR, a sterilization protocol to prevent the amplification of contaminants and highly specific primers should be applied. Compared to conventional PCR, DPO-PCR increases

the specificity and sensitivity of detection by blocking non-specific binding sites; therefore, it eliminates imperfect primer annealing^[13]. On the basis of the C¹³-urea breath test, *H. pylori* detection by DPO-PCR had a sensitivity of 87.5%, a specificity of 91.3%, a positive predictive value of 84.0%, a negative predictive value of 93.3%, and an accuracy of 90.0%^[14].

However, in clinical practice, false-negative results can be a more significant problem. When the detection of *H. pylori* infection initially fails in patients with *H. pylori*-associated disease, additional biopsies and endoscopic procedures are required, which would be burdensome for clinicians and patients. Particularly in patients with recent upper gastrointestinal bleeding, the diagnosis of *H. pylori* infection can be discouraging, and its prevalence in bleeding peptic ulcers is usually underestimated^[15]. Therefore, a diagnostic test at some point after the bleeding episode would be a good tool to diagnose of *H. pylori* infection^[16,17]. From this point of view, our results are promising and the DPO-PCR test on the samples taken for RUT can reduce medical costs.

RUT is a convenient and inexpensive way to diagnosis *H. pylori* infection and is used worldwide clinically and for research. After interpretation, the biopsy specimen utilized for RUT is usually discarded. DPO-PCR is more expensive than RUT. It is highly dependent on the activity and equipment of the laboratory in which the test is performed. However, PCR tests using gastric biopsy specimens from the RUT kit can reduce the need for re-endoscopic examination with biopsy. Particularly when the RUT is negative and there is a suspicion of *H. pylori*, our method will greatly lighten the burdens of both clinicians and patients. In addition, when clarithromycin is the first-choice drug or in countries with high prevalence of primary clarithromycin resistance, our test will alleviate the social and economic costs of medical treatment.

Primary resistance to clarithromycin significantly affects the efficacy of eradication therapy and is considered to be a strong predictive factor for treatment failure^[18,19]. The eradication rate could be increased to an ideal level by conducting a test for clarithromycin resistance. The A2142G and A2143G mutations of the 23S rRNA gene in *H. pylori* are associated with clarithromycin resistance^[11,12]. Using the rapid and inexpensive DPO-based multiplex PCR test to detect clarithromycin resistance, clinicians can select the best regimen before eradication therapy. The DPO primer system differs from a conventional system by including a poly(I) linker between 2 unequal segments of primer sequences, which increases the specificity sufficiently to discriminate single-base changes by using 1-step PCR and allows accurate multiplex PCR. Therefore, there is no need for additional steps, expensive equipment, or specialized skills. In a previous clinical study, DPO-PCR was shown to be an alternative to culture and testing for clarithromycin resistance to *H. pylori*. The sensitivity of DPO-PCR was 97.7% and specificity was 83.1%, considering culture as

the reference test^[13]. Our results show that the frequency of clarithromycin resistance was 20%, but this result was not conclusive because of the small number of enrolled patients. A previous report from South Korea revealed that the antibiotic resistance rates for amoxicillin, metronidazole, and clarithromycin were 0%, 40.6%, and 5.9%, respectively, prior to 2000^[20]. However, these rates increased to 18.5%, 66.2%, and 13.8%, respectively, in 2003^[21]. Between 2003 and 2009, the resistance rates to amoxicillin and metronidazole decreased to 4.5% and 29.7%, respectively, but the resistance rate for clarithromycin increased drastically to 32.0%^[22]. The recent Maastricht III consensus report recommended that the clarithromycin not be used or that a susceptibility test be performed when the resistance to this antibiotic is $\geq 20\%$ ^[23]. Currently, DPO-based multiplex PCR can detect clarithromycin resistance before eradication therapy and help in the selection of the appropriate regimen. Hopefully, this process can prevent exposure to unnecessary antibiotics and increase the eradication rate.

Gastric biopsy specimens stored in a gel of the RUT kit can be used to confirm the diagnosis of *H. pylori* infection and to test clarithromycin susceptibility despite having been stored at room temperature for 30 d^[24]. *H. pylori* DNA can be detected by PCR on gastric biopsy specimens processed by the RUT kit. The contents of RUT are bacterial agar containing urea, phenol red (phenolsulfonphthalein), and sodium phosphate. These materials do not damage DNA. We combined the rationales for DPO-PCR and RUT and designed this study to determine the diagnostic accuracy of a DPO-PCR test using tissue specimens previously processed by a RUT kit. DNA testing is becoming a popular method of clinical diagnosis. Furthermore, DNA profiling is being used more often and can provide individual medical information. However, DNA testing can result in ethical or legal issues if informed consent is not obtained. In clinical practice, an institutional device or method to prevent inadvertent disclosure of personal information should be established.

In conclusion, our results demonstrate that DPO-based multiplex PCR using tissue samples analyzed by RUT is appropriate for detecting of *H. pylori* and clarithromycin resistance. Particularly in patients with RUT-negative results, this test could be helpful for diagnosing *H. pylori* infection. Moreover, it would be beneficial in economical aspect. Further experience and large-scale studies are needed to compare the various diagnostic methods.

COMMENTS

Background

In diagnosing *Helicobacter pylori* (*H. pylori*) infection, the CLOtest alone is unreliable. PCR has the advantage of providing diagnostic results that are highly sensitive and specific. The authors aim to investigate whether tissue samples previously processed by the CLOtest kit are suitable for dual-priming oligonucleotide-based multiplex polymerase chain reaction (DPO-PCR) to detect *H. pylori*.

Research frontier

A reliable diagnosis of infection is crucial in patients with *H. pylori*-related diseases, but there is no single test that can be considered the gold standard. Recently, a commercial DPO primer has been developed to detect SNPs using a 1-step PCR assay. It achieves accurate and rapid detection using the specific primers. Moreover, DPO-PCR provides information about clarithromycin resistance.

Innovations and breakthroughs

Authors' results demonstrate that DPO-PCR using a tissue sample previously analyzed by the CLOtest kit is appropriate for detecting *H. pylori* and clarithromycin resistance, particularly in patients with CLO-negative results.

Applications

In diagnosing *H. pylori* infection, the DPO-PCR test is accurate and economical. Further experience and large-scale studies are needed to compare the various diagnostic methods.

Terminology

DPO-PCR is a novel, commercial dual-priming oligonucleotide-based multiplex PCR method used to detect *H. pylori*.

Peer review

This manuscript investigates whether tissue samples from the CLOtest kit are suitable for DPO-PCR to detect *H. pylori*. Although it is possible that the small sample size could affect the results, the DPO-PCR test could be helpful in diagnosing *H. pylori* infection.

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Investigation of cholecystokinin receptors in the human lower esophageal sphincter

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Abstract

AIM: To compare the binding of cholecystokinin (CCK)-8 to CCK receptors in sling and clasp fibers of the human lower esophageal sphincter.

METHODS: Esophageal sling and clasp fibers were isolated from eight esophagectomy specimens, resected for squamous cell carcinoma in the upper two thirds of the esophagus, which had been maintained in oxygenated Krebs solution. Western blot was used to measure CCK-A and CCK-B receptor subtypes in the two muscles. A radioligand binding assay was used to determine the binding parameters of ³H-CCK-8S to the CCK receptor subtypes. The specificity of binding was determined by the addition of proglumide, which blocks the binding of CCK to both receptor subtypes.

RESULTS: There was no significant difference between the sling and clasp fibers of the human lower esophageal sphincter in the amount of CCK-A [integrated

optical density (IOD) value: 22.65 ± 0.642 vs 22.328 ± 1.042 , $P = 0.806$] or CCK-B receptor protein (IOD value: 13.20 ± 0.423 vs 12.45 ± 0.294 , $P = 0.224$) as measured by Western blot. The maximum binding of radio-labeled CCK-8S was higher in the sling fibers than in the clasp fibers (595.75 ± 3.231 cpm vs 500.000 ± 10.087 cpm, $P < 0.001$) and dissociation constant was lower (K_d : 1.437 ± 0.024 nmol/L vs 1.671 ± 0.024 nmol/L, $P < 0.001$). The IC_{50} of the receptor specific antagonists were lower for the CCK-A receptors than for the CCK-B ($P < 0.01$).

CONCLUSION: CCK binding modulates the contractile function of the lower esophageal sphincter through differential binding to the CCK-A receptor on the sling and clasp fibers.

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Key words: Cholecystokinins; Cholecystokinins-A receptor; Cholecystokinins-B receptor; Radioligand binding; Lower esophageal sphincter; Sling fibers; Clasp fibers

Core tip: We isolated the sling and clasp muscles which help form the human lower esophageal sphincter. The expression of cholecystokinin (CCK)-A and CCK-B receptors was measured in the two muscles. The binding of ³H-CCK-8S to the CCK receptors was studied to determine the binding characteristics of the hormone to the receptor subtypes. It is concluded that the CCK-A receptor probably plays a more important role than the CCK-B receptor in mediating the contractile function of lower esophageal sphincter, through a combination of more receptors and a stronger binding affinity.

Liu JF, Zhang J, Liu XB, Drew PA. Investigation of cholecystokinin receptors in the human lower esophageal sphincter. *World J Gastroenterol* 2014; 20(21): 6554-6559 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6554.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6554>

INTRODUCTION

The lower esophageal sphincter (LES) is the incompressible muscle bundle located at the esophagogastric junction, and includes the sling fibers from the greater curvature and clasp fibers from the lesser curvature of the stomach^[1]. The LES can open to allow liquids or solids to enter the stomach, or to permit vomiting or belching. At other times the basal tone of the LES is intended to prevent abnormal reflux of gastric contents into the esophagus^[2]. The regulation of the LES is complex, involving interplay between the nervous and hormonal systems, as well as local myogenic influences^[3,4]. In particular, gastrointestinal peptide hormones play important roles in its regulation^[5,6].

The cholecystokinins (CCK) are a family of peptide hormones which have important roles in regulating gastrointestinal motility and the delivery of nutrients to the small intestine^[7-10]. The individual members are identified by the number of amino acids in the hormone following post-translational modification of the CCK gene product, preprocholecystokinin (*e.g.*, CCK-58, CCK-8). The receptors for CCK are divided into two subtypes, CCK-A and CCK-B, based on their affinities to CCK analogues, gastrin and specific antagonists. Gastrin and CCK are similar in structure, and both bind to CCK-B receptors. We have previously shown that human sling and clasp fibers contract in response to both gastrin and CCK-8, but the sling fibers have stronger contractions than the clasp fibers^[6].

Structural and functional abnormalities of the LES may result in esophageal diseases^[11,12]. An incompetent LES permits gastro-esophageal reflux, which damages the esophageal epithelial lining and may lead to complications such as esophagitis, Barrett's esophagus or cancer^[13,14]. Currently, antireflux surgery, typically Nissen fundoplication, is the mainstay treatment to prevent reflux, but it is invasive and has a number of potential side-effects. A better understanding of the physiology of the LES may lead to medical interventions to prevent or reduce reflux, avoiding the need for surgery. In this study we investigated if the differential response of sling and clasp fibers to CCK-8 correlates with differential binding characteristics of CCK-8 to the CCK receptors in these fibers.

MATERIALS AND METHODS

Tissues

Specimens of the esophagogastric junction were obtained from 5 men and 3 women (mean age 53 years, range 45-75 years) who underwent esophagectomy for squamous cell carcinoma in the upper two-thirds of the esophagus. Patients with heartburn symptoms or motility disorders, such as achalasia of the esophagus or dermatosclerosis, were excluded from the study. Immediately after removal from the patient the esophagogastric junction tissue was placed in oxygenated Krebs' solution and transported to the laboratory. Frozen section histol-

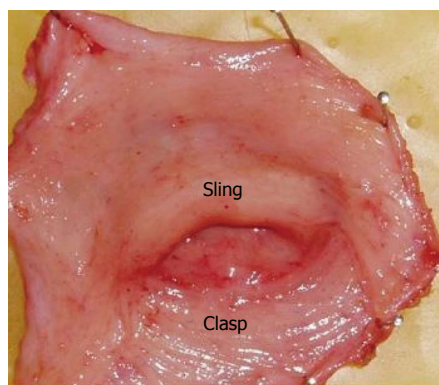


Figure 1 Arrangement of the sling and clasp muscles in the human gastroesophageal junction viewed from the distal luminal aspect after resection of the submucosa.

ogy was performed to confirm absence of tumor in the specimens. The sling and clasp fibers (Figure 1) were separated as described previously^[6]. The experimental protocol was approved by the Ethics Committee of the Fourth Hospital, Hebei Medical University.

Western blot

Membrane proteins were isolated using the Eukaryotic Membrane Protein Extraction kit (Pierce, Rockford, IL, United States), following the manufacturer's instructions. The CCK-A and CCK-B receptors in the membrane protein preparation were detected by Western blot as previously described^[15]. A goat polyclonal antibody to human CCK-A receptor (1:400 dilution) and a goat polyclonal antibody to human CCK-B receptor (Santa Cruz, United States) were each used at a dilution of 1:400. The secondary antibody was a donkey anti-goat IgG conjugated to peroxidase, used at a 1:2000 dilution and developed with DAB. The integrated optical density (IOD) of each band was determined using the Gel-Pro analyzer software package (Media Cybernetics, United States).

Binding studies

The binding studies were carried out as described by Salvatore *et al.*^[16]. The membrane protein isolate was incubated, at a final concentration of 0.15 mg/mL with serial dilutions of ³H-CCK-8S (Sigma, United States), ranging from 6.4 nmol/L to 0.05 nmol/L in a total volume of 200 μ L for 10 h at 4 $^{\circ}$ C with shaking every 30 min. The specificity of binding was determined by the addition of 5 μ mol/L proglumide, which blocks the binding of CCK to both receptor subtypes. Bound ligand was isolated by filtration under vacuum on Whatman GF/B filters which were then washed three times with ice-cold HEPES buffer (130 mmol/L NaCl, 5 mmol/L MgCl₂ and 10 mmol/L HEPES, pH 7.4). Bound radioactivity was measured by liquid scintillation counting (Model LS 6500, Beckman Instruments, Fullerton, CA, United States). Nonspecific binding, defined as the binding of radiolabelled ligand in the presence of 10 mmol/L CCK, was subtracted from the total binding measured in each assay. Competitive inhibition was determined using 7.5

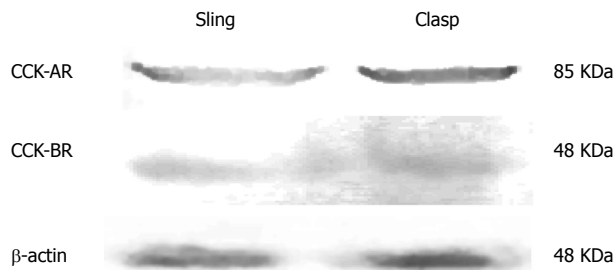


Figure 2 Western blots of the cholecystokinin-A and cholecystokinin-B receptors in the human sling and clasp muscles. CCK: Cholecystokinin.

| Table 1 Expression of cholecystokinin-A and cholecystokinin-B receptors in human sling and clasp muscles | | | |
|--|----------|----------------|---------------|
| | <i>n</i> | CCK-AR | CCK-BR |
| Clasp fibers | 8 | 22.328 ± 1.042 | 12.45 ± 0.294 |
| Sling fibers | 8 | 22.65 ± 0.642 | 13.20 ± 0.423 |
| <i>t</i> | | 0.263 | 1.439 |
| <i>P</i> value | | 0.806 | 0.224 |

The data are integrated optical densities from Western blots and are expressed as mean ± SD.

nmol/L ³H-CCK-8S with CR1409 as an antagonist to the CCK-A receptor or CR2945 to the CCK-B receptor (Sigma, United States). Data were analyzed and competitive curves constructed from the mean of triplicate measurements. The dissociation constant of the radioligand receptor complex (*K_d*) and the maximum binding value (*B_{max}*) were calculated using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, United States). The inhibitory binding constant *K_i* was calculated from the *IC₅₀* according to the Cheng-Prusoff equation, *K_i* = *IC₅₀*/(1 + *L/K_d*), where *L* is the concentration of the radioligand, *IC₅₀* is the concentration of drug causing 50% inhibition of the specific radioligand binding, and *K_d* is dissociation constant^[17].

Statistical analysis

Data are expressed as mean ± SD, and groups were compared by the paired Student's *t* test using the SPSS statistical program. Differences were considered statistically significant when *P* < 0.05.

RESULTS

Receptor expression

Both CCK-A and CCK-B receptors were measured in the membrane protein extract of the sling and the clasp muscle fibers by Western blot (Figure 2). The results in Table 1 show that the IOD for the beta-actin loading control did not differ between the fibers. There were no significant differences between the sling and clasp fibers in the IOD for the CCK-A (*t* = 0.263, *P* = 0.806) or the CCK-B (*t* = 1.439, *P* = 0.224) receptors.

Binding studies

The binding of ³H-CCK-8S to the CCK receptor was

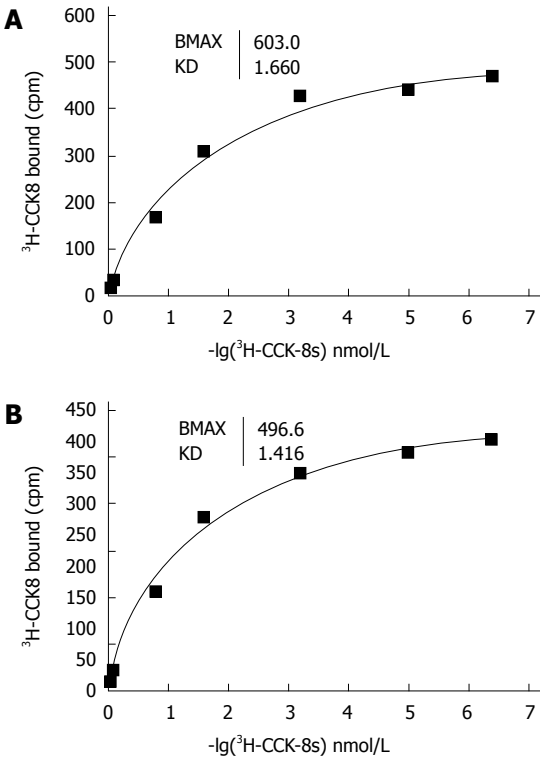


Figure 3 Representative saturation isotherms of the binding of ³H-cholecystokinin-8S to membrane proteins isolated from the human sling (A) and clasp (B) fibers. CCK: Cholecystokinin.

specific in both the sling and clasp fibers. A typical saturation isotherm plot for the binding of ³H-CCK-8S to human cell membrane protein extract is shown in Figure 3. The mean *B_{max}* and *K_d* values for all eight sling and clasp muscle preparations analysed (clasp fibers: 500.00 ± 10.09 *vs* 1.671 ± 0.024; sling fibers: 595.75 ± 3.23 *vs* 1.437 ± 0.024; *t*: 9.040 *vs* 6.898) differed significantly between the sling and the clasp fibers (each *P* < 0.001). The results in Figure 4 show the competitive inhibition curves of the specific CCK-A receptor antagonist, CR1409, and the specific CCK-B receptor antagonist, CR2945, for the binding of ³H-CCK-8S to the membrane protein extract from the sling and clasp fibers. The mean *IC₅₀* values for CR1409 and CR2945 for all eight sling and clasp muscle preparations analysed are shown in Table 2. There were no significant differences between the sling and clasp fibers in the *IC₅₀* for CR1409 (*t* = 1.72, *P* = 0.161) or CR2945 (*t* = 1.93, *P* = 0.126). In both the sling and clasp fibers the *IC₅₀* for CR1409 was significantly higher than that for CR2945 (*P* = 0.001 and *P* < 0.001 respectively). The *pK_i* values were also greater for CR1409 than for CR2945 in both the sling and clasp fibers (Table 2) (each *P* < 0.001).

DISCUSSION

The gastrointestinal hormone CCK plays an important role in the regulation of gastrointestinal motility. We have previously shown that the sling and clasp fibers, which contribute to the tone of the LES, contract in re-

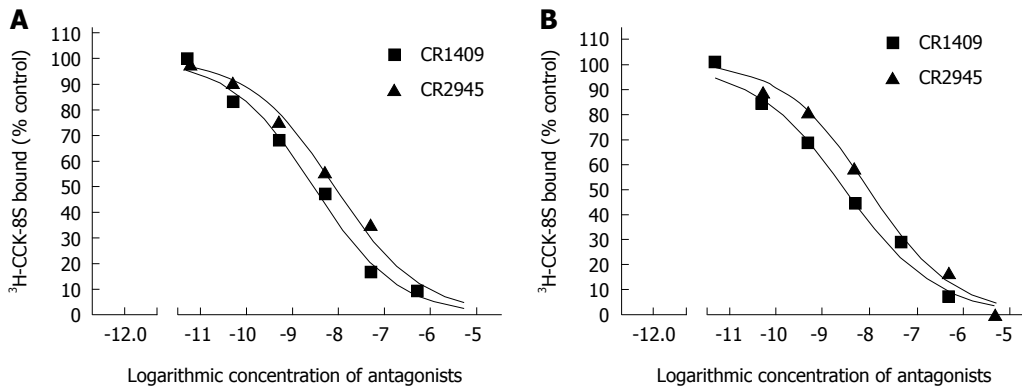


Figure 4 Representative competitive inhibition curves of the binding of ^3H -cholecystokinin-8S to membrane proteins isolated from the human sling (A) and clasp (B) fibers. The antagonists used were CR-1409 [selective for cholecystokinin (CCK)-A receptors] and CR-2945 (CCK-B receptors). Binding is expressed as the percentage of radioactivity specifically bound in the absence of antagonists.

Table 2 IC_{50} values and pK_i values

| | <i>n</i> | CR1409 | CR2945 | <i>t</i> | <i>P</i> value |
|-------------------------------|----------|-------------------|-------------------|----------|----------------|
| IC₅₀ values | | | | | |
| Clasp fibers | 8 | 3.165 ± 0.187 | 9.583 ± 0.501 | 11.99 | < 0.001 |
| Sling fibers | 8 | 2.798 ± 0.104 | 8.147 ± 0.551 | 9.53 | 0.001 |
| <i>t</i> | | 1.72 | 1.93 | | |
| <i>P</i> value | | 0.161 | 0.126 | | |
| pK_i values | | | | | |
| Clasp fibers | 8 | 8.476 ± 0.065 | 8.018 ± 0.028 | 12.69 | 0.001 |
| Sling fibers | 8 | 8.556 ± 0.022 | 8.090 ± 0.042 | 11.73 | 0.001 |
| <i>t</i> | | 1.653 | 2.754 | | |
| <i>P</i> value | | 0.156 | 0.058 | | |

The IC_{50} values and pK_i values for the CCK-A receptor antagonist CR1409 and the CCK-B receptor antagonist CR2945 in human sling and clasp fibers ($\times 10^{-9}$ mol/L; mean \pm SD).

sponse to CCK. The strength of the response differs between these fibers, with the sling fibers contracting more strongly than the clasp fibers^[6]. We report here that the binding of CCK-8 to the CCK receptor subtypes differs between these two types of fibers, which may provide an explanation for the difference in their response to the hormone.

The expression and distribution of the two CCK receptor subtypes, CCK-A and CCK-B, vary between tissues and organs, and within different parts of the same organ, within a species^[15]. CR1409 is a selective antagonist of the CCK-A receptor^[18], with concentration dependent inhibitory effects on CCK-8 mediated responses *in vivo*, such as satiety or contraction of the smooth muscle of the ileum or gallbladder^[19]. CR-2945 is a potent, selective and reversible non-peptide antagonist of CCK-B receptor. It is a candidate new generation, non-sedative anxiolytic, as well being considered a treatment for acid-related dyspeptic symptoms, where visceral motility and acid exposure play a role^[20]. We found that the IC_{50} for CR1409 was less than that for CR2945 in both the sling and clasp muscles, with the pK_i for CR1409 greater than that for CR2945. There were no significant differences in the IC_{50} of CR1409 and CR2945 between the clasp fibers and sling fibers, implying that there were no differences in the affinity of each antagonist to its

receptor between the two fibers. The difference in the B_{max} for the binding of the radiolabelled CCK-8S between the sling fibers and clasp fibers suggest that there are more CCK receptors in the sling fibers than in the clasp fibers. Our results are consistent with the ability of CCK to induce contraction of the sling and clasp fibers by binding to both receptor subtypes, with induction of stronger contractions in the sling fibers because of higher binding to the CCK-A receptor.

A number of studies show that CCK can reduce LES pressure, most commonly following a meal, when plasma levels of the hormone are significantly elevated. Boulant *et al*^[21] found in dogs that CCK infusion increased transient LES relaxations (TLESRs), and that a CCK-A receptor antagonist (but not a CCK-B receptor antagonist) prevented this increase, without affecting the basic tone of the LES. Ledeboer *et al*^[22] demonstrated in humans that the TLESRs following a fat meal, which are CCK mediated, are reduced by the CCK-A receptor antagonist lorglumide. Masclee *et al*^[23] reported that ingestion of cholestyramine in humans resulted in a reduction of LES pressure, again an effect which could be abolished by lorglumide, and similar findings following infusion of a triglyceride meal were reported by Trudgill *et al*^[11]. The postprandial reduction in LES pressure is complex, involving at least CCK and nitric oxide^[12,22,24]. Salapatek

et al.^[25] found in cats that CCK induced LES contraction through a preganglionic cholinergic mechanism involving a nicotinic synapse, but induction of relaxation occurred predominantly at a postganglionic site involving adrenergic modulation. Based on these experiments it was proposed that there is animal-to-animal variability in the balance of excitatory and inhibitory mechanisms to the LES, which determines the effect of a mediator which is capable of activating both mechanisms^[25].

The most likely explanation for the difference in action of CCK described in these reports and our study is that we studied the effect of the hormone on contraction of the isolated sling and clasp fibers *in vitro*, whereas in the other studies the LES pressure was measured *in vivo*^[25]. The location of a high pressure zone at the gastroesophageal junction and the asymmetric distribution of the pressure, as measured by esophageal manometry, are in concordance with the position and arrangement of the two bundles of fibers^[26]. Thus, while it is clear that the sling and clasp fibers contribute to the generation of the LES pressure, they are not the only muscles involved. Additionally, *in vitro* studies permit the measurement of effects due to CCK alone, whereas with *in vivo* studies it is difficult to control for all the variables which may impact on a function as complex as that which regulates LES pressure.

In conclusion, we found no difference in the amount of CCK-A or CCK-B receptors between human sling and clasp fibers, as measured by Western blot, but the sling fibers bound more radiolabelled CCK-8S than the clasp fibers. Our binding and inhibition data are consistent with the CCK-A receptor playing an important role in mediating the contractile function of the LES, acting through differential effects on the sling and clasp fibers.

COMMENTS

Background

The sling fibers of the human lower esophageal sphincter contract more strongly in response to gastrin and cholecystokinin (CCK)-8 than the clasp fibers. Authors investigate a possible explanation that the binding of CCK-8 to CCK receptor subtypes differs between these fibers.

Research frontiers

It is well known that the muscles comprising the lower esophageal sphincter have specific structural and physiological characteristics. The regulation of the lower esophageal sphincter is complex, involving an interplay between the nervous and hormonal systems, as well as local myogenic influences. In particular, gastrointestinal peptide hormones play an important role in the regulation of the sphincter. Authors have previously shown that the human sling fibers have stronger contractions than the clasp fibers in response to gastrin and CCK-8. In this study they show that this may result at least in part differences in binding of CCK-8 to the CCK receptor subtypes between the sling and clasp fibers of the human lower esophageal sphincter.

Innovations and breakthroughs

The majority of published studies on the lower esophageal sphincter have involved animals such as guinea pigs, cats, and dogs. The studies also generally involve the sphincter as a whole, with no investigation of the individual characteristics or separate contributions of the sling or clasp fibers to the function of the sphincter. This is the first report of the binding characteristics of CCK-8S to CCK receptor subtypes in isolated human sling and clasp fibers.

Applications

Binding of CCK to CCK-2 receptors modulates motility in the upper gut (including

the esophagus, lower esophageal sphincter and the stomach). CCK antagonists may have therapeutic value in the prevention or treatment of gastro-esophageal reflux disease including esophagitis, Barrett's esophagus and esophageal adenocarcinoma.

Peer review

The results are interesting and suggest that CCK binding modulates the contractile function of the lower esophageal sphincter through differential binding to the CCK-Areceptor on the sling and clasp fibers.

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Colon cancer-associated B2 *Escherichia coli* colonize gut mucosa and promote cell proliferation

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determined by PCR. Adhesion and invasion experiments were performed with I-407 intestinal epithelial cells using gentamicin protection assay. Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression in T84 intestinal epithelial cells was measured by enzyme-linked immunosorbent assay and by Western Blot. Gut colonization, inflammation and pro-carcinogenic potential were assessed in a chronic infection model using CEABAC10 transgenic mice. Cell proliferation was analyzed by real-time mRNA quantification of *PCNA* and immunohistochemistry staining of Ki67.

RESULTS: Analysis of mucosa-associated *E. coli* from colon cancer and diverticulosis specimens showed that whatever the origin of the *E. coli* strains, 86% of cyclomodulin-positive *E. coli* belonged to B2 phylogroup and most harbored polyketide synthase (*pks*) island, which encodes colibactin, and/or cytotoxic necrotizing factor (*cnf*) genes. *In vitro* assays using I-407 intestinal epithelial cells revealed that mucosa-associated B2 *E. coli* strains were poorly adherent and invasive. However, mucosa-associated B2 *E. coli* similarly to Crohn's disease-associated *E. coli* are able to induce CEACAM6 expression in T84 intestinal epithelial cells. In addition, *in vivo* experiments using a chronic infection model of CEACAM6 expressing mice showed that B2 *E. coli* strain 11G5 isolated from colon cancer is able to highly persist in the gut, and to induce colon inflammation, epithelial damages and cell proliferation.

CONCLUSION: In conclusion, these data bring new insights into the ability of *E. coli* isolated from patients with colon cancer to establish persistent colonization, exacerbate inflammation and trigger carcinogenesis.

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Key words: B2 *Escherichia coli*; Carcinoembryonic antigen-related cell adhesion molecule 6; Cell proliferation;

Abstract

AIM: To provide further insight into the characterization of mucosa-associated *Escherichia coli* (*E. coli*) isolated from the colonic mucosa of cancer patients.

METHODS: Phylogroups and the presence of cyclomodulin-encoding genes of mucosa-associated *E. coli* from colon cancer and diverticulosis specimens were

Colon cancer; Polyketide synthase genomic island

Core tip: Tumors and mucosa of patients with colon cancer are abnormally colonized by *Escherichia coli* (*E. coli*) belonging to B2 phylogroup. The aim of the present study was to provide further insight into the characterization of colon cancer-associated *E. coli*. Despite their poor ability to adhere to and to invade intestinal epithelial cells *in vitro*, we showed that colon cancer-associated *E. coli* induce carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression, a receptor involved in adhesion of pathogenic *E. coli*. These bacteria were also able to persist and promote low grade inflammation and cell proliferation, in a chronic infection model of CEACAM6 expressing mice, highlighting their oncogenic potential.

Raisch J, Buc E, Bonnet M, Sauvanet P, Vazeille E, de Vallée A, Déchelotte P, Darcha C, Pezet D, Bonnet R, Bringer MA, Darfeuille-Michaud A. Colon cancer-associated B2 *Escherichia coli* colonize gut mucosa and promote cell proliferation. *World J Gastroenterol* 2014; 20(21): 6560-6572 Available from: URL: <http://www.wjnet.com/1007-9327/full/v20/i21/6560.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6560>

INTRODUCTION

Colorectal cancer (CRC) is the fourth leading cause of cancer death and is responsible for about 610000 deaths per year worldwide^[1]. Although many etiologic genetic changes are associated with progression from adenomatous lesions to invasive carcinoma^[2], the specific causative factors in the development of sporadic CRC remain unclear. Accumulating evidence supports that inflammation and gut microbial communities influence the development of colorectal carcinoma^[3-5]. Two theories have emerged to explain the contribution of bacteria in CRC: (1) the “alpha bug” concept, wherein select members of a microbial community with virulence and pro-carcinogenic features are capable of remodeling the microbiome as a whole to drive pro-inflammatory immune responses and colonic epithelial cell transformation leading to cancer^[6]; and (2) the “driver-passenger” concept, wherein certain indigenous intestinal bacteria, termed “bacteria drivers”, initiate CRC by inducing epithelial DNA damages: the resulting tumorigenesis induces intestinal niche alterations that promote the proliferation of passenger opportunistic bacteria with a growth advantage in the tumour microenvironment^[7].

Dysbiosis of the intestinal microbiota has been observed in CRC patients. Recent pyrosequencing data of CRC-associated bacterial microbiota have revealed, in particular, over-representation of some bacteria such as *Bacteroides/Prevotella*, *Faecalibacterium* and *Fusobacterium*^[8,9]. In addition, independent studies show that colonic adenomas, carcinomas and the mucosa of CRC patients are abnormally colonized by high numbers of adherent *Es-*

cherichia coli (*E. coli*) compared to controls^[10-12]. It has been suggested that the role of *E. coli* in CRC promotion and development is related to chronic inflammation. Inflammation can result from bacterial infection, *via* its effects on both the host and the microbiota, in particular by promoting the expansion of *E. coli*, which actively contribute to the accumulation of mutations resulting from DNA damages induced by genotoxins, or by downregulating host DNA mismatch repair proteins^[3,11,13]. In particular, *E. coli* strains harboring the polyketide synthase (*pks*) genotoxic island, which are found in a significantly high percentage of inflammatory bowel disease (IBD) and CRC patients, can promote invasive carcinoma in mono-colonized azoxymethane (AOM)-treated *I10^{-/-}* mice^[3]. In addition, certain pathogenic bacteria can also be involved in cancer development, like, for example enterotoxigenic *Bacteroides fragilis* (ETBF), a common human commensal bacterium that is associated with colon cancer^[14]. ETBF-induced chronic inflammation and tumorigenesis in *Ap^{c^{Min/+}}* mice (a mouse model of familial adenomatous polyposis) involve the induction of the polyamine catabolic enzyme spermine oxidase, which causes DNA damages and uncontrolled cell proliferation in intestinal epithelial cells^[15].

Patients with IBD have an increased risk of colon cancer and small bowel adenocarcinoma^[16,17]. As in colon cancer patients, dysbiosis toward selected micro-organisms and decreased complexity of commensal bacteria have been observed in patients with Crohn's disease (CD) and ulcerative colitis (UC), but it is not clear whether dysbiosis contributes to the development of IBD or is instead a consequence of the disease. Patients with IBD, compared to healthy controls, have fewer bacteria with anti-inflammatory properties and/or more bacteria with pro-inflammatory properties. Several metagenomic-based studies reported that members of the phyla Bacteroidetes and Firmicutes were reduced in patients with CD or UC^[18-20]. Among the Firmicutes, *Faecalibacterium prausnitzii* has anti-inflammatory properties; its numbers are reduced in patients with CD and associated with a risk of post-resection recurrence of ileal CD^[20]. In contrast, a greater relative abundance of *Enterobacteriaceae*, mostly *E. coli* belonging to the B2 phylogenetic group, has been reported in CD patients more notably on mucosa-associated microbiota than in fecal samples^[10,18,21-24]. Intestinal colonization by *E. coli* correlates with bacterial adhesion of CD-associated *E. coli* strains to intestinal epithelial cells^[10,25]. CD-associated *E. coli* share abilities to adhere to and to invade intestinal epithelial cells and to survive within macrophages^[26,27] and they are termed accordingly adherent-invasive *E. coli* (AIEC). The abnormal colonization of CD mucosa by AIEC involves abnormal expression of a host receptor, the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)^[28]. Interestingly, CEACAM6 is not only abnormally expressed in the ileum of patients with CD^[28] but expression of this molecule is also up-regulated in proliferating cells in adenomas and colorectal tumors^[29,30]. However, the origin

of CEACAM6 surexpression in colon cancer is not yet clearly understood.

The aim of the present study was to provide further insight into the characterization of mucosa-associated *E. coli* isolated from the colonic mucosa of cancer patients. We determined their ability to interact with intestinal epithelial cells, with a particular focus on biofilm formation and the presence of cyclomodulin-encoding genes, and to induce CEACAM6 expression in intestinal epithelial cells. Finally, using CEABAC10 transgenic mice expressing human CEACAMs, we assessed the effects of long-term chronic infection by the colon cancer-associated *E. coli* strain 11G5 for its ability to colonize the gut, to potentiate inflammation and to induce cell proliferation.

MATERIALS AND METHODS

Ethical considerations

Ethical approval for the study was granted by the Clermont-Ferrand research ethics committee. This IRB allowed for the waiver of written consent and approved the process of obtaining verbal consent from potential subjects, because the research involved no procedures for which written consent is normally required outside of the research context and presents no risk of harm to subjects. The biological samples were collected from colon resections, which were required for the treatment of patients. The investigators explained the study to the potential subject verbally, providing all pertinent information such as purpose, procedures and putative risks. Following this verbal explanation, the potential subject was provided with a study information sheet. After allowing the potential subject time to read the study information sheet, the investigators answered any additional questions the subject may have. A verbal agreement to participate in the research was obtained for all patients included in the study. The dates of verbal consent were tracked in a non-identifiable manner.

Patients

Eighty-one patients were studied between March 2007 and July 2010 at the University hospital of Clermont-Ferrand, France, 48 with colon cancer (adenocarcinoma), and 33 with diverticulosis. For ethical considerations no surgical specimens from healthy patients were included and diverticulosis specimens were used as non-neoplastic controls. Among patients with diverticulosis, we excluded those with acute or chronic inflammation at the time of surgery, and those with stenosis to avoid potential consequences of inflammation on gut microbiota. Sex ratio (M/F) was 1.22 and 0.74 for CRC and diverticulosis patients respectively. The age range was 35-95 years for cancer patients (median age, 70 years and average age, 67 years) and 34-81 years for controls (median age, 58 years and average age, 60 years). Biopsies were taken on non-involved mucosa near the site of malignant tumors in resected colon. Pathologic analysis confirmed the neoplastic features of the samples. Bowel preparation was

by oral sodium picosulfate or oral polyethylene glycol the evening before surgery. All resection patients had received cefoxitin (2 g intravenously) at the time of incision and none had received antibiotics in the 4 wk before sampling. Ethical approval for the study was granted by the Clermont-Ferrand Research Ethics Committee.

Biopsy treatment for determination of associated *E. coli* numbers

The mucosal biopsy specimens were transported on ice to the laboratory. The samples were weighed (50 to 100 mg each) and washed thoroughly three times in 10 mL PBS to remove most of the fecal bacteria. To determine the number of associated bacteria, samples were crushed (Ultra-Turrax, IKA) and incubated for 15 minutes in the presence of 0.1% Triton X-100. Ten-fold dilutions of the lysates were then plated on Drigalski agar and chromogenic agar chromID CPS3® (bioMérieux), which allow the identification of *E. coli* isolates. Colony forming units (CFUs) of *E. coli* isolates were collected after 24 h of incubation at 37 °C and the identification of bacteria was confirmed with the automated Vitek II® (bioMérieux) system. When possible a maximum of 96 *E. coli* isolates per sample were collected for molecular typing. The bacteria were subcultured for 24 h at 37 °C in 96-well plates in Luria Bertani medium, supplemented with 15% glycerol and then stored at -80 °C.

Molecular phylogenetic grouping and PCR assay for cyclomodulin and adhesin-encoding genes

Ten isolates per sample were typed with molecular methods to identify the *E. coli* isolates (*E. coli* genotypes) colonizing the samples. Two genotyping methods were used: an “Enterobacterial Repetitive Intergenic Consensus” sequence (ERIC)-PCR using primer ERIC2 (5’ AAGTAAGTGACTGGGGTGAGCG 3’) and a “Random Amplified Polymorphic DNA” (RAPD)-PCR using primer 1283 (5’ GCGATCCCCA 3’)^[31,32]. For each isolate, one representative isolate was subsequently analysed and stored at -80 °C in Luria-Bertani medium supplemented with 15% glycerol. *E. coli* isolates were then classified according to the *E. coli* Reference Collection system into phylogenetic groups A, B1, B2, and D using a multiplex PCR technique^[33]. Strain RS218, which harbors all the genes targeted by the multiplex PCR, was used as positive control. To investigate the presence of cyclomodulin (*pks* genomic island, *cdt*, *cnf*, and *cjf*)-, adhesin (*afa*, *afa/dr* and *aaf*)-, or intimin (*eae*)-encoding genes, PCR assays were performed using primers listed in Table 1.

Cell culture

The intestinal epithelial cell lines T84 (ATCC, CCL-248) and Intestine-407 (I-407; ATCC, CCL-6) were maintained in an atmosphere containing 5% CO₂ at 37 °C in the culture medium recommended by ATCC. For infection assays, cells were seeded in 24-well plates at a density of 2 × 10⁵/cm².

Table 1 List of primers used for PCR assays

| Primer name | Sequence (5'-3') | Region specific for | Ref. |
|--------------------------|---|---------------------|------|
| afa-f | CGGCTTTTCTGCTGAAGTGGCAGGC | <i>afaC</i> | [49] |
| afa-r | CCGTCAGCCCCACGGCAGACC | | |
| afa1 | GCTGGGCAGCAAACGTATAACTCTC | <i>afaBC</i> | |
| afa2 | CATCAAGCTGTTTGTTCGTCGCCCG | | |
| afaE-f ¹ | TTAGACCGTACTGTTGTGTACCCC | | |
| | C | | |
| afaE1-r | CATCGCCCGTCGCAGAGCCCAT | <i>afaE1</i> | |
| afaE2-r | GTTTCCAGTAGACTGGAATGAAG | <i>afaE2</i> | |
| | C | | |
| afaE3-r | CCCTATTGTTGTCGCTGATCAGGAA | <i>afaE3</i> | |
| | G | | |
| daaE-r | CGGCTAGTCATATATAGATTGTGCG | <i>daaE</i> | |
| | C | | |
| afaE5-f | TCAACTCACCCAGTAGCCCCAG | <i>afaE5</i> | |
| afaE5-r | AGGAAGTGGTAGCACCGGTACG | | |
| afaE7-f | GCTAAATCAACTGTTGATGTT | <i>afaE7</i> | |
| afaE7-r | GGACAATCCAAATGGCGAATTA | | |
| afaE8-f | CTAACTTGCCATGCTGTGACAGTA | <i>afaE8</i> | |
| afaE8-r | TTATCCCTGCGTAGTGTGAATC | | |
| aggR1 | CTAATTGTACAATCGATGTA | <i>aggR</i> | |
| aggR2 | CTGAAGTAATCTGTGAA | | [50] |
| pksORF9-10.1KJ | ATTTCGATAGCGTACCCCAAC | <i>clbK-clbJ</i> | |
| pksORF9-10.2KJ | TAAGCGTCTGGAATGCAGTG | | [51] |
| CNF-1s | GGGGGAAGTACAGAAGAATTA | <i>cnf1</i> | |
| CNF-1as | TTGCCGTCCACTCTCACCAGT | | |
| CNF-2s | TATCATACGGCAGGAGGAAGCACC | <i>cnf2</i> | |
| CNF-2as | GTCACAATAGACAATAATTTCCG | | |
| CNF3-3D | TAACGTAATTAGCAAAGA | <i>cnf3</i> | |
| CNF-3as | GTCTTCATTACTACAGT | | |
| CDT-s1 | GAAAGTAAATGGAATATAAAATGTC | <i>cdtB- II ,</i> | [51] |
| | CG | <i>cdtB- III ,</i> | |
| CDT-as1 | AAATCACCAAGAATCATCCAGTTA | <i>cdtB- V</i> | |
| CDT- II as ² | TTTGTGTGCGCGCGCTGGTGAAA | <i>cdtB- II</i> | |
| CDT- III as ² | TTTGTGTGCGTGCAGCAGGGAAAA | | |
| CDT-s2 | GAAAATAAATGGAACACACATGTC | <i>cdtB- I ,</i> | |
| | CG | <i>cdtB- IV</i> | |
| CDT-as2 | AAATCTCCTGCAATCATCCAGTTA | | |
| CDT- I s | CAATAGTCGCCACAGGA | <i>cdtB- I</i> | |
| CDT- I as | ATAATCAAGAACACCACCAC | | |
| CDT- IV s | CCTGATGGTTCAGGAGGCTGGTTC | <i>cdtB- IV</i> | |
| CDT- IV as | TTGCTCCAGAATCTATACCT | | |
| P105 | GTCAACGAACATTAGATTAT | <i>cdtC- V</i> | |
| c2767r | ATGGTCATGCTTTGTTATAT | | |
| cif-int-s | AACAGATGGCAACAGACTGG | <i>cif</i> | |
| cif-int-as | AGTCAATGCTTTATGCGTCAT | | [51] |
| clbQ-F | TTGTATAGTTACACAACATATTTC | | |
| clbQ-R | CCTGTTAGCTTTCGTTTC | | |
| | | This study | |
| MiclbQaa-dA7-F | CATTAAATCATCAAATTAAC-GAATTCTATTACACAACAAGGAGT-GGGACGCACTGGCATTTAATAAC-GCGTC | | |
| MiclbQaa-dA7-R | GATGATGGAACAGCCATATCTATT-GCTCCTTGTATAGTTACACAAC-TATTTTAAATCACTTTACTTTTATC | | |
| | | | |

¹The afa-f and afa-r primers detect all *afa*-positive strains, irrespective of the *afaE* subtype and the binding properties of the adhesins (Afa/Dr+ and Afa/Dr-). Specific detection of strains encoding Afa/Dr+ adhesins was performed using afa-1 and afa-2 primers; ²Primers used with CDT-s1 primer.

Adhesion and invasion assays

I -407 cells were infected at a multiplicity of infection (MOI) of 10 bacteria per cell. Adhesion and invasion assays were performed as previously described^[27]. For adhesion assays, monolayers were washed five times in PBS after 3 h of incubation at 37 °C. To determine the numbers of intracellular bacteria (invasion assay), cell culture medium containing gentamicin at a concentration of 200 µg/mL was added for 1 h to kill extracellular bacteria. The epithelial cells were then lysed with 1% Triton X-100 in deionized water. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 min. Samples were diluted and plated onto LB agar plates to determine the number of CFU.

Biofilm formation assays

Biofilm formation assays on abiotic surface were performed using a previously described method^[34]. Biofilm measurements were calculated using the formula SBF = (AB-CW)/G, in which SBF is the specific biofilm formation, AB the OD_{570nm} of the attached and stained bacteria, CW the OD_{570nm} of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values), and G is the OD_{630nm} of cell growth in broth. Assays were performed in triplicate.

Biofilm formation assays were also performed using PFA-fixed I -407 cells^[34]. Briefly, confluent I -407 monolayers were fixed for 15 min in 3.7% PFA-PBS. The fixed cells were washed and infected with bacteria in M63 minimal medium and incubated overnight at 30 °C without shaking. For visualization, infected epithelial cells were fixed for 15 min in 3.7% PFA-PBS and permeabilized in PBS-0.1% Triton X-100. Coverslips were incubated with goat anti-*E. coli* polyclonal antibodies (dilution 1/100, AbD serotec) and Alexa 488-labeled anti-goat antibodies (dilution 1/300, Invitrogen). Actin cytoskeleton was stained using TRITC-labelled-phalloidin (Sigma). The slides were examined with a Zeiss LSM 510 Meta confocal microscope (ICCF platform, Clermont-Ferrand, France).

Mouse model infection

CEABAC10 transgenic mice (heterozygote^[35]) were housed in specific pathogen-free conditions in the animal care facility at Université d'Auvergne, Clermont-Ferrand, France). Mice from the same generation were used for experimentation. Animal protocols were approved by the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

A total of 22 female 10 wk-old CEABAC10 mice were divided into three groups: non-infected control group (*n* = 6), 11G5-infected group (*n* = 7) and AIEC LF82-infected group (*n* = 9). The animals were pretreated once before the first infection cycle by oral administration of the broad-spectrum antibiotic streptomycin (20 mg intragastric per mouse) to disrupt normal resident bacterial flora in the intestinal tract and received a dose of 0.25%

Table 2 Histological grading of intestinal inflammation

| Symptoms | Characteristics |
|---|--|
| Infiltration of inflammatory cells | |
| 0 | Rare inflammatory cells in the lamina propria |
| 1 | Increased numbers of inflammatory cells, including neutrophils in the lamina propria |
| 2 | Confluence of inflammatory cells extending into the submucosa |
| 3 | Transmural extension of the inflammatory cell infiltrate |
| Infiltration of epithelium by polynuclear cells | |
| 0 | No infiltration |
| 1 | Surface |
| 2 | Inside the crypt |
| 3 | Cryptic abscess |
| Severity of epithelial damage | |
| 0 | Absence of mucosal damage |
| 1 | Lymphoepithelial lesions |
| 2 | Mucosal erosion/ulceration |
| 3 | Extensive mucosal damage and extension through deeper structures of the bowel wall |
| Surface of epithelial damage | |
| 0 | Normal |
| 1 | Focal |
| 2 | Wide |

Table 3 List of primers used for RT qPCR assays

| Primer name | Sequence (5'-3') | Region specific for | Ref. |
|-------------|-----------------------|---------------------|------------|
| mmu-26s-FW | TGTCATTTCGGAACATTGTAG | S26 | This study |
| mmu-26s-RV | GGCTTTGGTGGAGGTC | | |
| mmu-PCNA-FW | CCACATTGGAGATGCTGTG | PCNA | |
| mmu-PCNA-RV | CAGTGGAGTGGCTTTTGTGA | | |

(wt/vol) of dextran sulfate sodium (DSS; molecular mass = 36000-50000 daltons; MP Biomedicals) in drinking water 3 d before infection to increase the accessibility of bacteria to the surface of the epithelial layer. The administration of 0.25% DSS did not affect the body weight of mice and did not induce clinical symptoms of colitis^[36]. The mice were subjected to 8 consecutive cycles of infection. For each infection cycle, they were orally challenged twice a week by intra-gastric gavage with 2×10^8 bacteria for a 3-wk period. This infection period was followed by a 1-wk recovery period without infection. For each cycle, 5 d after the last oral bacterial infection, fresh fecal pellets (100-200 mg) were collected and suspended in PBS to evaluate colonization. After serial dilution, bacteria were enumerated by plating on LB agar medium containing 50 µg/mL of kanamycin and 50 µg/mL of ampicillin isolate 11G5 bacteria or 100 µg/mL of ampicillin and 20 µg/mL of erythromycin to isolate LF82 bacteria, and incubated at 37 °C overnight.

Histological grading of intestinal inflammation and epithelial damages

After mouse sacrifice, the entire colon was excised and rolls of the proximal colon were fixed in buffered 4% formalin, paraffin-embedded, cut into 5-µm slices, and stained with hematoxylin/eosin/safranin. The histological severity of colitis was graded in a blinded fashion by a GI pathologist. The tissue samples were assessed for

the extent and depth of inflammation and the extent of epithelial damages, as presented in Table 2. The histology score corresponds to the sum of each item.

Immunohistochemistry

For immunohistochemical staining of mouse Ki-67, heat-induced epitope retrieval was performed using sodium citrate buffer (pH 6.0). Ki-67 antigen was detected using anti-mouse Ki-67 polyclonal antibodies (Leica) and revealed with Vectastain ABC kit (Vector) and DAB detection kit (Invitrogen). The sections were counterstained using Gill's hematoxylin (Vector).

Real-time mRNA quantification

Total RNAs were extracted from tissue using a Nucleospin® RNA/Protein extraction kit (Macherey-Nagel GmbH & Co). RNA samples were subjected to reverse transcription using High-Capacity cDNA Reverse Transcription Kit and non-specific random hexamer primers (Applied Biosystems) and quantification was performed using FastStart SYBR® Green Master kit (Roche Applied Science). The primer sequences used are given in Table 3. Gene expression values were calculated based on the $\Delta\Delta C_t$ method.

Enzyme-linked immunosorbent assay

T84 colon epithelial cells were infected with bacteria for 6h at a MOI of 100 bacteria per cell. The amount of CEACAM6 on whole cell protein extracts was determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (R and D systems).

Western immunoblotting

T84 colon epithelial cells were infected for 6h at a MOI of 100 bacteria per cell. Whole-cell protein extracts were prepared by adding NP-40 lysis buffer. Protein concen-

Table 4 Distribution of *Escherichia coli* strains producing various cyclomodulins according to phylogroups and specimen origins *n* (%)

| Phylogroups | | <i>E. coli</i> strains exhibiting cyclomodulin-encoding genes | | | |
|--|---------------------|---|------------|------------|------------|
| | | <i>pks</i> | <i>cnf</i> | <i>cdt</i> | <i>cif</i> |
| Colon cancer-associated <i>E. coli</i> strains (<i>n</i> = 88) ¹ | A (<i>n</i> = 20) | 0 (0) | 0 (0) | 0 (0) | 2 (2) |
| | B1 (<i>n</i> = 14) | 0 (0) | 1 (1) | 1 (1) | 1 (1) |
| | B2 (<i>n</i> = 38) | 23 (26) | 16 (18) | 4 (11) | 0 (0) |
| | D (<i>n</i> = 16) | 0 (0) | 1 (1) | 1 (1) | 0 (0) |
| Diverticulosis-associated <i>E. coli</i> strains (<i>n</i> = 46) ² | A (<i>n</i> = 17) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | B1 (<i>n</i> = 3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| | B2 (<i>n</i> = 15) | 6 (13) | 4 (9) | 0 (0) | 0 (0) |
| | D (<i>n</i> = 11) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

¹Isolated from 48 colon cancer patients; ²Isolated from 33 patients with diverticulosis. *E. coli*: *Escherichia coli*.

Table 5 Phylogroup distribution of cyclomodulin-positive *Escherichia coli* strains according to specimen origins *n* (%)

| | Phylogroups | | | |
|---|-------------|-------|---------|-------|
| | A | B1 | B2 | D |
| Cyclomodulin-positive <i>E. coli</i> strains isolated from colon cancer patients | 2 (6) | 2 (6) | 26 (84) | 1 (3) |
| Cyclomodulin-positive <i>E. coli</i> strains isolated from patients with diverticulosis | 0 (0) | 0 (0) | 6 (100) | 0 (0) |
| Cyclomodulin-positive <i>E. coli</i> strains | 2 (5) | 2 (5) | 32 (86) | 1 (3) |

E. coli: *Escherichia coli*.

trations were determined by Bradford assay. Total proteins were subjected to SDS-PAGE on 12% gels.

Proteins were electroblotted onto nitrocellulose membranes (Amersham International), and the membranes were immunoblotted for CEACAM6 (mouse anti-CEACAM6, dilution 1/1.000, Genovac) and GAPDH (rabbit anti-GAPDH; dilution 1/1.000, Cell Signaling). Immunoreactants were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibodies, ECL reagents (Amersham Biosciences) and autoradiography.

Statistical analysis

Where appropriate, nonparametric data were expressed by median value (range). Normally distributed data were expressed as means. Error bars represent SEM. Statistical analysis was done using ANOVA (Histopathological score and RT-qPCR), Mann Whitney (adhesion, invasion and biofilm assays) using GraphPad prism5 software. A *P* value of 0.05 was considered significant.

RESULTS

Most cyclomodulin-producing *E. coli* strains associated with colon cancer and diverticulosis belong to B2 phylogroup

The analysis of the presence of cyclomodulin-encoding genes, *pks* island coding for colibactin, and/or *cnf* and/or *cdt* and/or *cif* genes coding for cytotoxic necrotizing factor (CNF), cytolethal distending toxin (CDT), and cycle-inhibiting factor (Cif) indicated that, whatever the origin of the *E. coli* strains, either from colon cancer or diverticulosis samples, 86% of cyclomodulin-positive *E. coli* belonged to B2 phylogroup (Tables 4 and 5). Among *E. coli* strains isolated from colon cancer specimens, B2 *E. coli*

strains harboring *pks*, *cnf* and *cdt* genes represented 26%, 18% and 11% of the total strains isolated, respectively (Tables 4 and 6). Among *E. coli* strains isolated from patients with diverticulosis, *pks*-positive B2 *E. coli* strains represented 13%, and *cnf*-positive B2 *E. coli* strains 9% of the total strains isolated. Although a higher prevalence of B2 *E. coli* strains harboring *pks* or *cnf* genes was observed in colon cancer patients than in patients with diverticulosis, this was not significant (*P* = 0.06 for both *pks* and *cnf* genes). Of interest, all but two *cnf* positive strains also harbored *pks* and all *cnf*- and *pks*-positive *E. coli* strains belonged to the B2 phylogroup.

Low level of adhesion and invasion but high ability to form biofilm of B2 *E. coli* strains isolated from colon cancer or diverticulosis patients

The analysis of the ability of *E. coli* strains to adhere to and to invade intestinal epithelial cells was restricted to B2 *E. coli*, which were the main cyclomodulin producers in our study. Of note, due to cytolytic activity of hemolysin on cultured cells, hemolysin-positive *E. coli* strains were not tested. Results showed that B2 phylogroup *E. coli* strains isolated from colon cancer and from diverticulosis displayed low levels of adhesion to I-407 intestinal epithelial cells (Figure 1A). Compared to the adhesion level of the AIEC reference strain LF82, for which a mean adhesion index of 53.23 ± 6.63 was observed, the adhesion levels of all *E. coli* strains isolated from colon cancer (except *E. coli* strain 14H4, which had a mean adhesion level of 25.76 ± 5.06) or from diverticulosis ranged from 0.15 ± 0.02 to 4.04 ± 1.24 or from 0.10 ± 0.04 to 9.17 ± 3.40 , respectively. Microscopy examination after Giemsa staining showed a diffuse adhesion pattern (data not shown), and we therefore searched for adhesive factor-encoding genes associated with diffusely adhering

Table 6 Hemolysin expression and presence of cyclomodulin- and adhesin-encoding genes in B2 phylogroup *Escherichia coli* strains

| <i>E. coli</i> strains | Haemolytic phenotype ¹ | Cyclomodulin-encoding genes | | | | Adhesin-encoding genes | | |
|------------------------|-----------------------------------|-----------------------------|-------------|----------------|------------|------------------------|--------------------|-------------|
| | | <i>pks</i> | <i>cnf</i> | <i>cdtB</i> | <i>cif</i> | <i>afa</i> | <i>dra</i> | <i>aagR</i> |
| Colon cancer | | | | | | | | |
| 1C12 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 1D2 | - | + | - | - | - | - | - | - |
| 1F8 | - | - | - | - | - | - | - | - |
| 2D5 | - | - | - | - | - | - | - | - |
| 2F8 | + | + | <i>cnf1</i> | <i>cdtB-IV</i> | - | - | - | - |
| 2G2 | + | - | - | - | - | - | - | - |
| 4A9 | - | - | - | - | - | - | - | - |
| 6A8 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 6G8 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 6G10 | - | - | - | <i>cdtB-IV</i> | - | - | - | - |
| 6G11 | - | - | - | - | - | - | - | - |
| 7G1 | - | - | - | - | - | - | - | - |
| 7G2 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 8A9 | + | + | - | - | - | - | - | - |
| 8A10 | - | - | - | - | - | - | - | - |
| 8F1 | + | - | <i>cnf1</i> | - | - | - | - | - |
| 8G8 | - | - | - | - | - | - | - | - |
| 9G5 | + | + | <i>cnf1</i> | - | - | + | - | - |
| 10D12 | - | + | - | - | - | - | - | - |
| 10E9 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 11F1 | - | - | - | - | - | - | - | - |
| 11G5 | - | + | - | - | - | - | - | - |
| 12B1 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 13H2 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 14H4 | - | + | - | - | - | + | + (<i>afaE5</i>) | - |
| 15D1 | + | - | - | - | - | - | - | - |
| 15D3 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 16C1 | - | + | - | - | - | - | - | - |
| 17G3 | + | - | <i>cnf1</i> | - | - | - | - | - |
| 18C3 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 18C5 | - | - | - | - | - | - | - | - |
| 18H5 | - | + | - | - | - | - | - | - |
| 19D12 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 19G1 | - | + | - | - | - | - | - | - |
| 19H2 | + | + | <i>cnf1</i> | <i>cdtB-IV</i> | - | - | - | - |
| 20B6 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 20C3 | - | + | - | <i>cdtB- I</i> | - | - | - | - |
| 20D5 | - | - | - | - | - | - | - | - |
| Diverticulosis | | | | | | | | |
| 1D5 | - | - | - | - | - | - | - | - |
| 4D5 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 9D7 | - | - | - | - | - | - | - | - |
| 9F1 | - | - | - | - | - | - | - | - |
| 9F4 | - | - | - | - | - | - | - | - |
| 11D9 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 12H1 | - | + | - | - | - | - | - | - |
| 13D1 | + | + | <i>cnf1</i> | - | - | - | - | - |
| 15C1 | - | - | - | - | - | - | - | - |
| 16A4 | - | + | - | - | - | - | - | - |
| 16A8 | - | - | - | - | - | - | - | - |
| 17C1 | - | - | - | - | - | - | - | - |
| 17F2 | - | - | - | - | - | - | - | - |
| 17E1 | - | - | - | - | - | - | - | - |
| 18E6 | + | + | <i>cnf1</i> | - | - | - | - | - |

¹Alpha-hemolysin expression was analysed after over-night growth of bacteria on agar plates containing sheep blood. *cnf*: Cytotoxic necrotizing factor; *cdt*: Cytolethal distending toxins; *cif*: Cycle-inhibiting factor; *afa*: Afimbrial adhesion. *E. coli*: *Escherichia coli*.

E. coli (DAEC) strains (*i.e.*, Afa and Afa/Dr adhesin-encoding genes). None of the B2 *E. coli* strains tested was positive for *afa* or *afa/dr* genes except the highly adherent *E. coli* strain 14H4 isolated from colon cancer (Table 6). Of note, none of the B2 *E. coli* strains tested was positive

for *eae* gene coding for intimin of enteropathogenic *E. coli* or for *aaf* gene coding for the adhesive factor AAF of enteroaggregative *E. coli*, indicating that B2 *E. coli* strains studied do not belong to these *E. coli* pathovars. Analysis of the ability of bacteria to invade I-407 cells showed

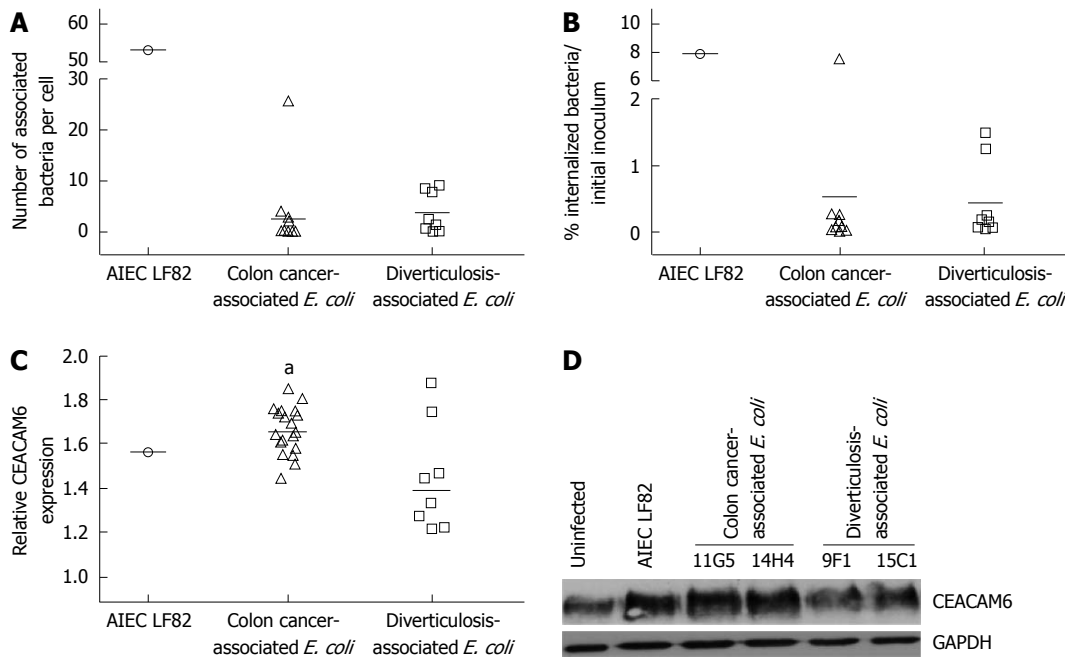


Figure 1 Adhesion, invasion and ability to induce carcinoembryonic antigen-related cell adhesion molecule 6 expression of B2 *Escherichia coli* strains. A and B: Ability of colon cancer- and diverticulosis-associated B2 *Escherichia coli* (*E. coli*) strains and AIEC strain LF82 to adhere to and to invade I-407 intestinal epithelial cells. A: Adhesion. Results are expressed as number of associated bacteria per cell after 3 h of infection; B: Invasion. Results are expressed as percentage of inoculum surviving after 3 h of infection and 1 h of gentamicin treatment; C and D: Induction of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression in colon epithelial T84 cells infected for 6 h with colon cancer- and diverticulosis-associated B2 *E. coli* strains and AIEC strain LF82. C: Quantitative dosage of CEACAM6 by ELISA. Results are expressed as amounts of CEACAM6 in stimulated or infected cells relative to untreated cells. ^a $P \leq 0.05$ vs diverticulosis associated *E. coli*. D: CEACAM6 expression analysis by Western Blot. *E. coli* strains 11G5 and 14H4 were isolated from colon cancer patients and *E. coli* strains 9F1 and 15C1 from patients with diverticulosis.

that whatever the origin of the B2 *E. coli* strains their invasion levels were very low, ranging from 0.02% to 1.49%, except strain 14H4, for which invasion level was similar to that of the AIEC strain LF82 (Figure 1B).

We also investigated the ability of B2 *E. coli* isolated from colon cancer to induce CEACAM6 expression as abnormal CEACAM6 expression was shown to promote gut colonization by AIEC^[36] and AIEC bacteria were reported to be able to induce increased CEACAM6 expression in intestinal epithelial cells^[28]. A quantitative analysis of the level of CEACAM6 expression by T84 intestinal epithelial cells in response to B2 *E. coli* infection was determined by ELISA (Figure 1C) and Western blot (Figure 1D). Interestingly, we observed that most of B2 *E. coli* strains isolated from colon cancer induced increased expression of CEACAM6 to a level similar to that of AIEC strain LF82. Of note, B2 *E. coli* strains isolated from diverticulosis induced no or very low expression of CEACAM6 in T84 cells.

Another important bacterial trait involved in the colonization of the intestinal mucosa by gut resident bacteria is their ability to form biofilm. This property was investigated both on abiotic and on fixed intestinal epithelial cells. The level of biofilm formation on abiotic surface was evaluated by calculating the specific biofilm formation index (SBF). An SBF index of 3.13 ± 0.23 was obtained for AIEC strain LF82 compared to 0.99 ± 0.22 for the non-pathogenic K-12 *E. coli* strain C600 (Figure 2A). We observed that 7/19 (37%) B2 *E. coli* strains isolated

from colon cancer and 2/8 (25%) B2 *E. coli* strains isolated from diverticulosis harbored SBF index similar to that of the biofilm producer AIEC strain LF82 ($P \geq 0.05$). Biofilm formation on fixed I-407 intestinal epithelial cells was evaluated by confocal microscopy (Figure 2B), which confirmed that 6/9 B2 *E. coli* strains having a high SBF index on abiotic surface were able to form a strong biofilm on fixed I-407 cultured cells. Combining the two methods of biofilm formation assessment, 16/27 B2 *E. coli* strains tested were able to form biofilm. This shows that even B2 *E. coli* strains have a low ability to adhere to intestinal epithelial cells, at least half of them were able to form biofilm to a level similar to that of CD-associated *E. coli* strain LF82 known to form a strong biofilm and no difference was observed between B2 *E. coli* strains isolated from colon cancer patients or from patients with diverticulosis.

Colonization of colon mucosa in CEACAM-expressing mice by colon cancer-associated B2 *E. coli* strain 11G5: induction of inflammation and enhanced epithelial intestinal cell proliferation

CEABAC10 mice harboring a bacterial artificial chromosome that contains part of the human CEA family gene cluster including the *CEACAM6* gene were infected with AIEC reference strain LF82 or B2 *E. coli* strain 11G5 isolated from colon cancer patient. To assess bacterial colonization, the levels of bacteria in the stools were determined 5 d after the last infection of each cycle, over the

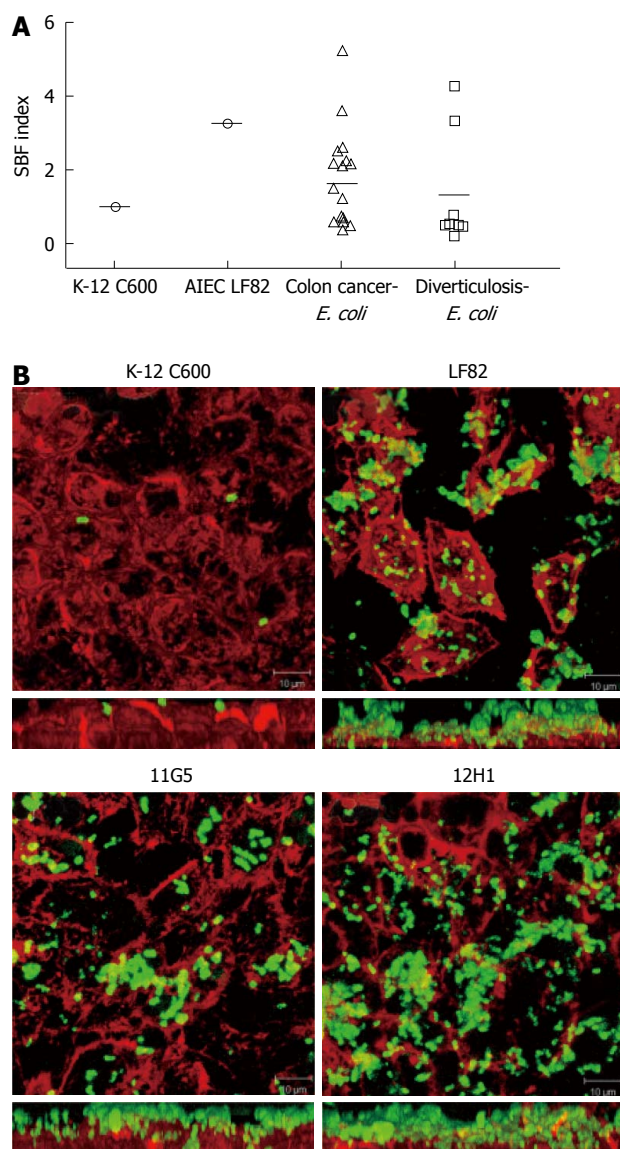


Figure 2 Ability of B2 *Escherichia coli* strains to form biofilm. Biofilm formation of colon cancer-associated and diverticulosis-associated B2 *Escherichia coli* (*E. coli*) were compared to that of the non-pathogenic K-12 *E. coli* strain C600 and the biofilm producer AIEC strain LF82. A: Biofilm formation on abiotic surface. Results are expressed as specific biofilm formation (SBF) index; B: Biofilm formation on human I-407 intestinal epithelial cells. *E. coli* strain 11G5 was isolated from a patient with colon cancer and *E. coli* strain 12H1 from a patient with diverticulosis. Bacteria were stained using goat anti-*E. coli* polyclonal antibodies (green) and I-407 cells were labeled for actin cytoskeleton using TRITC-labeled phalloidin (red). Y- and Z-stack projections are presented.

8 consecutive cycles of infection. Analysis of the number of bacteria recovered in the stools at cycle 4 and cycle 8 revealed similar ($P = 0.78$) colonization levels for mice infected with AIEC LF82 and colon cancer-associated 11G5 bacteria (Figure 3A).

On macroscopic examination, no sign of tumor development such as neoplasia or polyp was observed in the colon of mice infected with AIEC strain LF82 or colon cancer-associated 11G5 *E. coli* strain. Histological analysis showed a similar colonic histological score for inflammation and epithelial damages for mice infected with AIEC strain LF82 and *E. coli* strain 11G5 ($P \geq 0.05$)

(Figure 3B). Mice infected with *E. coli* strains LF82 and 11G5 exhibited infiltration of polynuclear cells in crypts, larger numbers of crypt abscesses and large and multifocal erosion plates (Figure 3C).

The level of *proliferating cell nuclear antigen* (PCNA) mRNA was measured in the colonic mucosa of infected mice to determine the proliferative index (Figure 3D). Significant ($P \leq 0.05$) 2.5-fold and 2.9-fold increases in PCNA mRNA levels were observed in the colonic mucosa of mice infected with the *E. coli* strain 11G5 compared to those of control mice or mice infected with AIEC strain LF82, respectively. This finding was confirmed by Ki67 immunostaining on colonic mucosa tissue. 11G5-infected mice had higher numbers of proliferative epithelial cells in crypts than control mice and mice infected with AIEC strain LF82 (Figure 3E). This indicates that colonic mucosa cells undergo accelerated proliferation in response to infection by B2 *E. coli* strain 11G5 associated with colon cancer.

DISCUSSION

Accumulating evidence supports the involvement of infectious agents in the development of cancer, especially in organs that are continuously exposed to microorganisms such as the colon. Remodeling of the colonic microbiota due to environmental changes is thought to contribute to the pathogenesis of colon cancer by suppressing the growth of cancer-protective bacterial species and allowing the emergence or expansion of bacterial species with oncogenic potential. It has been suggested that the role of *E. coli* in CRC promotion and development is linked to chronic inflammation, which can result from bacterial infection *via* its effects on both the host and the microbiota, in particular that of promoting the expansion of certain bacteria, such as pro-inflammatory *E. coli*^[37] or ETBF^[38,39]. In parallel, two different studies have reported that between 71% and 82% of patients with colonic adenoma or carcinoma^[10,12] are highly colonized by mucosa-associated *E. coli* compared to controls. The aim of the present study was to provide further insight into the characterization of the *E. coli* colonizing the mucosa of colon cancer patients.

It is well documented that B2 *E. coli* harbors genes coding for cyclomodulins such as colibactin, which is encoded by the *pks* genomic island, CDT, CNF or Cif, which can act as genotoxic agents and/or can modulate cellular differentiation, apoptosis, and proliferation^[13,40,41]. In the present study, we observed that 86% of cyclomodulin-positive *E. coli* isolated from colon cancer and diverticulosis specimens belonged to B2 phylogroup. Of interest, all but two *cnf* positive strains also harbored *pks* and all *cnf*- and *pks*-positive *E. coli* strains belonged to the B2 phylogroup. Our results are in good agreement with those reported by Arthur *et al.*^[3], who observed that 66.7% of patients with CRC and 20.8% of controls harbored *pks*-positive *E. coli*.

E. coli strains belonging to B2 phylogroup have a

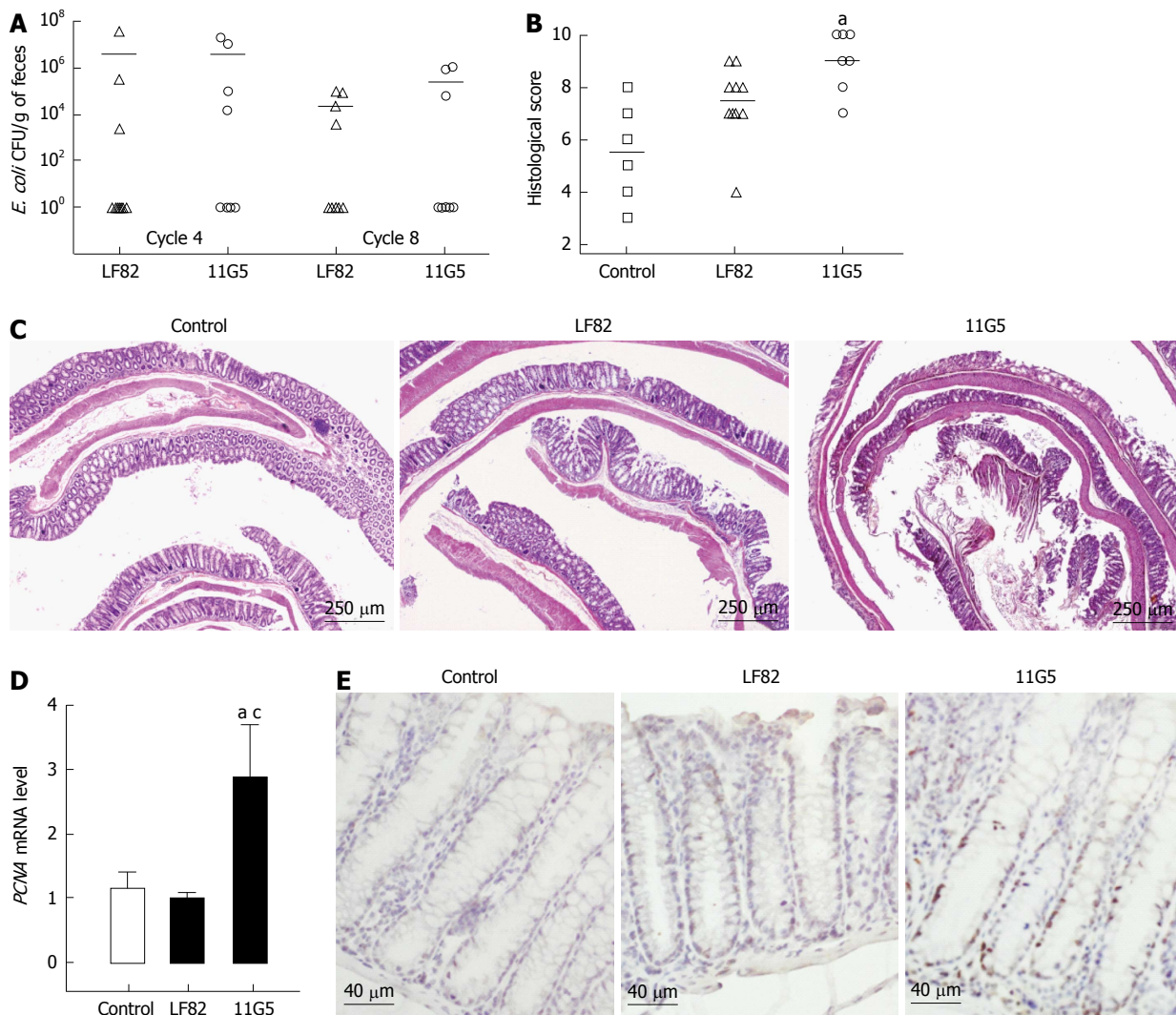


Figure 3 Impact of CEABAC10 mice colonization by B2 *Escherichia coli* strain on inflammation and cell proliferation. CEABAC10 mice transgenic for human CEACAMs, including CEACAM6, were subjected to 8 consecutive cycles of infection with AIEC LF82 or B2 phylogroup *Escherichia coli* (*E. coli*) strain 11G5. Control mice received PBS. A: Quantification of the number of bacteria in the feces of mice at cycle 4 and cycle 8; B: Histopathological scoring for several parameters of inflammation and epithelial damages (see Table 2) was performed at the end of the 8th cycle. ^a $P \leq 0.05$ vs control; $P = \text{NS}$ vs LF82; C: Hematoxylin/eosin/safran (HES) staining of colonic tissue sections; D: Total RNAs from colons were extracted at the end of the 8th cycle. PCNA and S26 mRNA levels were measured by RT-qPCR. PCNA amount relative to S26 is presented. ^a $P \leq 0.05$ vs control; ^c $P \leq 0.05$ vs LF82; E: Immunohistochemistry examination of Ki67 on colonic tissue sections. NS: Not significant.

greater ability to colonize the human gut, due, at least in part, to accumulation of genes encoding fitness factors such as pili and adhesins^[42,43]. In addition, an increased proportion of mucosa-associated *E. coli* expressing hemagglutinins was observed in CD patients (39%) and colon cancer patients (38%) compared to controls (4%), in correlation with the ability of bacteria to adhere to I-407 and HT-29 intestinal epithelial cells^[10]. However in our study, analysis of the adhesive abilities of B2 *E. coli* isolated from colon cancer or diverticulosis revealed that the strains were poorly adherent to I-407, even if the majority of them were able to form biofilm. However some B2 *E. coli* strains isolated from colon cancer induced increased expression of CEACAM6 to a level similar to that of AIEC strain LF82 associated with CD, indicating that colon cancer-associated *E. coli* could influence carcinogenesis, since CEACAM6 has been implicated in cel-

lular adhesiveness, invasiveness, and metastatic behavior of tumor cells^[30,44]. In addition, this result indicates that, in agreement with what we previously reported for AIEC strains isolated from CD patients^[28], colon cancer-associated *E. coli* strains could have the ability to promote their own colonisation since CEACAM6 serves as a receptor for mediating adherence and/or cell entry of pathogenic bacteria such as *Neisseria* bacteria^[45], diffusely-adhering *E. coli* (DAEC)^[46] or AIEC^[28].

Experiments of long-term colonization of CEABAC10 mice revealed that an *E. coli* strain isolated from colon cancer (strain 11G5) was able to persist in the gut of CEABAC10 transgenic mice expressing human CEACAMs, including CEACAM6 and to exacerbate colonic inflammation. Whether colonisation of the intestinal mucosa of colon cancer patients by B2 *E. coli* is a cause or a consequence of malignant transformation is a ques-

tion that has yet to be addressed. We show here that B2 phylogroup *E. coli* isolated from colon cancer increased the proliferative index of epithelial cells in crypts in the chronic infection model of CEABAC10 mice. This indicates that colonic mucosa cells undergo accelerated proliferation in response to infection by B2 *E. coli*. The ability to induce cell proliferation is a common trait of various pathogens involved in carcinogenesis. Indeed, *Bacteroides fragilis* enterotoxin induces *c-myc* transcription and translation and persistent cellular proliferation ensues, mediated in part by β -catenin/T-cell factor-dependent transcriptional activation^[47]. Another example is *Helicobacter pylori* (*H. pylori*), which increases the proliferation of gastric cancer cells. This process is dependent on the LPS-TLR4 pathway since *H. pylori* LPS induces the proliferation of gastric cancer cells and the use of neutralizing antibody against TLR4 almost completely abrogates the proliferative activities of cancer cells^[48]. Some cyclomodulins, such as CNF, which are mostly produced by B2 *E. coli*, induce epithelial cell proliferation^[49]. In our study the B2 *E. coli* strain 11G5 did not harbor the *cnf* genes and was able to promote cell proliferation as observed in infected CEABAC10 mice. This effect could be related to *E. coli*-derived LPS, which was previously reported to have a more remarkable cancer proliferative activity than *H. pylori*-derived LPS^[48]. Because *E. coli* inhabits the host colon as normal intestinal flora, owing to host tolerance toward *E. coli*, it is likely that *E. coli* LPS stimulates the host cellular immune response to prevent cancer progression. However, we can hypothesize that when too great a load of *E. coli* colonize the colonic mucosa, as observed in 11G5-infected CEABAC10 mice, potent tumor proliferative activity is no longer effectively repressed. The cell proliferation observed in 11G5-infected CEABAC10 mice could also result from the presence of colibactin. Colibactin with its genotoxic activity promotes DNA damages, which leads to carcinogenesis and cell proliferation.

In conclusion, B2 *E. coli* abnormally colonized the mucosa of colon cancer patients, indicating that microbiota remodeling had occurred promoting their expansion. Together with previous findings reported by Arthur *et al.*^[3], this study on a larger cohort of patients confirms the high prevalence of B2 *pks*-positive or *pks-cnf*-positive *E. coli* in colon cancer patients. The study also indicates that, these bacteria can promote low grade inflammation and cell proliferation, as shown in the CEABAC10 infected mouse model. Analyses to determine whether these bacteria take advantage of the tumor microenvironment to colonize the gut or promote their own colonization may be an important step in understanding their role in carcinogenesis and in the development of therapeutic strategies.

ACKNOWLEDGMENTS

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COMMENTS

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, and is the fourth leading cause of cancer death worldwide. Inflammation and changes in composition and function of gut microbial communities are suspected to be causative factors in the development of sporadic CRC.

Research frontiers

The authors and other researchers have reported abnormal colonization of tumors and mucosa of colon cancer patients by *Escherichia coli* (*E. coli*) belonging to B2 phylogroup.

Innovations and breakthroughs

To date, there has been a limited number of studies analyzing interaction of colon cancer-associated *E. coli* to intestinal epithelial cells. The authors showed that colon cancer-associated *E. coli* induce expression of the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) receptor in intestinal epithelial cells, and that these bacteria were able to persist in a chronic infection model of CEACAM6 expressing mice and had oncogenic potential.

Applications

The authors have analyzed the ability of colon cancer-associated *E. coli* to colonize gut mucosa and influence carcinogenesis. Analyses to determine whether these bacteria take advantage of the tumor microenvironment to colonize the gut or promote their own colonization may be an important step in understanding their role in carcinogenesis and in the development of therapeutic strategies.

Terminology

CEACAM6 molecule serves as a receptor for mediating mucosa colonization by pathogenic bacteria.

Peer review

This study provides evidence supporting the hypothesis that colon cancer-associated *E. coli* are able to colonize gut mucosa and to induce cell proliferation in a mouse model with overexpression of human CEACAM6.

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Role of cystatin C and renal resistive index in assessment of renal function in patients with liver cirrhosis

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Abstract

AIM: To evaluate the clinical significance of cystatin C and renal resistive index for the determination of renal function in patients with liver cirrhosis.

METHODS: We conducted a study of 63 patients with liver cirrhosis. A control group comprised of 30 age and gender-matched healthy persons. Serum cystatin C was determined in all study subjects and renal Doppler ultrasonography was made. Estimated glomerular filtration rate from serum creatinine (GFR_{Cr}) and cystatin C (GFR_{Cys}) was calculated.

RESULTS: We confirmed significant differences in val-

ues of cystatin C between patients with different stages of liver cirrhosis according to Child-Pugh ($P = 0.01$), and a significant correlation with model of end stage liver disease (MELD) score ($r_s = 0.527$, $P < 0.001$). More patients with decreased glomerular filtration rate were identified based on GFR_{Cys} than on GFR_{Cr} ($P < 0.001$). Significantly higher renal resistive index was noted in Child-Pugh C than in A ($P < 0.001$) and B stage ($P = 0.001$). Also, a significant correlation between renal resistive index and MELD score was observed ($r_s = 0.607$, $P < 0.001$). Renal resistive index correlated significantly with cystatin C ($r_s = 0.283$, $P = 0.028$) and showed a negative correlation with GFR_{Cys} ($r_s = -0.31$, $P = 0.016$).

CONCLUSION: Cystatin C may be a more reliable marker for assessment of liver insufficiency. Additionally, cystatin C and renal resistive index represent sensitive indicators of renal dysfunction in patients with liver cirrhosis.

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Key words: Liver cirrhosis; Cystatin C; Renal resistive index

Core tip: Early diagnosis of renal dysfunction is important to prevent serious complications. We conducted a study of 63 patients with liver cirrhosis and 30 healthy controls. Serum cystatin C (CysC) was measured and renal Doppler ultrasonography was performed. More patients with decreased glomerular filtration rate (GFR) were identified based on CysC GFR than on creatinine GFR. Higher renal resistive index in advanced disease by Child-Pugh and model of end stage liver disease was noticed. Renal resistive index (RRI) correlated with CysC and negatively correlated with glomerular filtration rate from serum cystatin C. Cystatin C may be a more reliable marker for liver insufficiency assessment. CysC and RRI represent sensitive indicators of renal dysfunction in liver cirrhosis.

Ćulafić Đ, Štulić M, Obrenović R, Miletić D, Mijač D, Stojković M, Jovanović M, Ćulafić M. Role of cystatin C and renal resistive index in assessment of renal function in patients with liver cirrhosis. *World J Gastroenterol* 2014; 20(21): 6573-6579 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6573.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6573>

INTRODUCTION

Cirrhosis of the liver is often accompanied by functional renal failure particularly in advanced stages of liver disease. Hemodynamic alterations with reduced effective arterial blood volume and peripheral vasodilation are followed by activation of vasoconstrictive hormones (renin-aldosterone, vasopresin, endothelin) and neurohumoral systems (including increased activity of nervous system)^[1,2]. The most common functional renal abnormalities in patients with cirrhosis are an impaired ability to excrete sodium and water and a reduction of renal blood flow and glomerular filtration rate, the latter two being secondary to vasoconstriction of the renal circulation^[3]. Hence renal failure is directly linked to the mortality rate of cirrhotic patients, it is of a great clinical importance to monitor renal function closely in order to estimate the prognosis and determine the optimal therapeutic option^[4].

Whereas patients with a significantly impaired glomerular filtration rate can be diagnosed easily by elevated serum creatinine (Cr) concentrations, moderately reduced renal function may go unnoticed by this conventional parameter. Nevertheless, the protease inhibitor cystatin C (CysC) has been proposed as a specific marker of glomerular filtration rate (GFR) and an early indicator of impaired renal function^[5].

CysC is a non-glycosylated 13 kDa protein, produced at a constant rate by all nucleated cells, freely filtered by the glomeruli and subsequently metabolized in the proximal tubules^[6]. Opposed to Cr, CysC is independent of gender, age, and muscle mass and not influenced by serum bilirubin, inflammation, or malignancy^[7,8].

The aim of the study was to evaluate the clinical significance of CysC and renal blood flow for the determination of renal function in patients with liver cirrhosis.

MATERIALS AND METHODS

Subjects

We conducted a study of 63 patients, aged 18 years and above, with alcoholic or viral liver cirrhosis examined and treated between December 2011 and September 2013 at the Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, Belgrade. A healthy control group comprised of 30 age and gender-matched subjects. Diagnostic approach was based on clinical clues from the patient's medical history (*e.g.*, consumption of pure alcohol more than 50 g/d over a five-year period), physical examination, laboratory tests, abdominal ultrasonography and upper endoscopy; liver biopsy was performed in 15 (23.8%)

patients. Laboratory analyses included tests of hepatocyte integrity, cholestasis, synthetic liver function tests and etiological tests.

The degree of liver insufficiency was assessed according to the Child-Pugh classification and divided into three stages: A, B and C (score $A \leq 6$, $B 7-9$, and $C \geq 10$)^[9]. The diagnosis of hepatic encephalopathy was based on clinical criteria, and the severity of hepatic encephalopathy was based on the West Haven Criteria for grading of mental status^[10]. The model of end stage liver disease (MELD) has also been used to assess patients with liver cirrhosis^[11].

All respondents were evaluated for any superimposed conditions such as infection, intrinsic renal disease, chronic obstructive pulmonary disease, congestive heart failure, thyroid dysfunction, and diabetes mellitus. The following exclusion criteria were applied: patients with hepatocellular carcinoma, gastrointestinal bleeding, or hepatorenal syndrome (HRS). Patients receiving corticosteroids, antiviral agents, angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors, aminoglycosides, nonsteroidal anti-inflammatory drugs, or aminoacids L-arginine and L- ornithine were also excluded from the study.

Biochemistry

Diuretics were stopped in all patients, at least 24 h before laboratory testing. Patients were advised to adopt a low-sodium diet (less than 40 mmol/d). Serum samples were obtained on the day of urine collection. Venous blood samples were collected in vacutainers without additives, centrifuged at 3500 rpm (about 2000 g) and preserved at -80 °C after separation. CysC serum concentration was determined by the PENIA method (Particle-Enhanced Nephelometric Immuno-Assay), using the SIEMENS (Marburg, Germany) tests, on a laser nephelometer (BN IIdadeBehring). CysC referent value was 0.59-1.04 mg/L. Cr was determined according to the kinetic Jaffe's method, using an automated biochemical analyzer (Olympus AU 400) and commercially available assay kits by the same manufacturer. Cr referent value for men was 59-104 μmol/L and for women 45-84 μmol/L. Creatinine clearance (CL_{Cr}) was calculated as a product of urinary Cr and 24-h urine volumen divided by serum Cr (μmol/L) and multiplied by 1440. Referent values for 24-h urinary creatinine excretion were 23 mg/kg ideal body weight for men and of 17 mg/kg ideal body weight for women^[12].

Estimated GFR was calculated from serum Cr using the Modification of Diet in Renal Disease (MDRD) equation: $eGFR = 186 \times sCr^{-1.154} \times age^{-0.203} \times 1.212$ (if African American) $\times 0.742$ (if female)^[13], and from serum CysC using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation: $eGFR = 127.7 \times CysC^{-1.17} \times age^{-0.13} \times 0.91$ (if female) $\times 1.06$ (if African American)^[14]. Serum sodium (sNa⁺) referent value was 135-148 mmol/L, and urine sodium (uNa⁺) referent value for men was 40-220 mmol/L and for women 27-287 mmol/L.

Table 1 Characteristics of enrolled patients based on Child Pugh score (*n* = 63)

| Characteristic | A | B | C | <i>P</i> value |
|--|-------|-------|------|----------------|
| Bilirubin (μmol/L) | 17.7 | 33.5 | 55.1 | < 0.001 |
| AST (U/L) | 34 | 39 | 57 | 0.035 |
| ALT (U/L) | 32 | 29 | 40 | 0.187 |
| ALP (U/L) | 112 | 109 | 113 | 0.888 |
| GGT (U/L) | 103 | 73 | 51 | 0.153 |
| Albumin (g/L) | 36 | 32 | 25 | < 0.001 |
| sNa ⁺ (mmol/L) | 139 | 137 | 135 | 0.008 |
| Cr (μmol/L) | 67 | 70 | 74 | 0.540 |
| CL _{Cr} (mL/min) | 120.7 | 114 | 104 | 0.823 |
| GFR _{Cr} (mL/min per 1.73 m ²) | 129.9 | 116.1 | 111 | 0.476 |
| CysC (mg/L) | 0.83 | 1.09 | 1.12 | 0.010 |
| GFR _{Cys} (mL/min per 1.73 m ²) | 93.3 | 67.1 | 64.7 | 0.017 |
| RRI | 0.63 | 0.7 | 0.75 | < 0.001 |

All data are presented as median values. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Gamma-glutamyl transferase; sNa⁺: Serum sodium; Cr: Creatinine; CL_{Cr}: Creatinine clearance; GFR_{Cr}: Glomerular filtration rate based on creatinine; CysC: Cystatin C; GFR_{Cys}: Glomerular filtration rate based on cystatin C; RRI: Renal resistive index.

Abdominal and renal Doppler ultrasonography

Ultrasonography (Toshiba Core Vision, with Doppler duplex convex probe, 3.5 MHz) was performed to examine the liver size, echo structure of the hepatic parenchyma and possible focal changes, spleen diameter, and presence of ascites. Renal color Doppler duplex ultrasonography was used to evaluate renal resistive index (RRI). The renal arteries were evaluated bilaterally of the distal arcuate branches. RRI equals peak systolic velocity minus the final diastolic velocity divided by the peak systolic velocity. RRI less than 0.7 is considered normal and was calculated based on the mean value of renal arteries^[15,16].

Statistical analysis

One-sample Kolmogorov-Smirnov and Shapiro-Wilk tests were performed to determine whether the data showed normal distribution. *t* test or Mann-Whitney test was applied to assess the differences in investigated parameters. Analysis of variance (ANOVA) or Kruskal-Wallis test was applied to assess the influence of the investigated parameters. After assessing overall effects of a factor by means of ANOVA, post-hoc multiple comparison procedures with Bonferonni correction were performed to determine individual differences between the groups. Pearson's (*r*) or Spearman's correlation (*r_s*) procedures were performed to evaluate the relationship between different variables. McNemar's test was used to assess the differences in detecting renal function using two different approaches. The Statistical Package for Social Sciences version 15 (SPSS Inc., Chicago, IL, United States) was used for statistical analyses, at the 0.05 level of significance.

Ethical considerations

The study was conducted in accordance with Guidelines

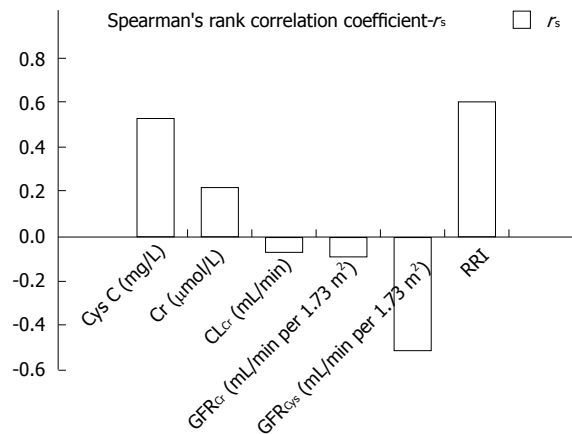


Figure 1 Correlation of renal parameters with model of end stage liver disease score. *r_s*: Spearman's rank correlation coefficient; CysC: Cystatin C (*P* < 0.001); Cr: Creatinine (*P* = 0.091); CL_{Cr}: Creatinine clearance (*P* = 0.557); GFR_{Cr}: Glomerular filtration rate based on creatinine (*P* = 0.460); GFR_{Cys}: Glomerular filtration rate based on cystatin C (*P* < 0.001); RRI: Renal resistive index (*P* < 0.001).

for Good Clinical Practice, the Declaration of Helsinki, and local laws and regulations. The protocol was approved by joint Research and Ethics Committee of the Clinical Center of Serbia, Belgrade, filed under number 2385/5. Written informed consent was obtained from all the participants in the study.

RESULTS

The patient group comprised 47 (74.6%) males and 16 (25.4%) females. The average age of the patients was 50.8 ± 13.5. Alcoholic cirrhosis was diagnosed in 41 (65.1%) and viral cirrhosis in 22 (34.9%) patients.

The average value of CysC measured in patients with liver cirrhosis was 1.09 ± 0.42 mg/L, while it was 0.88 ± 0.12 mg/L in the control group. Comparing these groups, we have confirmed significantly higher CysC in patients with cirrhosis (*P* = 0.036). Increased values of CysC were observed in 23 (40%) patients.

The liver insufficiency degree, determined by generally accepted Child-Pugh classification, was divided into three stages: A in 23 (36.5%), B in 21 (33.3%) and C in 19 (30.2%) patients. Patients' characteristics based on Child-Pugh score are presented in Table 1. MELD score ranged from 8 to 26.

Post-hoc comparisons showed statistically significant differences in values of CysC between Child-Pugh A and B (*P* = 0.014) and between A and C (*P* = 0.007) stages, while there was no difference between B and C stages (*P* > 0.05) (Table 1). Moreover, we confirmed a statistically significant correlation between CysC and MELD score (Figure 1).

Increased Cr values were detected in 7 (11.1%) patients, and all of them had increased values of CysC. Mean values for 24-h urinary creatinine excretion for men was 18.3 mg/kg, and for women 16.3 mg/kg. Decreased creatinine values were detected in 32/47 (68%) men and 9/16 (56.3%) women. There were no significant differ-

ences in Cr values between the stages of liver cirrhosis in regards to Child-Pugh classification. Furthermore, we detected no significant correlation between Cr and MELD score (Figure 1).

Decreased CL_{Cr} was detected in 24 (38%) patients with an average value of 57.2 ± 21.2 mL/min. Reduced CL_{Cr} in 9 out of 23 (39.1%) patients with Child-Pugh A, 8 out of 21 (38.1%) with B and 7 out of 19 (36.8%) with stage C were confirmed. No correlation was observed between CL_{Cr} and MELD score (Figure 1). Also we detected no statistically significant differences in CL_{Cr} between Child-Pugh stages. Additionally, CL_{Cr} negatively correlated with CysC and Cr ($r_s = -0.415$, $P = 0.001$; $r_s = -0.511$, $P < 0.001$, respectively).

There was a strong negative correlation between increased CysC and decreased sNa^+ in patients ($r = -0.9$, $P = 0.002$). In contrast, increased Cr values did not show a significant correlation with sNa^+ concentration ($P > 0.05$). In patients with decreased urinary excretion of sodium, CysC correlated negatively with uNa^+ concentration ($r = -0.748$, $P = 0.05$), while Cr showed no significant relation with uNa^+ ($P > 0.05$).

The mean GFR estimated using Cr (GFR_{Cr}) was 113.5 mL/min per 1.73 m^2 . Lower values of $GFR_{Cr} < 90$ mL/min per 1.73 m^2 were observed in 13 (20.6%) patients, and there was no statistically significant difference between Child-Pugh stages. Lower values of $GFR_{Cr} < 60$ mL/min per 1.73 m^2 were observed in 7 (11.1%) patients ($P > 0.05$). No significant correlation was observed in GFR_{Cr} between Child-Pugh stages (Table 1). Moreover, no significant correlation was detected between GFR_{Cr} and MELD score (Figure 1).

Mean GFR based on CysC (GFR_{Cys}) was 77.6 mL/min per 1.73 m^2 . Lower values of $GFR_{Cys} < 90$ mL/min per 1.73 m^2 were observed in 40 (63.5%) patients and $GFR_{Cys} < 60$ mL/min per 1.73 m^2 in 16 (25.4%) patients, with no statistically significant difference between Child-Pugh stages ($P > 0.05$).

Mean GFR_{Cys} in patients with normal values of CysC was significantly higher than that in patients with increased values of CysC (94.8 ± 15.0 mL/min per 1.73 m^2 vs 53.5 ± 14.5 mL/min per 1.73 m^2 , $P < 0.001$, respectively). Statistically significant differences in GFR_{Cys} between Child-Pugh stages were observed (Table 1). Post-hoc comparisons showed differences in GFR_{Cys} between Child-Pugh stages A and B ($P = 0.006$) and between A and C ($P = 0.005$). Also, a significant correlation between GFR_{Cys} and MELD was determined (Figure 1). A moderate degree of correlation was found between GFR_{Cys} and GFR_{Cr} in patients with liver cirrhosis ($r_s = 0.64$, $P < 0.001$). We identified significantly more patients with decreased GFR based on CysC than on Cr ($P < 0.001$).

We noticed that RRI was significantly higher in cirrhotic patients than in controls ($P = 0.005$). RRI was already more increased in 27 (43%) patients with ascites when compared to 36 (57%) without ascites ($P = 0.005$).

Also, RRI was significantly influenced by Child-Pugh stage (Table 1). Comparisons showed markedly higher

RRI in Child-Pugh stage C than in A ($P < 0.001$) and B stage ($P = 0.001$). Also, a significant correlation was noted between RRI and MELD (Figure 1).

RRI correlated significantly with CysC ($r_s = 0.283$, $P = 0.028$) and showed a significant negative correlation with GFR_{Cys} ($r_s = -0.31$, $P = 0.016$). However, we detected no relationship between RRI and Cr, CL_{Cr} , or GFR_{Cr} .

DISCUSSION

Early diagnosis of impaired renal function, particularly decreased GFR, is very important to prevent serious complications^[5]. The gold standard for determining GFR is to measure the clearance of an exogenous substances such as chromium-51 labeled ethylenediamine tetraacetic acid (^{51}Cr -EDTA) and inulin. Procedures determining GFR using exogenous substances are invasive and carry a risk for patients, usually are considered too expensive and time consuming for routine clinical use^[17]. Moreover, procedure for measuring inulin clearance is impractical because of the necessity for steady-state infusion, a urine bladder catheter, and possible interference from blood glucose^[18].

The endogenous marker of GFR most commonly used in routine clinical and laboratory practice is serum Cr. However, in liver cirrhosis, specific non-renal factors may influence Cr concentration. Protein-calorie malnutrition, muscle wasting, and impaired liver function will directly reduce Cr production^[19]. Moreover, ascites and peripheral edema can also decrease the Cr due to larger area for distribution^[20,21]. We report that reduced urinary creatinine excretion in cirrhosis correlates with anthropometrically estimated muscle mass and is not related to reduced liver function. Our results are consistent with a previous study conducted by Pirlich *et al*^[22]. Given the fact that serum Cr systematically overestimates renal function, mild degree of renal insufficiency may go unnoticed as Cr level may remain in the normal range despite a major decline in GFR^[4,23]. Moreover, several studies have shown that CL_{Cr} overestimates true GFR about 13 mL/min per 1.73 m^2 compared to inulin clearance in patients with cirrhosis^[24,25]. Variation in creatinine excretion exists during the day, making estimation of GFR, even from a valid 24-hour urine collection, incorrect^[25].

Some studies have indicated that serum CysC could be proposed as a marker of liver disease stage and a more sensitive indicator of renal function in patients with cirrhosis than serum Cr level^[26-28].

Particularly in patients with Child-Pugh class C, CysC determination is a valuable tool for the early diagnosis of moderately impaired renal function^[5].

Significant differences were observed in CysC values but not Cr values, between Child-Pugh class A, B, and C. The finding suggests that CysC may indirectly reflect the degree of liver dysfunction^[22,29]. Woitas *et al*^[30] found that CysC was significantly higher in Child-Pugh B and C patients when compared to Child-Pugh A patients. Still no difference was observed between patients with

Child-Pugh B and C. Similar to the previous findings, we confirmed that the values of CysC were significantly increased in advanced stages (Child-Pugh B and C) compared to early stage (Child-Pugh A) of liver cirrhosis, although there was no significant difference between B and C stages. Additionally, we report a significant correlation between CysC and MELD score, advocating CysC as a valid marker of liver insufficiency. Considering a variety of non-renal factors influencing serum creatinine levels, and exclusion of patients with HRS from the study, no significant correlation between Cr and MELD score was noted. Prognostic and clinical significance of CysC is also shown in a recent study stating that CysC, serum sodium and prothrombin time were independent factors for predicting survival in patients with cirrhosis^[31].

In our study, unlike Cr, CysC levels and serum and urinary concentration of sodium demonstrated a strong negative correlation, suggesting a clinical relevance of CysC. These findings may be especially useful for monitoring patients with decompensated liver cirrhosis.

A study comparing GFR_{Cys} formula to GFR_{Cr} formula showed that CysC was more likely to predict the patients' GFR below or above 60 mL/min per 1.73 m²^[32]. Furthermore, the CysC showed a more significant correlation than serum Cr with GFR by ^{99m}Tc-DTPA technique^[4,33,34]. Also, Coll *et al*^[35] reported that serum CysC levels started to increase when GFR was 88 mL/min per 1.73 m², while serum Cr level began to increase when GFR was 75 mL/min per 1.73 m².

However, Xirouchakis *et al*^[36] compared ⁵¹Cr-EDTA with GFR_{Cr} and GFR_{Cys} formulas in 74 patients with cirrhosis, candidates for liver transplantation. They reported that estimated GFR in cirrhosis is not better based on CysC formulas compared with creatinine ones.

In contrast, we identified significantly more patients with decreased glomerular filtration based on GFR_{Cys} compared to GFR_{Cr}. In regards to our findings, we suggest GFR_{Cys} as a more sensitive parameter for assessment of renal function in patients with liver cirrhosis.

The RRI is a sensitive marker of intrarenal hemodynamics and it has been reported to increase even in non-azotemic patients with cirrhosis^[37]. Moreover, data suggest that normal serum Cr levels may be associated with a significantly decreased glomerular filtration rate and that more than 50% of patients with end-stage liver disease and increased RRI have normal serum creatinine levels^[38,39].

A recently published study reported that RRI was significantly higher in Child-Pugh C patients than in Child-Pugh B or A patients^[23]. Findings from our research showed that RRI significantly increased from Child-Pugh stages A to C. Also, the RRI increased with an increase of MELD score. These results indicate that RRI is directly influenced by liver insufficiency degree.

In cirrhotic patients fluid accumulation can occur (in form of pedal edema, minimal ascites and/or diuretic-sensitive ascites), and renal blood flow is expected to decrease with GFR maintained at normal levels by in-

creased filtration fraction. The recognition and identification of these patients are particularly important for the early intervention and prevention of progression of renal diseases^[40]. Accordingly, we aimed to examine this hemodynamic disturbance by measuring RRI.

To our knowledge, a small number of studies have been published on possible correlations between CysC, GFR and arterial renal blood flow resistance. Cystatin C was compared to RRI in patients with viral C cirrhosis and authors reported significant positive correlations^[22]. Ustundag *et al*^[29] noted that serum CysC, but not serum creatinine or RRI measurement, correlated with GFR (GFR was estimated by technetium (99m)-diethylene triamine pentaacetic acid renal scintigraphy), in each stage of liver failure. Interestingly, our data showed that RRI significantly correlated with GFR_{Cys}, but not with GFR_{Cr}.

In conclusion, CysC may be a more reliable marker for liver insufficiency assessment. Additionally, RRI and CysC represent sensitive indicators of renal dysfunction in patients with liver cirrhosis.

COMMENTS

Background

Early diagnosis of impaired renal function in cirrhotic patients is very important to prevent serious complications. Renal blood flow decrease is common, while glomerular filtration rate (GFR) remains normal because the filtration fraction increases. The protease inhibitor (CysC) has been proposed as a specific marker of GFR and an early indicator of impaired renal function.

Research frontiers

Some studies have indicated that serum CysC could be proposed as a marker of liver disease stage and a more sensitive indicator of renal function in patients with cirrhosis. Opposed to creatinine (Cr), CysC is independent of gender, age, and muscle mass and not influenced by serum bilirubin, inflammation, or malignancy. The renal resistive index (RRI) is proposed to be a sensitive marker of intrarenal hemodynamics in patients with cirrhosis. The research hotspot is to evaluate the clinical significance of cystatin C and RRI in early detection of renal dysfunction in cirrhotic patients.

Innovations and breakthroughs

Previous studies have shown different results. Some confirmed a significant correlation with CysC based GFR but not with Cr based GFR, when compared to technetium 99m-diethylene triamine pentaacetic acid renal scintigraphy technique. However, others compared GFR_{Cr} and GFR_{Cys} with chromium-51 labeled ethylenediamine tetraacetic acid in patients with cirrhosis and found no significant difference. These procedures that estimated GFR based on exogenous substances are invasive and carry a risk for patients, usually are considered too expensive and time consuming for routine clinical use. In order to eliminate these invasive and costly procedures, the authors measured RRI to evaluate renal blood flow, and examined correlations with GFR_{Cys} and GFR_{Cr}. RRI correlated significantly with CysC and showed a significant negative correlation with GFR_{Cys}. Moreover, the authors detected no relationship between RRI and Cr, CL_{Cr}, or GFR_{Cr}.

Applications

The study results suggest that RRI and CysC represent sensitive indicators of renal dysfunction in patients with liver cirrhosis.

Terminology

Cystatin C: CysC is a non-glycosylated 13 kDa protein, produced at a constant rate by all nucleated cells, freely filtered by the glomeruli and subsequently metabolized in the proximal tubules. Renal resistive index: RRI equals peak systolic velocity minus the final diastolic velocity divided by the peak systolic velocity. RRI less than 0.7 is considered normal and was calculated based on the mean value of renal arteries.

Peer review

The manuscript written by Ćulafić *et al* describes that cystatin C and renal

resistive index may be more reliable markers for assessment of liver and renal dysfunction in patients with liver cirrhosis. Conventionally, renal dysfunction is assessed by serum Cr or GFR_{Cr}. However, cystatin C and renal resistive index are more sensitive than those markers. The manuscript provides important information in the management of patients with liver cirrhosis.

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Prognostic factors in stage IB gastric cancer

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Abstract

AIM: To identify the subset of patients with stage IB gastric cancer with an unfavorable prognosis.

METHODS: Overall survival (OS) rates were examined in 103 patients with stage IB (T1N1M0 and T2N0M0) gastric cancer between January 2000 and December 2011. Univariate and multivariate analyses were performed to identify risk factors using a Cox proportional hazards model.

RESULTS: The OS rates of patients with T1N1 and T2N0 cancer were 89.2% and 94.1% at 5-years, re-

spectively. Both univariate and multivariate analyses demonstrated that tumor location was the only significant prognostic factor. The OS rate was 81.8% at 5-years when the tumor was located in the upper third of the stomach and was 95.5% at 5-years when the tumor was located in the middle or lower third of the stomach ($P = 0.0093$).

CONCLUSION: These data may suggest that tumor location is associated with survival in patients with stage IB gastric cancer.

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Key words: Gastric cancer; Stage IB; Prognostic factor

Core tip: This study identified the subset of patients with stage IB gastric cancer with an unfavorable prognosis. Overall survival (OS) rates were examined in 103 patients with stage IB gastric cancer. Both univariate and multivariate analyses demonstrated that tumor location was the only significant prognostic factor. The OS rate was 81.8% at 5-years when the tumor was located in the upper third of the stomach and was 95.5% at 5-years when the tumor was located in the middle or lower third of the stomach ($P = 0.0093$). Our data may suggest that tumor location is associated with survival in patients with stage IB gastric cancer.

Aoyama T, Yoshikawa T, Fujikawa H, Hayashi T, Ogata T, Cho H, Yamada T, Hasegawa S, Tsuchida K, Yukawa N, Oshima T, Oba MS, Morita S, Rino Y, Masuda M. Prognostic factors in stage IB gastric cancer. *World J Gastroenterol* 2014; 20(21): 6580-6585 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6580.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6580>

INTRODUCTION

Gastric cancer is the fourth most common malignant

disease and the second most frequent cause of cancer-related death worldwide^[1]. East Asia has the highest occurrence of gastric cancer. It was shown from mass-screening programs and examinations that stage I gastric cancer has increased by up to 50% over the past two decades in Japan and South Korea^[2].

Stage I gastric cancer is divided into IA and IB according to the third English edition of the Japanese Classification of Gastric Carcinoma. Five-year survival rate was reportedly 95.1% in stage IA and 88.9% in stage IB following surgery alone (90.2% in T2N0 and 87.6% in T1N1)^[3]. Standard treatment for stage I is defined as surgery alone according to Japanese gastric cancer treatment guidelines^[4]. Although the prognosis of stage IB is excellent, some patients recur even after curative surgery. Once recurrence has developed, the prognosis is limited, and is up to 1 year^[5,6].

As a result of the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) phase III trial, S-1 became the first anti-cancer drug to have efficacy as adjuvant chemotherapy for stages 2 and 3 after curative gastrectomy for gastric cancer^[7]. In updated results of the ACTS-GC phase III trial, the 5-year overall survival of stage 2 was 71.3% in the surgery alone group, but reached 84.2% in the S-1 group^[8]. In the subset analyses, hazard ratio was lower in stage 2 than stage 3 and in node-negative disease than node-positive disease. Thus, S-1 was more effective, especially for relatively early disease. If we know which patients with stage IB recur after surgery, S-1 could prevent recurrence. However, prognostic factors of stage IB have not yet been fully clarified. The aim of the present study was to identify the subset of patients with stage IB gastric cancer with an unfavorable prognosis.

MATERIALS AND METHODS

Patients

The patients were selected from the prospective database of the Kanagawa Cancer Center, Department of Gastrointestinal Surgery, Yokohama, Japan, according to the following criteria: (1) the patients had histologically-proven gastric adenocarcinoma; (2) the patients underwent curative resection for gastric cancer as a primary treatment between January 2000 and December 2011; (3) the patients with stage IB (T1N1M0 or T2N0M0) disease were diagnosed pathologically according to the third English edition of the Japanese Classification of Gastric Carcinoma^[9]; and (4) the patients did not receive any adjuvant chemotherapy after surgery.

Surgery and follow up

All patients underwent total or distal gastrectomy with D1+ or D2 lymph node dissection in accordance with the Japanese gastric cancer treatment guidelines published in 2010 (ver. 3)^[4]. In distal gastrectomy, resected nodes included # 1, 3, 4sb, 4d, 5, 6, 7, 8a, and 9 in D1+ plus # 11p and 12a in D2. In total gastrectomy, resected

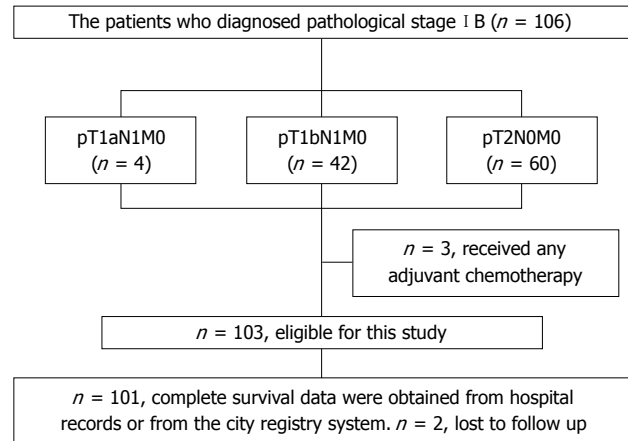


Figure 1 Flow diagrams and details of the 106 patients diagnosed with stage IB cancer according to the third English edition of the Japanese Classification of Gastric Carcinoma.

Table 1 Comparison of patient's characteristics in the pT1N1M0 group and pT2N0M0 group

| Characteristics | pT1N1M0 group (n = 45) | pT2N0M0 group (n = 58) | P value |
|-----------------------------|---------------------------|---------------------------|---------|
| Age (yr) | | | 0.433 |
| < 70 | 32 | 37 | |
| ≥ 70 | 13 | 21 | |
| Gender | | | 0.510 |
| Male | 30 | 35 | |
| Female | 15 | 23 | |
| Performance status (ECOG) | | | 0.208 |
| 0 | 45 | 56 | |
| 1 | 0 | 2 | |
| Site of tumor | | | 0.719 |
| Upper third | 11 | 16 | |
| Middle third | 24 | 22 | |
| Lower third | 10 | 20 | |
| Maximal tumor diameter (mm) | | | 0.281 |
| ≤ 20 | 7 | 4 | |
| > 20, ≤ 40 | 22 | 27 | |
| > 40 | 16 | 27 | |
| Histological type | | | 0.931 |
| Differentiated | 19 | 24 | |
| Undifferentiated | 26 | 34 | |
| Lymphatic invasion | | | 0.010 |
| Negative | 26 | 47 | |
| Positive | 19 | 11 | |
| Vascular invasion | | | 0.256 |
| Negative | 32 | 35 | |
| Positive | 13 | 23 | |

nodes included # 1, 2, 3, 4sa, 4sb, 4d, 5, 6, 7, 8a, 9, and 11p in D1+ plus #10, 11d, and 12a in D2. In principal, D1+ lymphadenectomy was indicated for patients with cT1N0 tumors other than those for whom endoscopic mucosal resection or endoscopic submucosal dissection were recommended. D2 lymphadenectomy was indicated for patients with potentially curable T2-T4 tumors, as well as for those with cT1N+ tumors.

The patients received follow-up visits at outpatient clinics. Hematological tests and physical examinations were performed at least every three months for five

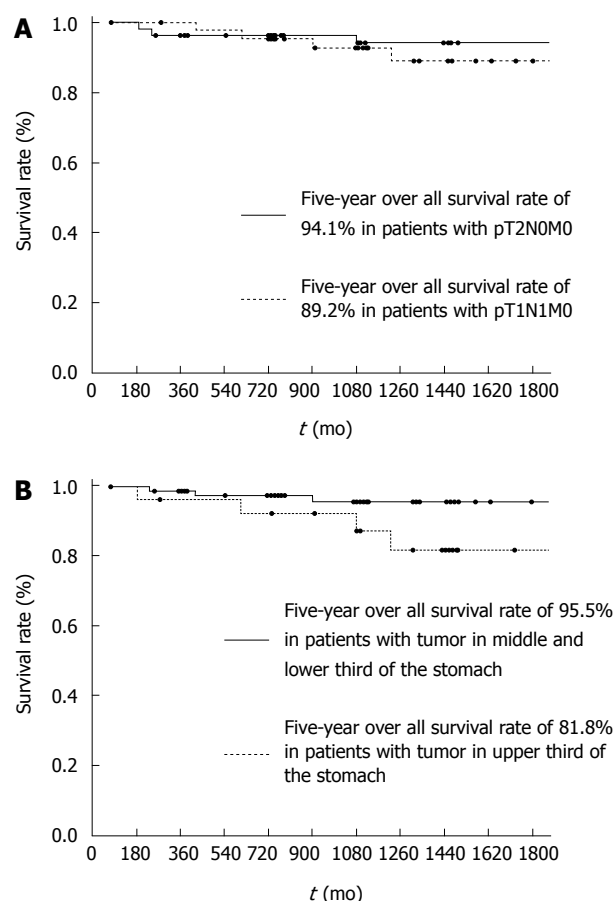


Figure 2 Overall survival curves. A: Overall survival curves in patients with stage T1N1M0 and stage T2N0M0 gastric cancer; B: Overall survival curves in patients with pathological tumors in the upper third of the stomach and tumors in the middle and lower third of the stomach.

years after surgery. CEA and CA19-9 tumor marker levels were determined at least every three months for five years. The patients underwent a computer tomography examination every six months during the first three years after surgery, and then every year for a further two years. The patients who received distal gastrectomy underwent an endoscopic examination every year for five years after the surgery.

Evaluation and statistical analyses

The staging and clinicopathological characteristics of the tumors were based on the third English edition of the Japanese Classification of Gastric Carcinoma^[9]. Overall survival (OS) was defined as the period between surgery and the occurrence of death. The data for patients who did not experience an event were treated as censored cases on the date of the final observation.

The OS curves were calculated using the Kaplan-Meier method and compared by the log-rank test. Cox's proportional hazard model was used to perform univariate and multivariate analyses. The survival data were obtained from hospital records or from the city registry system. A *P* value of < 0.05 was defined as statistically significant. The SPSS software package (v11.0J Win, SPSS, Chicago, IL, United States) was used for all statis-

Table 2 Comparison of recurrence-free survival rate by patient's characteristics

| Characteristics | No. of patients | 3-yr rate | 5-yr rate | <i>P</i> value |
|----------------------------------|-----------------|-----------|-----------|----------------|
| Age (yr) | | | | 0.0637 |
| < 70 | 69 | 97.0% | 94.7% | |
| ≥ 70 | 34 | 89.7% | 85.2% | |
| Gender | | | | 0.1512 |
| Female | 38 | 100% | 95.2% | |
| Male | 65 | 91.8% | 89.8% | |
| Tumor location | | | | 0.0565 |
| L | 29 | 91.7% | 91.7% | |
| M | 47 | 97.7% | 97.7% | |
| U | 27 | 88.8% | 84.1% | |
| Pathological tumor diameter (mm) | | | | 0.6106 |
| ≤ 20 | 11 | 90.9% | 90.9% | |
| > 20, ≤ 40 | 49 | 95.1% | 91.8% | |
| > 40 | 43 | 95.1% | 91.8% | |
| Histological type | | | | 0.4419 |
| Differentiated | 43 | 92.5% | 92.5% | |
| Undifferentiated | 60 | 94.6% | 91.0% | |
| Number of lymph node metastases | | | | 0.9013 |
| 0 | 58 | 94.1% | 94.1% | |
| 1 | 33 | 93.4% | 89.1% | |
| 2 | 12 | 90.9% | 90.9% | |
| Lymphatic invasion | | | | 0.1645 |
| Negative | 73 | 95.4% | 93.2% | |
| Positive | 30 | 89.3% | 89.3% | |
| Vascular invasion | | | | 0.7861 |
| Negative | 67 | 93.3% | 93.3% | |
| Positive | 36 | 93.5% | 89.0% | |

tical analyses.

RESULTS

Between January 2000 and December 2011, a total of 106 patients underwent surgical resection and were diagnosed with pathological stage IB gastric cancer. The details of the stage IB patients are shown in Figure 1. Among these patients, 103 were eligible for the present study. Three patients were excluded from the study because they received postoperative adjuvant chemotherapy. The patients' median age was 64 years (range 34 to 86 years). Sixty-five patients were male and 38 were female. The pathological stage was classified as T1N1 in 45 patients and T2N0 in 58 patients. Table 1 shows patient characteristics in the T1N1 group and T2N0 group. Fifty-four patients received D1+ lymph node resection and 52 patients received D2 lymph node resection. The median follow-up period was 49.1 mo (range 2.7 to 103.6 mo). None of the patients died within 30 d after surgery. Two patients were lost from follow up. Five patients died due to recurrence during the study period. Of 5 patients with metastases, lymph node metastasis was observed in 3 patients and liver metastasis in 2 patients.

The OS rates of the patients with T1N1 and T2N0 were 92.7% and 96.5% at 3-years, 89.2% and 94.1% at 5-years, respectively (Figure 2A). There was no significant difference between the two groups (*P* = 0.6738). Therefore, we grouped the patients with T1N1 and T2N0 disease together. Further prognostic analyses were then focused on the patients with stage T1N1 and T2N0

Table 3 Uni- and multivariate Cox proportional hazards analysis of clinicopathological factors

| Characteristics | Number | Univariate | | | Multivariate | | |
|----------------------------------|--------|------------|--------------|---------|--------------|--------------|---------|
| | | OR | 95%CI | P value | OR | 95%CI | P value |
| Age (yr) | | | | 0.078 | | | |
| < 70 | 69 | 1.000 | | | | | |
| ≥ 70 | 34 | 3.147 | 0.879-11.271 | | | | |
| Gender | | | | 0.186 | | | |
| Female | 38 | 1.000 | | | | | |
| Male | 65 | 4.118 | 0.506-33.527 | | | | |
| Tumor location | | | | 0.018 | | | 0.018 |
| M or L | 76 | 1.000 | | | 1.000 | | |
| U | 27 | 5.273 | 1.326-20.969 | | 5.273 | 1.326-20.969 | |
| Pathological tumor diameter (mm) | | | | 0.874 | | | |
| ≤ 40 | 60 | 1.000 | | | | | |
| > 40 | 43 | 1.123 | 0.268-4.704 | | | | |
| Histological type | | | | 0.482 | | | |
| Differentiated | 43 | 1.000 | | | | | |
| Undifferentiated | 60 | 1.608 | 0.428-6.041 | | | | |
| Number of lymph node metastases | | | | 0.902 | | | |
| 0 | 58 | 1.000 | | | | | |
| 1 | 33 | 1.293 | 0.287-5.830 | | | | |
| 2 | 12 | 1.563 | 0.169-14.495 | | | | |
| Lymphatic invasion | | | | 0.177 | | | |
| Negative | 73 | 1.000 | | | | | |
| Positive | 30 | 2.369 | 0.674-8.512 | | | | |
| Vascular invasion | | | | 0.786 | | | |
| Negative | 67 | 1.000 | | | | | |
| Positive | 36 | 1.192 | 0.334-4.253 | | | | |

cancer. When the OS rates were stratified according to each clinical factor, a marginal difference was observed for tumor location (Table 2). Each clinicopathological factor was categorized as shown in Table 3 and analyzed for prognostic significance. Both univariate and multivariate analyses of the OS rates demonstrated that tumor location was the only significant prognostic factor (Table 3). The OS rates were 87.3% at 3-years and 81.8% at 5-years when the tumors were located at the upper third of the stomach and were 95.5% at 3-years and 95.5% at 5-years when the tumors were located at the middle or lower third of the stomach ($P = 0.0093$) (Figure 2B). Recurrence was observed in 3 patients who had tumors located at the upper third of the stomach and in 2 patients with tumors located at the middle or lower third of the stomach.

DISCUSSION

The present study identified a subset of patients with stage IB gastric cancer who had unfavorable outcomes. We found that patients with stage T1N1 and T2N0 cancer had similar outcomes. Therefore, we grouped the patients with T1N1 and T2N0 disease together. Further prognostic analyses were then focused on patients with stage T1N1 and T2N0 cancer. In this study, the survival rates of patients with stage IB cancer were clearly associated with tumor location. When the tumors were located in the upper third of the stomach, the 5-year OS rate was only 81.8%, which was poorer than 84.2% in stage 2 patients who received S-1 adjuvant chemotherapy^[8]. Thus, surgery alone may not be justified for stage IB

when the tumors are located at the upper third of the stomach. According to the subset analysis of the ACTS-GC, S-1 was much more effective against stage II than stage IIIA or stage IIIB cancers^[7]. Considering that S-1 was more effective, especially for relatively early disease, adjuvant S-1 could be an option for these patients. Some authors have reported the significance of tumor location in terms of the prognosis of gastric cancer. For example, Piao *et al*^[10] evaluated 532 patients with gastric cancer, and reported that long-term survival was worse in patients with proximal disease than in those with distal tumors. The proximal stomach is a predominant site for undifferentiated-type tumors, which tend to have a poorer prognosis than differentiated-type tumors. Anatomically, the lymphatic drainage is complex, and tumors located in this region can metastasize to almost all lymph nodes except #5. Curative surgery for proximal tumors is D2 total gastrectomy with splenectomy, which is more invasive than that for distal cancer. Although the precise mechanism is unclear, multiple factors, including those described above, could explain why patients with proximal tumors had a poorer survival.

Yokoyama *et al*^[11] previously demonstrated that undifferentiated-type adenocarcinoma was the only risk factor for the recurrence of stage IB gastric cancer. However, there were some differences between the present study and Yokoyama^[11]'s study. First, the evaluation of staging was different. We classified stage using the third English edition of the Japanese Classification of Gastric Carcinoma, while Yokoyama *et al*^[11] used the second English edition of the Japanese Classification of Gastric Carcinoma. In addition, Yokoyama *et al*^[11] included T3N0 and

T1N2 cancers, which are now classified as stage II A. Ahn *et al*^[3] analyzed stage-specific survival using the third English edition of the Japanese Classification of Gastric Carcinoma, and reported that the five-year survival was 88.9% for stage IB (90.2% in T1N1 and 87.6% in T2N0, respectively) and 83.1% for stage II A (84.0% in T1N2 and 82.1% in T3N0, respectively). Thus, survival was worse in the latter cases than in the former. Second, Yokoyama *et al*^[11] included patients who received adjuvant chemotherapy in the analysis. Adjuvant chemotherapy could have affected survival^[12,13].

There are some limitations in the present study. First, this was a retrospective single center study with a small sample size. Our findings could have been observed by chance in this series. Moreover, age or lymphatic invasion, which was marginally significant in the present study, might become more significant by increasing the number of patients or by extending the follow-up period. The only way to draw a definite conclusion is to collect recent data from many hospitals. However, our data help to clarify which parameters should be included in such a study. Therefore, we believe that our study will have an important clinical impact. Second, there is bias regarding time in this study. The data were collected between 2000 and 2010 and surgical procedures and perioperative care have changed. For example, the patients received two types of perioperative care, including conventional care before May 2009 and enhanced recovery after surgery after June 2009^[14]. These factors might have affected our results.

In conclusion, our data may suggest that tumor location is associated with survival in patients with stage IB gastric cancer. As our study was a retrospective single-center study with a small sample size, a prospective multi-center study is necessary to confirm whether the patients with stage IB gastric tumors located in the upper third of the stomach have a poorer survival than those with tumors in other locations.

COMMENTS

Background

Stage I gastric cancer is divided into IA and IB according to the third English edition of the Japanese Classification of Gastric Carcinoma. Standard treatment for stage I is defined as surgery alone according to the Japanese gastric cancer treatment guidelines.

Research frontiers

Five-year survival rate is reportedly 95.1% in stage IA and 88.9% in stage IB following surgery alone (90.2% in T2N0 and 87.6% in T1N1). Although the prognosis of stage IB is excellent, some patients recur even after curative surgery. Once recurrence has developed, the prognosis is limited, and is up to 1 year. If the authors know which patients with stage IB recur after surgery, S-1 could prevent recurrence. However, prognostic factors of stage IB have not yet been fully clarified. The aim of the present study was to identify the subset of patients with stage IB gastric cancer with an unfavorable prognosis.

Innovations and breakthroughs

In this study, the survival rates of patients with stage IB cancer were clearly associated with tumor location. When the tumors were located in the upper third of the stomach, the 5-year overall survival rate was only 81.8% which was poorer than 84.2% in stage 2 patients who received S-1 adjuvant chemotherapy.

Applications

This study suggested that surgery alone may not be justified for stage IB when

tumors are located at the upper third of the stomach. According to the subset analysis of the ACTS-GC, S-1 was much more effective against stage II than stage IIIA or stage IIIB cancers. Considering that S-1 was more effective, especially for relatively early disease, adjuvant S-1 could be an option for these patients.

Terminology

S-1 is an oral fluoropyrimidine, consisting of tegafur (a prodrug of fluorouracil), 5-chloro-2, 4-dihydropyrimidine (CDHP), and potassium oxonate.

Peer review

There is still an open question what are the risk factors for the early recurrence of gastric cancer (even when it presents as a limited disease) as these patients might require additional treatment modalities besides the surgery, including but not limited to the adjuvant chemotherapy, intraperitoneal chemotherapy (EPIC, HIPEC), biological therapy, etc. There is a need for a systematic review of the available in the recent literature data on the prognostic factors associated with the early recurrences of early gastric cancer, and the research team has successfully contributed to this field by adding just another important paper.

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Conventional endoscopic features are not sufficient to differentiate small, early colorectal cancer

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Abstract

AIM: To evaluate the depth of invasion of small, early colorectal cancers (ECCs) using conventional endoscopic features.

METHODS: From January 2005 to September 2011, colonoscopy cohort showed that a total of 72 patients with small colorectal cancers with the size less than 20 mm underwent colonoscopy at the Yonsei University College of Medicine, Seoul, South Korea. Among them, 8 patients were excluded due to incomplete medical records. Finally, a total of 64 ECCs with submucosa (SM) invasion and size less than 20 mm were included. One hundred fifty-two adenomas with size less than 20 mm were included as controls. Nine endoscopic features, including seven morphological findings (*i.e.*, loss of lobulation, excavation, demarcated and depressed areas, stalk swelling, fullness, fold convergence, and bleeding ulcers), pit patterns, and non-lifting signs, were evalu-

ated retrospectively. All endoscopic features were evaluated by two experienced endoscopists who have each performed over 1000 colonoscopies annually for more than five years without knowledge of the histology.

RESULTS: Among the morphological findings, the size of deep submucosal cancers was bigger than that of superficial lesions (16.9 mm *vs* 12.3 mm, $P < 0.001$). Also, demarcated depressed areas, stalk swelling, and fullness were more common in deep SM cancers than in superficial tumors (demarcated depressed areas: 52.0% *vs* 15.7%, $P < 0.001$; stalk swelling: 100% *vs* 4.2%, $P < 0.001$; fullness: 25.0% *vs* 0%, $P = 0.001$). Among deep SM cancers, 96% of polyps showed invasive pit patterns, whereas 19.4% of superficial tumors showed invasive pit patterns ($P < 0.001$). A positive non-lifting sign was more common in deep SM cancers (85.0% *vs* 28.6%, $P < 0.001$). Diagnostic accuracy of invasive morphology, invasive pit patterns, and non-lifting signs for deep SM cancers were 71%, 82%, and 75%, respectively.

CONCLUSION: Conventional endoscopic findings were insufficient to discriminate small, deep SM cancers from superficial SM cancers by white light, standard colonoscopy.

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Key words: Colonoscopy; Colorectal neoplasms; Differential diagnosis

Core tip: This present study was designed to evaluate the depth of invasion of small, early colorectal cancers using conventional endoscopic features. This study exhibited that invasive pit patterns were a more accurate finding than morphological features or non-lifting signs to discriminate small, deep submucosa (SM) cancers from superficial SM cancers by a white light, standard colonoscopy. However, it also showed that conventional endoscopic findings, such as morphologi-

cal features, non-lifting sign, and invasive pit patterns are insufficient to discriminate deep SM cancers to determine therapeutic strategy under white light standard colonoscopy.

Park W, Kim B, Park SJ, Cheon JH, Kim TI, Kim WH, Hong SP. Conventional endoscopic features are not sufficient to differentiate small, early colorectal cancer. *World J Gastroenterol* 2014; 20(21): 6586-6593 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i21/6586.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6586>

INTRODUCTION

Endoscopic management for early colorectal neoplasm has been accepted as an effective method to treat or prevent colorectal cancer. Currently, colorectal neoplasms either confined to the mucosa or after invasion to submucosa (SM) with a size less than 1000 μm are considered good candidates for endoscopic treatment^[1-3]. Because 6%-12% of lymph node metastases have been reported in deep SM cancers, these lesions are not indicated for endoscopic treatment^[4]. Therefore, it is crucial to precisely evaluate the depth of invasion in advanced colorectal neoplasm for adequate therapeutic treatment^[5].

Size is one of the important indicators of the depth of invasion and for the choice of an adequate treatment for advanced colorectal neoplasms. A previous study reported that 7.4%-14% of colorectal polyps larger than 20 mm were submucosal carcinoma^[4], thus endoscopists must treat large colorectal neoplasms endoscopically to avoid an incomplete resection. However, during screening colonoscopy, most colorectal polyps are detected as small polyps less than 20 mm in size and can be resected by a simple polypectomy during the procedure^[4,6-10]. Although a previous report showed that only 0.07%-5.80% of polyps less than 20 mm in size are submucosal carcinoma^[4], recent advances in colonoscopy technology enable the frequent detection of small advanced colorectal neoplasms. However, it is difficult to determine whether those small polyps are invasive carcinoma prior to histologic evaluation. Generally, endoscopists remove these lesions using a simple polypectomy and fail to achieve complete resection.

Recent innovative technology has allowed endoscopists to differentiate advanced colorectal neoplasms during a colonoscopy. Magnifying chromoendoscopy or narrow-band imaging (NBI) has been widely studied to assess depth of invasion^[11-13]. However, most of studies have been focused on large colorectal neoplasms which are candidates for advanced endoscopic techniques, such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD)^[1-3]. Therefore, the present study evaluated conventional endoscopic findings, including morphological features, pit patterns, and no-lifting signs, to assess depth of invasion in advanced colorectal

neoplasms less than 20 mm in size using a standard colonoscopy for accurate diagnoses and treatment.

MATERIALS AND METHODS

Study population

From January 2005 to September 2011, colonoscopy cohort showed that a total of 72 patients with small colorectal cancers with the size less than 20 mm underwent a colonoscopy at the Yonsei University College of Medicine, Seoul, South Korea. Among them, eight patients were excluded from the present study due to incomplete medical records. Finally, a total of 64 small colorectal cancers with SM invasion were included; 25 (39%) deep submucosal cancers, and 39 (61%) superficial submucosal cancers. All lesions were histologically confirmed to be adenocarcinomas. 39 superficial submucosal cancers and 152 adenomas with high-grade dysplasia less than 20 mm were included as control.

Colonoscopic examination

Colonoscopy was performed after bowel preparation with 4 L polyethylene glycol solution (Colyte; Taejun, Seoul, South Korea or Colyte-F or Colonlyte; Dre-ampharma, Seoul, South Korea) by three experienced gastroenterologists. All colonoscopies were performed with a standard colonoscope (CF Q240L, CF Q240I, CF H260AI, CF Q260AI, or PCF Q260AI; Olympus Optical Co, Ltd, Tokyo, Japan). The shape, size, number, location, and histology of small advanced colorectal neoplasms were evaluated. The shape of small colorectal neoplasms was classified as either pedunculated or non-pedunculated (sessile or flat/depressed) type. Location was divided into the right colon (including the cecum, ascending colon, transverse colon, or splenic flexure) or left colon (including the descending colon, sigmoid colon, or rectum). Polyp size was estimated using a 7 mm diameter open-biopsy forceps.

We investigated nine endoscopic findings of the colorectal neoplasms, including seven morphological features (*i.e.*, loss of lobulation, excavation, demarcated depressed areas, stalk swelling, fullness, fold convergence, and bleeding ulcers), pit patterns, and non-lifting signs from the previously published literature^[14]. The definitions of the nine endoscopic findings were as follows (Figure 1). (1) loss of lobulation: loss of normal lobulation; (2) excavation: a crumbled, damaged area of the tumor that prevents observation of the surface structure; (3) demarcated depressed areas: depressed demarcations on the surface of the tumor; (4) stalk swelling: a thickened and expanded stalk; (5) fullness: a bursting appearance due to expansive growth of the tumor; (6) fold convergence: fold convergence towards the tumor; (7) bleeding ulcer; (8) Pit pattern: Sub-classified as invasive or non-invasive (Figures 2 and 3)^[12]. Non-invasive pattern: normal mucosa, star-shaped crypts (Kudo's type I or II), or regular crypts with or without demarcated areas or irregular pits without demarcated areas (Kudo's type III S, III

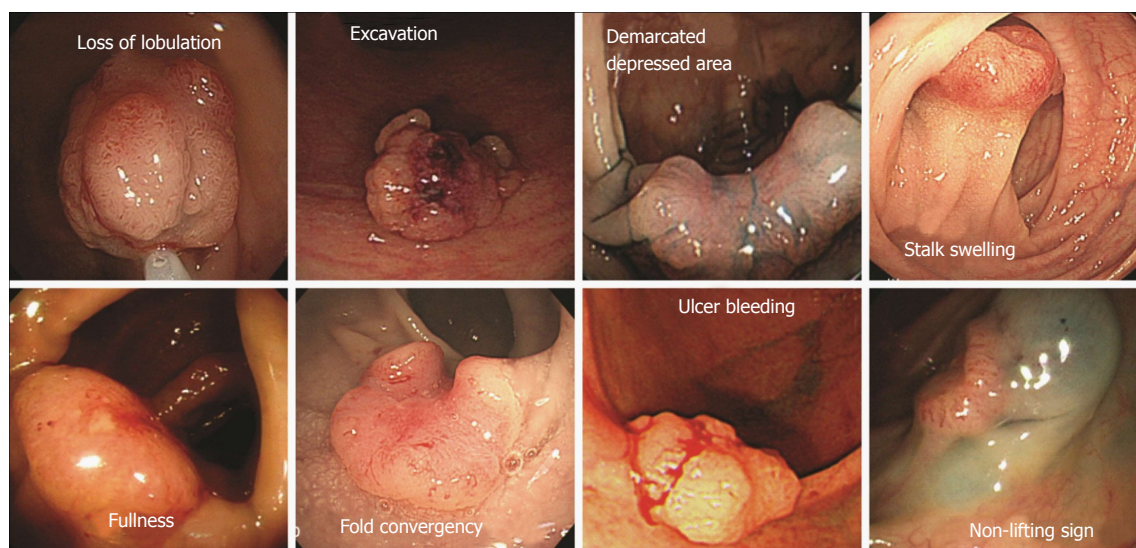


Figure 1 Seven morphological features and non-lifting sign for submucosal cancer.

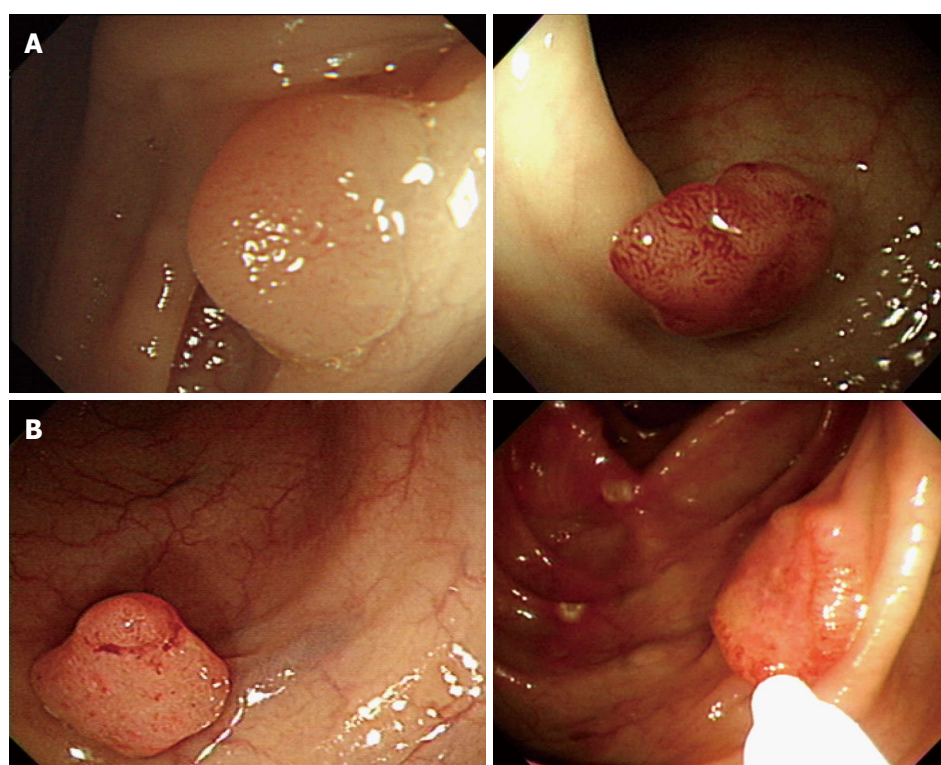


Figure 2 Pit-pattern classification; (A) non-invasive pattern, and (B) invasive pattern.

L, or IV); Invasive pattern: irregular and distorted crypts in demarcated areas (Kudo's type VN and VI); and (9) Non-lifting sign: SM injection was performed at a point approximately 2 mm from the edge of the lesion using a 23-gauge needle. A saline solution containing epinephrine (0.01 mg/mL) and 0.8% indigo carmine was injected into the submucosal layer to lift the lesion off the muscle layer. A non-lifting sign was defined as positive when the surrounding mucosa, but not the lesion, was elevated and negative when the lesion itself was elevated^[15].

All endoscopic features were evaluated retrospec-

tively by two experienced endoscopists who have each performed over 1000 colonoscopies annually for more than five years without knowledge of the histology. Final endoscopic features were determined after agreement between the two endoscopists.

Histopathology

Histopathological diagnoses were based on the Vienna classification by a highly experienced pathologist^[16]. A microscope with a built-in ruler was used to determine the depth of SM invasion. Superficial SM cancer was defined

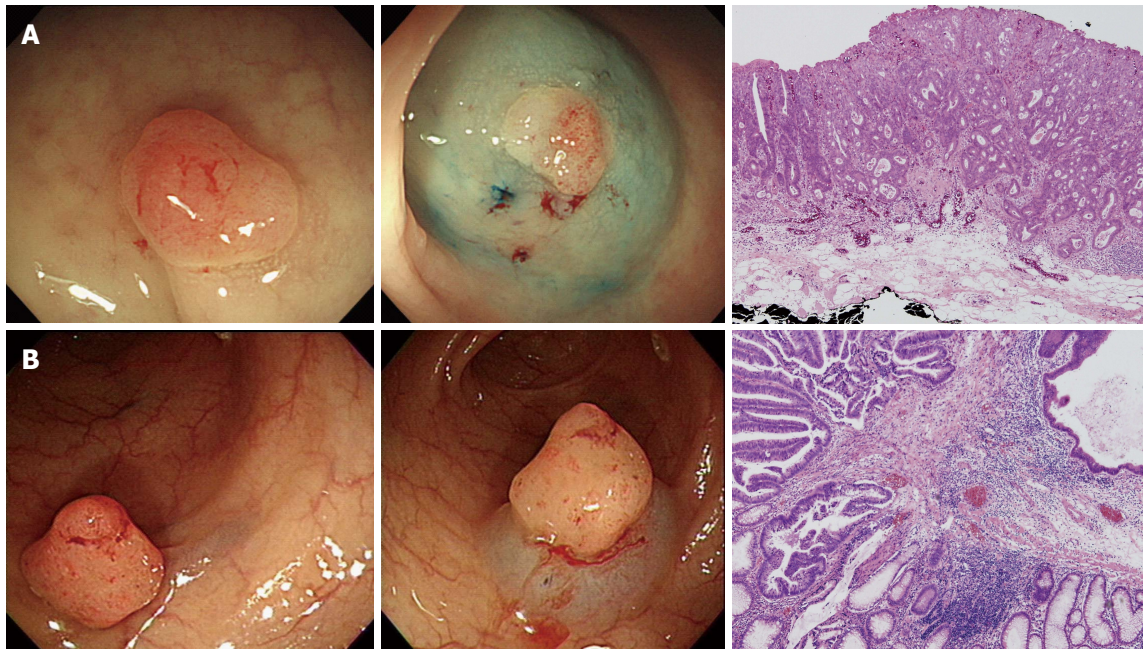


Figure 3 An 8-mm sessile polyp with negative non-lifting signs was treated by endoscopic mucosal resection (A); histology showed that cancer cells invaded the submucosa up to 200 μm (B); a 12-mm sized sessile polyp with negative non-lifting signs was treated by endoscopic mucosal resection. Histology showed that cancer cells invaded the submucosa to 2000 μm . The patient received additional surgery.

Table 1 Baseline characteristics and endoscopic findings according to the depth of invasion in submucosal cancer *n* (%)

| | Deep submucosal cancer (<i>n</i> = 25) | Superficial submucosal cancer (<i>n</i> = 39) | <i>P</i> value |
|---------------------------------|---|--|----------------|
| Age (mean \pm SD, yr) | 58.4 \pm 8.5 | 62.4 \pm 9.8 | 0.097 |
| Sex | | | 0.241 |
| Male | 13 (52.0) | 26 (66.7) | |
| Female | 12 (48.0) | 13 (33.3) | |
| Size (mm) | 16.88 \pm 3.28 | 15.28 \pm 3.64 | 0.080 |
| Shape | | | 0.397 |
| Sessile | 8 (32.0) | 18 (46.2) | |
| Pedunculated | 5 (20.0) | 4 (10.3) | |
| Superficial (Flat) | 12 (48.0) | 17 (43.6) | |
| Location | | | 0.581 |
| Right colon | 8 (32.0) | 10 (25.6) | |
| Left colon | 17 (68.0) | 29 (74.4) | |
| Endoscopic finding | | | |
| Morphological features | | | 0.084 |
| Any of them | 22 (88.0) | 27 (69.2) | |
| None of them | 3 (12.0) | 12 (30.8) | |
| (P, S) Loss of lobulation | 3/13 (23.1) | 7/22 (31.8) | 0.709 |
| (P, S) Excavation | 0/13 (0.0) | 1/22 (4.5) | 1.000 |
| (All) Demarcated depressed area | 13/25 (52.0) | 17/39 (43.6) | 0.511 |
| (P) Stalk swelling | 5/5 (100.0) | 2/4 (50.0) | 0.167 |
| (F) Fullness | 3/12 (25.0) | 0/17 (0.0) | 0.060 |
| (F) Fold convergency | 1/12 (8.3) | 0/17 (0.0) | 0.414 |
| (All) Ulcer bleeding | 1/25 (4.0) | 1/39 (2.6) | 1.000 |
| Pit pattern | | | 1.000 |
| Non-invasive | 1 (4.0) | 2 (5.1) | |
| Invasive | 24 (96.0) | 37 (94.9) | |
| Non-lifting sign | | | 0.010 |
| Positive | 17 (85.0) | 17 (48.6) | |
| Negative | 3 (15.0) | 18 (51.4) | |

Superficial tumors: Superficial submucosal cancer and adenoma; P: Pedunculated type; S: Sessile type; F: Flat/depressed type.

as invasion less than 1000 μm and deep SM cancer was defined as invasion greater than 1000 μm .

Statistical analyses

The primary outcome of the present study was to evaluate the different endoscopic findings between small, deep SM cancer and small, superficial SM cancers. Patients' baseline characteristics were analyzed using descriptive statistics. The differences of categorical variables were analyzed by Fisher's exact test. Continuous variables were analyzed by the Student's *t* test. The morphological features were analyzed according to shape. Stalk swelling was assessed for only the pedunculated type; loss of lobulation and excavation were assessed for both pedunculated and sessile types; fullness and fold convergence were assessed for the flat/depressed type; demarcated depressed areas were assessed for all three types. Continuous variables are expressed as the means \pm SD. *P* values of less than 0.05 were considered statistically significant. Statistical analyses were carried out using SPSS 18.0 (SPSS, Chicago, IL, United States) and SAS 9.2 (SAS Institute Inc., Cary, NC, United States).

RESULTS

SM cancers vs adenomas

Baseline characteristics and endoscopic findings between SM cancers and adenomas were described at the Table 1. The size of SM cancers was bigger than that of adenomas (15.91 mm vs. 11.47 mm, *P* < 0.001) and the superficial (flat) shape was frequently observed in SM cancer than adenomas (45.3% vs 13.2%, *P* < 0.001).

Table 2 Baseline characteristics and endoscopic findings according to the depth of invasion *n* (%)

| | Deep submucosal cancer (<i>n</i> = 25) | Superficial tumors (<i>n</i> = 191) | <i>P</i> value |
|---------------------------------|---|--------------------------------------|----------------|
| Age (mean ± SD, yr) | 58.4 ± 8.5 | 61.0 ± 9.4 | 0.191 |
| Sex | | | 0.179 |
| Male | 13 (52.0) | 128 (67.0) | |
| Female | 12 (48.0) | 63 (33.0) | |
| Size (mm) | 16.88 ± 3.28 | 12.25 ± 4.93 | < 0.001 |
| Shape | | | 0.005 |
| Sessile | 8 (32.0) | 106 (55.5) | |
| Pedunculated | 5 (20.0) | 48 (25.1) | |
| Superficial (Flat) | 12 (48.0) | 37 (19.4) | |
| Location | | | 1.000 |
| Right colon | 8 (32.0) | 57 (29.8) | |
| Left colon | 17 (68.0) | 134 (70.2) | |
| Endoscopic finding | | | |
| Morphological features | | | |
| Any of them | 22 (88.0) | 59 (30.9) | < 0.001 |
| None of them | 3 (12.0) | 132 (69.1) | |
| (P, S) Loss of lobulation | 3/13 (23.1) | 27/154 (17.5) | 0.705 |
| (P, S) Excavation | 0/13 (0.0) | 3/154 (1.9) | 1.000 |
| (All) Demarcated depressed area | 13/25 (52.0) | 30/191 (15.7) | < 0.001 |
| (P) Stalk swelling | 5/5 (100.0) | 2/48 (4.2) | < 0.001 |
| (F) Fullness | 3/12 (25.0) | 0/37 (0.0) | 0.001 |
| (F) Fold convergence | 1/12 (8.3) | 0/37 (0.0) | 0.310 |
| (All) Ulcer bleeding | 1/24 (4.0) | 2/189 (1.0) | 0.245 |
| Pit pattern | | | < 0.001 |
| Non-invasive | 1 (4.0) | 154 (80.6) | < 0.001 |
| Invasive | 24 (96.0) | 37 (19.4) | |
| Non-lifting sign | | | < 0.001 |
| Positive | 17 (85.0) | 22 (28.6) | |
| Negative | 3 (15.0) | 55 (71.4) | |

Superficial tumors: Superficial submucosal cancer and adenoma; P: Pedunculated type; S: Sessile type; F: Flat/depressed type.

Also, demarcated depressed areas, stalk swelling, and fullness were more common in SM cancers than adenomas (demarcated depressed areas: 46.9% *vs* 8.6%, *P* < 0.001; stalk swelling: 77.8% *vs* 0%, *P* < 0.001; fullness: 10.9% *vs* 0%, *P* = 0.001). Other morphological features (*i.e.*, loss of lobulation, excavation, fold convergence, and bleeding ulcers) were not statistically different between the two groups. Among SM cancers, 95.3% of polyps showed invasive pit patterns, while 0% of adenomas showed invasive pit patterns (*P* < 0.001). A positive non-lifting sign was more common in SM cancers than in adenomas (45.3% *vs* 11.9%, *P* < 0.001).

Deep SM cancers vs superficial SM cancers

Baseline characteristics of 64 submucosal cancer patients were as follows. Among 64 SM cancers, 6 cases (9%) were 10 mm or less in size, 33 cases (52%) were 11–15 mm in size, and 25 cases (39%) were 16–19 mm in size. Deep SM cancers were larger than superficial SM cancers, but it was not statistically significant (16.88 mm *vs* 15.28 mm, *P* = 0.080). The flat and sessile types were more common in two groups than pedunculated type. The distributions of cancers were similar in the two groups, more common in left colon (68.0% in deep SM, and 74.4% in superficial sm). Non-lifting sign was more common in deep SM can-

Table 3 Diagnostic accuracy of conventional endoscopic features

| | Sensitivity | Specificity | PPV | NPV | Accuracy |
|-----------------------------------|-------------|-------------|------|------|----------|
| Invasive morphology ¹ | 0.88 | 0.69 | 0.27 | 0.98 | 0.71 |
| Invasive pit pattern ¹ | 0.96 | 0.81 | 0.39 | 0.99 | 0.82 |
| Non-lifting sign ² | 0.85 | 0.73 | 0.46 | 0.95 | 0.75 |

¹Evaluated in all polyps; ²Evaluated in sessile and flat polyps. Deep submucosal cancer *vs* superficial tumors. PPV: Positive predictive value; NPV: Negative predictive value.

cers than in superficial SM cancers (85.0% *vs* 48.6%, *P* = 0.010).

Deep submucosal cancers vs superficial tumors

Baseline characteristics and endoscopic findings according to the depth of invasion were seen at the Table 2. When comparing endoscopic findings between deep SM cancers and superficial tumors, the size of deep submucosal cancers was bigger than that of superficial lesions (16.9 mm *vs* 12.3 mm, *P* < 0.001). Also, demarcated depressed areas, stalk swelling, and fullness were more common in deep SM cancers than superficial tumors (demarcated depressed areas: 52.0% *vs* 15.7%, *P* < 0.001; stalk swelling: 100% *vs* 4.2%, *P* < 0.001; fullness: 25.0% *vs* 0%, *P* = 0.001; Table 2). Other morphological features (*i.e.*, loss of lobulation, excavation, fold convergence, and bleeding ulcers) were not statistically different between the two groups. Among deep SM cancers, 96% of polyps showed invasive pit patterns, while 19.4% of superficial tumors showed invasive pit patterns (*P* < 0.001). A positive non-lifting sign was more common in deep SM cancers than in superficial tumors (85.0% *vs* 28.6%, *P* < 0.001).

Diagnostic accuracy of endoscopic features for deep SM cancer

When comparing deep SM cancers with superficial SM tumors, the sensitivity, specificity, PPV, and NPV of any of the invasive morphology were 88%, 69%, 27% and 98%, respectively (Table 3). The sensitivity, specificity, PPV, and NPV of invasive pit patterns were 96%, 81%, 46%, and 95%, respectively. The sensitivity, specificity, PPV, and NPV of non-lifting signs were 85%, 73%, 46% and 95% in sessile and flat polyps, respectively. The diagnostic accuracy of the presence of morphological features, invasive pit patterns, and non-lifting sign were 71%, 82% and 75%, respectively.

Treatment

Among 39 superficial SM cancers, 30 cases were initially treated with EMR (28 cases) or ESD (2 cases) and nine cases were treated with surgery (Figure 4). Among 25 deep SM cancers, 17 cases were initially treated with endoscopic techniques (polypectomy, 1 case; EMR, 15 cases; ESD, 1 case). Subsequently, 13 cases received further surgical treatment, and one case showed lymph node metastasis. Eight cases with deep SM cancers were ini-

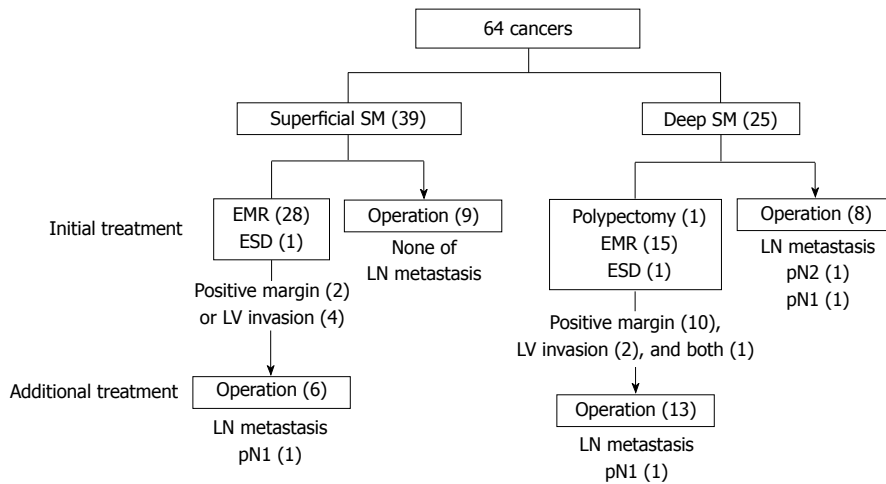


Figure 4 Diagram for the treatment of 64 early colorectal cancers. SM: Submucosa; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

tially treated with surgery.

DISCUSSION

A recent study showed that endoscopic treatment of SM cancers was safe and feasible with favorable long-term efficacy when the following conditions are satisfied: a lesion is determined histopathologically to be well differentiated; invasion of the SM layer is less than 1000 μm (superficial SM cancer); and the lesion is negative for both lymphovascular invasion and sprouting^[17]. Thus, it is important to estimate whether the depth of SM invasion is less than 1000 μm to determine appropriate treatment. However, the present study showed that conventional endoscopic features were insufficient to differentiate deep SM cancers less than 20 mm in size resulting in a diagnostic accuracy of 79%. Among 25 patients with deep SM cancers, 68% were initially under-treated.

During colonoscopy, endoscopists usually assess colorectal polyps according to morphological findings and choose a treatment. The present study evaluated seven morphological features of polyps and found that demarcated depression, fullness, and stalk swelling were typical findings of small deep SM cancers. A previous study, which included polyps larger than 20 mm in size, showed that a loss of lobulation was also a typical endoscopic finding^[14], but this observation was not confirmed by the present study. The diagnostic accuracy of the presence of any of the invasive morphological features was 71%, meaning that the morphological characteristics themselves are insufficient to assess depth of invasion of small colorectal neoplasms. In a previous study, Uno *et al.*^[15] reported the clinical usefulness of non-lifting signs to predict depth of invasion for colorectal neoplasms. Adenomas or superficial SM cancers are readily lifted by SM injection, thus the non-lifting signs are clinically used to determine the therapeutic strategy for advanced colorectal neoplasm. A previous study showed that the accuracy of non-lifting signs for deep SM cancers were 94.8%^[18]. However, this does not seem to be applied to

small polyps, as 15% of patients with deep SM cancers showed negative non-lifting signs and were treated with EMR. The present study revealed that the non-lifting signs were limited to predict deep SM invasion in polyps less than 20 mm in size. In this study, NPVs for SM deep cancers of invasive morphology, invasive pit pattern and non-lifting sign are over 95%. Surely, High NPVs useful for determination of treatment strategy. Nevertheless, the diagnostic accuracy for SM deep cancers was not sufficient and it leads to initial under-treatment.

Recently magnifying chromoendoscopy has been used to assess depth of invasion of colorectal polyps, overcoming the limitations of morphological features^[19]. Pit pattern classification of colorectal neoplasm, initially proposed by Kudo and modified by Kudo and Tsuruta, is reported to be related to the histologic characteristics of the lesions^[20]. A previous study demonstrated that invasive pit patterns are able to differentiate superficial SM cancers from deep SM cancers with the diagnostic accuracy of 98.8%^[12]. Under magnifying chromoendoscopy, Kudo's classification type V pit pattern is usually considered to be invasive to the SM, and type VN is strongly suggestive of deep SM cancer^[21]. Therefore, it is crucial to discriminate between type VI and type VN patterns to assess precisely the depth of invasion of colorectal polyps. Under non-magnifying colonoscopy, it is difficult to discriminate type VN; for that reason, the present study showed a low diagnostic accuracy of pit patterns for deep SM cancers. However, it is unrealistic for clinics to apply this method as magnifying chromoendoscopy is not a conventional or universal method for screening or simple surveillance colonoscopy.

The present study has several limitations. First, because of the retrospective study design, there were some cases with poor qualified photos which made it difficult to precisely evaluate all of the endoscopic features. Second, pit patterns were evaluated after the conventional endoscopic diagnoses, suggesting an influence by the morphological features of polyps. Finally, pit patterns were evaluated by only standard colonoscopy; thus, it was

impossible to discriminate the type VN pit pattern, which is strongly suggestive of deep SM cancer. Therefore, the results of the present study should not be simply compared to those of previous studies using magnifying chromoendoscopy in terms of clinical usefulness. From a different point of view, the present study is more realistic because magnifying chromoendoscopy is not usually used in screening or surveillance colonoscopy.

In conclusion, although the prevalence of SM invasion is low in small colorectal polyps, the present study showed that conventional endoscopic findings, such as morphological features, non-lifting sign, and invasive pit patterns, are insufficient to discriminate deep SM cancers to determine therapeutic strategy under white light standard colonoscopy. Further studies are mandatory to evaluate precisely the depth of invasion in small colorectal polyps, using magnifying chromoendoscopy or NBI.

COMMENTS

Background

At present, colorectal neoplasms either confined to the mucosa or after invasion to submucosa (SM) with a size less than 1000 μ m are considered good candidates for endoscopic treatment. In contrast, because 6%-12% of lymph node metastases have been reported in deep SM cancers, these lesions are not indicated for endoscopic treatment. Therefore, it is crucial to precisely evaluate the depth of invasion in advanced colorectal neoplasm for adequate therapeutic treatment.

Research frontiers

This present study was designed to evaluate the depth of invasion of small, early colorectal cancers using conventional endoscopic features. This study exhibited that invasive pit pattern was more accurate finding than morphological features or non-lifting sign to discriminate small, deep SM cancers from superficial tumors by white light, standard colonoscopy. In addition, it is insufficient to discriminate deep SM cancers to determine therapeutic strategy under white light standard colonoscopy only. Recent innovative technology has allowed endoscopists to differentiate advanced colorectal neoplasms during colonoscopy. Especially, magnifying chromoendoscopy or narrow-band imaging has been widely studied to assess depth of invasion. Nevertheless, these techniques have some barriers and unrealistic to apply to the clinic for the daily practice. Therefore, future research should aim to develop the practical method or technology to evaluate the precise depth of invasion of small colorectal neoplasms.

Innovations and breakthroughs

This study identified the limitation of white light standard colonoscopy in the depth of invasion evaluation of small colorectal neoplasms. Furthermore, this study suggests the further studies to develop the method or technology to evaluate the precise depth of invasion of small colorectal neoplasms.

Applications

This study could be helpful to establish the therapeutic plan when the small polyps detected in usual colonoscopy using white light standard endoscopy.

Peer review

It is insufficient to discriminate deep SM cancers to determine therapeutic strategy under white light standard colonoscopy only. These results are interesting and these findings arouse the necessity for the further studies to develop the practical method or technology to evaluate the precise depth of invasion of small colorectal neoplasms.

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Inflammatory markers as selection criteria of hepatocellular carcinoma in living-donor liver transplantation

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Abstract

AIM: To investigate that inflammatory markers can predict accurately the prognosis of hepatocellular carcinoma (HCC) patients in living-donor liver transplantation (LDLT).

METHODS: From October 2000 to November 2011, 224 patients who underwent living donor liver transplantation for HCC at our institution were enrolled in this study. We analyzed disease-free survival (DFS) and overall survival (OS) after LT in patients with HCC and designed a new score model using pretransplant neutrophil-lymphocyte ratio (NLR) and C-reactive protein (CRP).

RESULTS: The DFS and OS in patients with an NLR level ≥ 6.0 or CRP level ≥ 1.0 were significantly worse than those of patients with an NLR level < 6.0 or CRP level < 1.0 ($P = 0.049$, $P = 0.003$ for NLR and $P = 0.010$, $P < 0.001$ for CRP, respectively). Using a new score model using the pretransplant NLR and CRP, we can differentiate HCC patients beyond the Milan criteria with a

good prognosis from those with a poor prognosis.

CONCLUSION: Combined with the Milan criteria, new score model using NLR and CRP represent new selection criteria for LDLT candidates with HCC, especially beyond the Milan criteria.

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Key words: C-reactive protein; Hepatocellular carcinoma; Liver transplantation; Neutrophil-lymphocyte ratio; Selection criteria

Core tip: Although the Milan criteria are accepted as the standard selection criteria for liver transplantation candidates with hepatocellular carcinoma (HCC), they are so strict; New selection criteria are needed to predict more accurately the prognosis of patients with HCC; Using a new score model using pretransplant neutrophil-lymphocyte ratio (NLR) and C-reactive protein (CRP), we can differentiate HCC patients beyond the Milan criteria with a good prognosis from those with a poor prognosis; Combined with the Milan criteria, a new score model using pretransplant NLR and CRP may represent new selection criteria for living-donor liver transplantation candidates with HCC, especially beyond the Milan criteria.

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INTRODUCTION

Among the several treatment modalities for hepatocellular

lar carcinoma (HCC), liver transplantation (LT) and surgical resection are curative methods. Chronic liver disease is one of the main etiologies of HCC; approximately 80% of patients with HCC have cirrhosis^[1]. LT is an ideal treatment for selected patients with HCC because it targets not only the tumor but also the underlying liver disease^[2]. Since the introduction of the Milan criteria by Mazzaferro *et al.*^[3] in 1996, disease-free survival and overall survival after LT for patients with HCC meeting the Milan criteria have been equivalent to those of non-HCC patients. Although survival rates after LT have improved dramatically, HCC recurrence remains a significant problem. It has been demonstrated that about 10% of HCC patients within the Milan criteria experience HCC recurrence^[4]. In contrast, some patients with HCC beyond the Milan criteria may have favorable outcomes^[5]. There are some limitations to the Milan criteria that should be addressed in any new standard selection criteria for HCC patients. Inflammation is a critical component of tumor progression^[6]. Several inflammatory markers, such as the neutrophil-lymphocyte ratio (NLR) and C-reactive protein (CRP), have been suggested as surrogate markers of treatment response and survival in patients with HCC^[7-9]. However, few studies have examined the relationship between these factors and HCC recurrence after living-donor liver transplantation (LDLT).

The aim of the present study was to assess whether the pretransplant NLR and CRP levels can accurately predict disease-free survival and overall survival after LDLT in patients with HCC. Furthermore, we established a pretransplant score model that may assist in the selection of patients with HCC that would benefit from liver transplantation.

MATERIALS AND METHODS

Patients

From October 2000 to November 2011, a total of 243 patients underwent LDLT for HCC at our transplant center. Nineteen patients were excluded from the study: 7 for postoperative mortality within 30 d after transplantation, 6 for preoperative infection, 4 for undergoing pretransplant locoregional treatment within 1 mo before transplantation, and 2 for massive alimentary tract bleeding within 1 mo before transplantation. After excluding these nineteen cases, the medical records of 224 patients were reviewed retrospectively. This study was approved by the Institutional Review Board of our center.

Diagnosis and treatment strategy of HCC

All patients who were to undergo transplantation for HCC were evaluated preoperatively by dynamic liver computed tomography (CT) and enhanced magnetic resonance imaging (MRI). Chest CT, bone scan, and positron emission tomography CT (PET CT) were performed to exclude distant metastasis and other primary malignancies. Contraindications for LT in patients with HCC included a tumor thrombus in the main portal vein,

regional lymph node metastasis, and distant metastasis. The alpha-fetoprotein (AFP) level and the proteins induced by vitamin K absence or angiotensin- II (PIVKA-II) level were evaluated as tumor markers. Hepatitis viral markers and liver function tests were also assessed. Neutrophil and lymphocyte counts were routinely measured on the day before transplantation, with the NLR calculated by dividing the neutrophil count by the lymphocyte count. The serum CRP level was measured with a turbidimetric immunoassay (Wako Chemicals GmbH, Neuss, Germany). The pretransplant CRP level was not measured routinely until 2006 and then was checked routinely on the day before transplantation. When HCC was diagnosed, the treatment was based on the tumor stage and the patient's liver function. Patients who were eligible for transplantation underwent DDLT or LDLT according to the meeting the Milan criteria and the availability of liver donor. Pretransplant locoregional treatments include hepatic resection, transarterial chemoembolization (TACE), radiofrequency ablation, and percutaneous ethanol injection, which were selected according to the Barcelona Clinic Liver Cancer scoring system. TACE was the primary treatment modality among pretransplant locoregional treatment modalities.

Liver transplantation and post-transplant follow-up

LDLT was performed according to a standard technique using a modified right lobe with middle hepatic vein reconstruction. For patients with ascites, aspiration and cytology were performed before beginning the operation. When lymph node enlargement was present, or in cases with suspicious metastatic disease, an intraoperative biopsy was performed. The operation was performed only in cases with negative biopsy results.

Immunosuppression regimens consisted of a triple drug regimen that included tacrolimus or cyclosporin, mycophenolatemofetil (MMF), and prednisolone. The dose of tacrolimus was adjusted to maintain levels of 7-10 ng/mL for the first postoperative month and 5-7 ng/mL thereafter. The dose of cyclosporin was adjusted to maintain levels of 100-150 ng/mL for the first postoperative month and 50-100 ng/mL thereafter. Steroids were withdrawn 1 mo after surgery, and MMF was withdrawn 6 mo after surgery. An interleukin-2 receptor blocker was administered on both the day of surgery and the fourth postoperative day.

Patients were followed weekly after hospital discharge until they were stable and then monthly for the first year, every 2 mo for 5 years, and then every 3 mo. Tumor markers were measured monthly during the first year, and then every 2 mo thereafter. Abdomen CT, chest CT, and bone scintigraphy were routinely performed every 4 mo for the first year, every 6 mo for the next year, and then annually. When tumor recurrence was suspected, MRI and/or PET-CT were performed.

Statistical analysis

Continuous variables are reported as mean \pm SD and

Table 1 Clinical parameters of the study population *n* (%)

| Parameter | Value |
|--|----------------------------|
| Mean age (yr) ¹ | 51.9 ± 6.9 |
| Male | 184 (82.1) |
| Diagnosis | |
| Hepatitis B | 197 (87.9) |
| Hepatitis C | 13 (5.8) |
| Others | 14 (6.3) |
| GRWR ¹ | 1.21 ± 0.27 |
| Child pugh score ¹ | 8.2 ± 2.4 |
| MELD score ¹ | 12.8 ± 7.6 |
| AFP (ng/mL) ¹ | 183.4 ± 762.7 |
| PIVKA-II (mAU/mL) ¹ | 206.5 ± 1118.7 |
| C-reactive protein (mg/dL) ¹ | 1.36 ± 2.74 |
| Neutrophil lymphocyte ratio ¹ | 3.47 ± 4.68 |
| Pretransplant treatment for HCC | 167 (74.6) |
| Tumor number ¹ | 2.6 ± 2.4 |
| Maximum tumor size (cm) ¹ | 3.2 ± 3.1 |
| Microvascular invasion | 44 (21.3) |
| Edmondson-steiner grade (III-IV) | 81 (42.9) |
| Within the milan criteria | 133 (59.4) |
| Follow-up duration (mo) ¹ | 48.9 ± 37.3 |
| | (median: 68, range: 6-139) |

¹Values shown are mean ± SD except where stated otherwise. GRWR: Graft-to-recipient body weight ratio; MELD: Model for end-stage liver disease; AFP: Alpha-fetoprotein; PIVKA-II: Proteins induced by vitamin K antagonism or absence-II; HCC: Hepatocellular carcinoma.

were compared using the Student *t* test. Categorical variables were analyzed using the χ^2 test. To evaluate the risk factors for HCC recurrence, univariate analysis of risk factors was performed using the Kaplan-Meier method and evaluated using the log-rank test. Candidate predictors associated with a *P* value less than 0.2 on univariate analysis were entered into a multivariate analysis using Cox regression analysis. The CRP level was excluded from the multivariate analysis because the number of patients with an available CRP level was small compared with other clinico-pathological variables. Subgroup analysis by the Milan criteria was also conducted. Overall survival and disease-free survival were calculated using the Kaplan-Meier method and evaluated with the log-rank test. Statistical analysis was performed using SPSS (Chicago, IL, United States) 18.0 for Windows. A *P* value < 0.05 was considered to indicate statistical significance.

RESULTS

Patients' characteristics

Among the 224 patients, 184 (82.1%) were male, and the mean age was 51.9 ± 6.9 years. The most common cause for LT was hepatitis B (*n* = 197, 87.9%), followed by hepatitis C (*n* = 13, 5.8%) and other causes (*n* = 14, 6.3%). Pretransplant locoregional treatments for HCC were performed in 167 patients (74.6%). Of the 224 patients, 133 (59.4%) met the Milan criteria. The mean Child-Pugh score was 8.2 ± 2.4, and the mean Model for End-stage Liver Disease (MELD) score was 12.8 ± 7.6 (Table 1).

The median follow-up period was 68 mo (range, 6-139 mo). The 1, 3, and 5 year overall survival rates were

Table 2 Disease-free survival and overall survival according to neutrophil-lymphocyte ratio and C-reactive protein

| | Disease-free survival | | Overall survival | |
|---|-----------------------|----------------|------------------|----------------|
| | χ^2 | <i>P</i> value | χ^2 | <i>P</i> value |
| Neutrophil-lymphocyte ratio (<i>n</i> = 224) | | | | |
| NLR ≥ 1 (<i>n</i> = 195) | 1.041 | 0.308 | 0.125 | 0.724 |
| NLR ≥ 2 (<i>n</i> = 115) | 2.938 | 0.087 | 2.777 | 0.096 |
| NLR ≥ 3 (<i>n</i> = 70) | 0.746 | 0.388 | 3.308 | 0.069 |
| NLR ≥ 4 (<i>n</i> = 44) | 1.132 | 0.287 | 5.301 | 0.021 |
| NLR ≥ 5 (<i>n</i> = 34) | 2.383 | 0.123 | 7.257 | 0.007 |
| NLR ≥ 6 (<i>n</i> = 27) | 3.497 | 0.049 | 8.799 | 0.003 |
| NLR ≥ 7 (<i>n</i> = 22) | 1.379 | 0.240 | 6.411 | 0.001 |
| C-reactive protein (<i>n</i> = 145) | | | | |
| CRP ≥ 0.5 (<i>n</i> = 72) | 1.359 | 0.244 | 4.032 | 0.045 |
| CRP ≥ 1.0 (<i>n</i> = 42) | 6.653 | 0.010 | 12.604 | < 0.001 |
| CRP ≥ 2.0 (<i>n</i> = 25) | 6.974 | 0.008 | 6.728 | 0.009 |

NLR: Neutrophil-lymphocyte ratio; CRP: C-reactive protein.

88.5%, 78.0% and 76.6%, respectively. During the follow-up period, 50 patients (22.3%) died. The cause of death was HCC recurrence in 31 patients (62.0%), technical complications in nine patients (18.0%), sepsis in five patients (10.0%), graft failure in three patients (6.0%), and other causes in two patients (4.0%). The 1, 3, and 5 year disease-free survival rates were 88.3%, 83.3% and 81.6%, respectively. Most HCC recurrences (*n* = 30, 81.1%) occurred within 2 years, with 26 patients (70.3%) experiencing HCC recurrence within 1 year. Two patients (5.4%) experienced HCC recurrence 5 years after transplantation.

Correlation between NLR and HCC recurrence after LDLT

To determine whether an elevated NLR level was correlated with HCC recurrence after LDLT, we performed the Kaplan-Meier analysis with the log-rank test. Using NLR cut-offs of 1, 2, 3, 4, 5, 6 and 7 and comparing disease-free survival and overall survival rates, we showed that an NLR of 6 was the most significant, with a χ^2 value of 3.497 and a *P* value of 0.049 for disease-free survival and a χ^2 value of 8.799 and a *P* value of 0.003 for overall survival (Table 2). Of the 224 patients, 27 (12.1%) had an NLR ≥ 6.0. Total bilirubin level (*P* = 0.006), Child-Pugh score (*P* < 0.001), MELD score (*P* < 0.001), and CRP level (*P* = 0.035) were significantly different between the patients with an NLR level ≥ 6.0 and those with an NLR level < 6.0. Tumor number and maximum tumor size, tumor biologic factors such as microvascular invasion and tumor grade, and tumor markers were not significantly different between the two groups of patients. Also, the NLR level was significantly correlated with the total bilirubin level (*r* = 0.384, *P* < 0.001), Child-Pugh score (*r* = 0.268, *P* < 0.001), MELD score (*r* = 0.419, *P* < 0.001), and CRP level (*r* = 0.220, *P* = 0.008) (Table 3).

Correlation between CRP and HCC recurrence after LDLT

To determine whether the elevated CRP level was correlated with HCC recurrence after LDLT, we performed

Table 3 Clinicopathological characteristics according to Neutrophil-lymphocyte ratio and C-reactive protein *n* (%)

| Parameters | NLR < 6 (<i>n</i> = 197) | NLR ≥ 6 (<i>n</i> = 27) | <i>P</i> value | CRP < 1 (<i>n</i> = 103) | CRP ≥ 1 (<i>n</i> = 42) | <i>P</i> value |
|--------------------------------------|------------------------------|-----------------------------|----------------|------------------------------|-----------------------------|----------------|
| Mean age (yr) ¹ | 51.8 ± 7.0 | 52.2 ± 6.3 | 0.748 | 52.4 ± 6.9 | 52.4 ± 6.8 | 0.992 |
| Male | 164 (83.2) | 20 (74.1) | 0.243 | 81 (78.6) | 35 (83.3) | 0.522 |
| Etiology (HBV) | 172 (87.3) | 25 (92.6) | 0.345 | 90 (87.4) | 37 (88.1) | 0.991 |
| GRWR ¹ | 1.20 ± 0.27 | 1.24 ± 0.30 | 0.495 | 1.21 ± 0.29 | 1.23 ± 0.26 | 0.777 |
| Total bilirubin (g/dL) ¹ | 3.8 ± 6.8 | 11.1 ± 12.5 | 0.006 | 3.2 ± 5.8 | 11.5 ± 12.2 | < 0.001 |
| PT INR ¹ | 2.6 ± 15.3 | 1.7 ± 0.5 | 0.771 | 1.4 ± 0.3 | 6.6 ± 33.2 | 0.293 |
| Child Pugh score ¹ | 7.9 ± 2.3 | 9.8 ± 2.2 | < 0.001 | 7.8 ± 2.4 | 9.9 ± 1.9 | < 0.001 |
| MELD score ¹ | 11.7 ± 6.8 | 20.6 ± 8.8 | < 0.001 | 10.9 ± 6.0 | 19.4 ± 8.9 | < 0.001 |
| AFP (ng/mL) ¹ | 142 ± 366 | 490 ± 1995 | 0.383 | 128 ± 381 | 346 ± 1593 | 0.389 |
| NLR ¹ | 2.12 ± 1.19 | 13.3 ± 7.79 | < 0.001 | 2.54 ± 2.71 | 6.15 ± 7.37 | 0.003 |
| CRP (mg/dL) ¹ | 1.03 ± 2.13 | 3.55 ± 4.76 | 0.035 | 0.33 ± 0.29 | 3.87 ± 4.13 | < 0.001 |
| Tumor number ¹ | 2.5 ± 2.3 | 3.3 ± 2.8 | 0.162 | 2.7 ± 2.4 | 2.4 ± 2.1 | 0.352 |
| Maximal tumor size ¹ | 3.10 ± 2.37 | 4.03 ± 6.13 | 0.441 | 2.9 ± 1.5 | 4.1 ± 5.8 | 0.475 |
| Microvascular invasion (+) | 38 (21.0) | 6 (23.1) | 0.800 | 20 (21.1) | 6 (15.0) | 0.225 |
| E-S grade (III-IV) | 94 (57.3) | 14 (56.0) | 0.901 | 38 (44.7) | 17 (45.9) | 0.415 |
| Beyond the Milan criteria | 69 (36.5) | 14 (51.9) | 0.125 | 39 (39.0) | 15 (36.6) | 0.899 |
| Pretransplant locoregional treatment | 146 (74.1) | 21 (77.8) | 0.682 | 81 (78.6) | 30 (71.4) | 0.352 |

¹Values shown are mean ± SD. NLR: Neutrophil-lymphocyte ratio; CRP: C-reactive protein; HBV: Hepatitis B virus; GRWR: Graft-to-recipient body weight ratio; PT INR: Prothrombin time international normalized ratio; MELD: Model for end-stage liver disease; AFP: Alpha-fetoprotein; E-S grade: Edmondson grade.

the Kaplan-Meier analysis with the log-rank test. Using CRP cut-offs of 0.5, 1.0 and 2.0 and comparing disease-free survival and overall survival rates, we showed that a CRP of 1.0 was the most significant, with a χ^2 value of 6.653 and a *P* value of 0.010 for disease-free survival and a χ^2 value of 12.604 and a *P* value of less than 0.001 for overall survival (Table 2). Of the 145 patents, 42 (29.0%) had a CRP ≥ 1.0. Total bilirubin level (*P* < 0.001), Child-Pugh score (*P* < 0.001), MELD score (*P* < 0.001), and NLR level (*P* = 0.003) were significantly different between patients with a CRP level ≥ 1.0 and those with a CRP level < 1.0, as with the results for NLR. Tumor number and maximum tumor size, tumor biologic factors such as microvascular invasion and tumor grade, and tumor markers were not significantly different between the two groups of patients. Also, the CRP level was significantly correlated with the total bilirubin level (*r* = 0.207, *P* = 0.012), Child-Pugh score (*r* = 0.216, *P* = 0.009), MELD score (*r* = 0.272, *P* = 0.001), the tumor number (*r* = 0.415, *P* < 0.001), and the NLR level (*r* = 0.220, *P* = 0.008) (Table 3).

Prognostic factors for disease free survival and overall survival after LDLT

On univariate analysis, the NLR level ≥ 6.0 (*P* = 0.049), CRP level ≥ 1.0 (*P* = 0.010), AFP ≥ 100 (*P* = 0.015), pretransplant locoregional treatment (*P* = 0.017), tumor size ≥ 5 cm (*P* < 0.001), and microvascular invasion (*P* = 0.024) were significantly associated with HCC recurrence after LDLT. According to multivariate analysis, AFP ≥ 100 (HR = 2.588, 95%CI: 1.187-5.645, *P* = 0.017) and tumor size ≥ 5 cm (HR = 6.014; 95%CI: 2.432-14.869, *P* < 0.001) were independent risk factors for HCC recurrence after LDLT. An NLR level ≥ 6.0 was a significant risk factor for tumor recurrence with marginal significance

(HR = 2.512; 95%CI: 0.987-6.391, *P* = 0.053). Also, an NLR level ≥ 6.0 (*P* = 0.003), CRP level ≥ 1.0 (*P* < 0.001), AFP ≥ 100 (*P* = 0.048), pretransplant locoregional treatment (*P* = 0.023), and tumor size ≥ 5 cm (*P* = 0.001) were significantly associated with overall survival after LDLT on univariate analysis. An NLR level ≥ 6.0 was an only independent prognostic factor for poor survival on multivariate analysis (HR = 2.896; 95%CI: 1.399-5.996, *P* = 0.004) (Table 4).

We analyzed disease-free survival and overall survival for patients according to the Milan criteria. For 133 patients (59.4%) within the Milan criteria, an NLR level ≥ 6.0 was associated with decreased overall survival (*P* = 0.037) but was not associated with disease-free survival (*P* = 0.541). A CRP level ≥ 1.0 was not associated with disease-free survival (*P* = 0.797) but was associated with decreased overall survival, but with only marginal significance (*P* = 0.054). A tumor size ≥ 5 cm was an only independent prognostic factor for HCC recurrence on multivariate analysis (HR = 6.980; 95%CI: 1.497-32.535), *P* = 0.013) (Table 5).

For 91 patients (40.6%) beyond the Milan criteria, NLR level ≥ 6.0 (HR = 3.973; 95%CI: 1.288-12.249, *P* = 0.016) and AFP ≥ 100 (HR = 3.619; 95%CI: 1.184-11.063, *P* = 0.024) were independent risk factors for HCC recurrence after LDLT. An NLR level ≥ 6.0 was an only independent prognostic factor for poor survival on multivariate analysis (HR = 4.685; 95%CI: 1.607-13.657, *P* = 0.005). A CRP level ≥ 1.0 was significantly associated with both disease-free survival (*P* = 0.004) and overall survival (*P* = 0.001) on univariate analysis (Table 5).

Pretransplant prognostic score model using NLR and CRP

We established a pretransplant new prognostic factor

Table 4 Prognostic factors for disease free survival and overall survival in the whole study population

| | Disease free survival | | | Overall survival | | |
|--------------------------------------|-----------------------|-----------------------|----------------|------------------|-----------------------|----------------|
| | Univariate | Multivariate analysis | | Univariate | Multivariate analysis | |
| | <i>P</i> value | HR (95%CI) | <i>P</i> value | <i>P</i> value | HR (95%CI) | <i>P</i> value |
| Age \geq 60 | 0.404 | | | 0.723 | | |
| Male gender | 0.196 | 2.086 (0.595-7.307) | 0.250 | 0.411 | | |
| Etiology (HBV) | 0.977 | | | 0.775 | | |
| GRWR \geq 1.0 | 0.551 | | | 0.639 | | |
| Child C | 0.978 | | | 0.810 | | |
| AFP \geq 100 | 0.015 | 2.588 (1.187-5.645) | 0.017 | 0.048 | 1.567 (0.822-2.987) | 0.172 |
| NLR \geq 6 | 0.049 | 2.512 (0.987-6.391) | 0.053 | 0.003 | 2.896 (1.399-5.996) | 0.004 |
| Pretransplant locoregional treatment | 0.017 | 4.604 (1.074-19.740) | 0.040 | 0.023 | 1.946 (0.804-4.713) | 0.140 |
| Tumor number \geq 2 | 0.604 | | | 0.913 | | |
| Tumor size \geq 5 cm | < 0.001 | 6.014 (2.432-14.869) | < 0.001 | 0.001 | 2.206 (0.968-5.028) | 0.060 |
| Microvascular invasion (+) | 0.024 | 1.369 (0.599-3.132) | 0.456 | 0.074 | 1.554 (0.769-3.141) | 0.220 |
| E-S grade (III-IV) | 0.172 | 1.040 (0.470-2.302) | 0.923 | 0.082 | 1.371 (0.715-2.630) | 0.342 |
| CRP \geq 1.0 (<i>n</i> = 145) | 0.010 | | | < 0.001 | | |

HBV: Hepatitis B virus; GRWR: Graft-to-recipient body weight ratio; AFP: Alpha-fetoprotein; NLR: Neutrophil lymphocyte ratio; E-S grade: Edmondson grade; CRP: C-reactive protein.

Table 5 Prognostic factors for disease free survival and overall survival in patients with hepatocellular carcinoma according to the Milan criteria

| | Disease free survival | | | Overall survival | | |
|--------------------------------------|-----------------------|-----------------------|----------------|------------------|-----------------------|----------------|
| | Univariate | Multivariate analysis | | Univariate | Multivariate analysis | |
| | <i>P</i> value | HR (95%CI) | <i>P</i> value | <i>P</i> value | HR (95%CI) | <i>P</i> value |
| Within the Milan criteria | | | | | | |
| AFP \geq 100 | 0.475 | | | 0.471 | | |
| NLR \geq 6 | 0.541 | | | 0.037 | 2.509 (0.945-6.658) | 0.065 |
| Pretransplant locoregional treatment | 0.193 | 2.126 (0.587-7.710) | 0.251 | 0.257 | | |
| Tumor number \geq 2 | 0.764 | | | 0.459 | | |
| Tumor size \geq 5 cm | < 0.001 | 6.980 (1.497-32.535) | 0.013 | 0.681 | | |
| Microvascular invasion (+) | 0.619 | | | 0.307 | | |
| E-S grade (III-IV) | 0.835 | | | 0.592 | | |
| CRP \geq 1.0 (<i>n</i> = 145) | 0.797 | | | 0.054 | | |
| Beyond the Milan criteria | | | | | | |
| AFP \geq 100 | 0.058 | 3.619 (1.184-11.063) | 0.024 | 0.082 | 2.456 (0.888-6.793) | 0.083 |
| NLR \geq 6 | 0.034 | 3.973 (1.288-12.249) | 0.016 | 0.045 | 4.685 (1.607-13.657) | 0.005 |
| Pretransplant locoregional treatment | 0.096 | | 0.972 | 0.086 | | 0.971 |
| Tumor number \geq 2 | 0.001 | 0.429 (0.108-1.701) | 0.228 | 0.016 | 0.493 (0.121-2.001) | 0.322 |
| Tumor size \geq 5 cm | < 0.001 | 3.753 (0.901-15.626) | 0.069 | 0.003 | 2.354 (0.587-9.449) | 0.227 |
| Microvascular invasion (+) | 0.226 | | | 0.318 | | |
| E-S grade (III-IV) | 0.083 | 2.191 (0.697-6.884) | 0.179 | 0.067 | 2.618 (0.895-7.651) | 0.079 |
| CRP \geq 1.0 (<i>n</i> = 145) | 0.004 | | | 0.001 | | |

HBV: Hepatitis B virus; GRWR: Graft-to-recipient body weight ratio; AFP: Alpha-fetoprotein; NLR: Neutrophil lymphocyte ratio; E-S grade: Edmondson grade; CRP: C-reactive protein.

score model based on the results for NLR and CRP. Each factor (NLR level \geq 6.0 or CRP level \geq 1.0) was given a score of 1, after which patients were divided into three groups according to prognostic scores: those with NLR level < 6.0 and CRP level < 1.0 [score 0, *n* = 97 (66.9%)], those with NLR level \geq 6.0 or CRP level \geq 1.0 [score 1, *n* = 35 (24.1%)], and those with NLR level \geq 6.0 and CRP level \geq 1.0 [score 2, *n* = 13 (9.0%)]. The disease-free survival for patients with a score of 1 or 2 was significantly lower than that for patients with a score of 0 (*P* = 0.045 and *P* = 0.006, respectively). The overall survival for patients with a score of 1 or 2 was also significantly lower than that for patients with a score of 0 (*P* = 0.001 and *P* = 0.002, respectively). For patients

meeting the Milan criteria, the disease-free survival and the overall survival were not significantly different according to the prognostic score model using NLR and CRP. For patients beyond the Milan criteria, the disease-free survival and the overall survival were significantly superior in patients with a score of 0 compared to those in patients with a score of 2 (*P* = 0.004 and *P* = 0.001, respectively) (Figure 1).

DISCUSSION

LT is considered an ideal treatment for selected patients with HCC because it can treat not only the tumor but also the underlying liver disease. Since the introduction

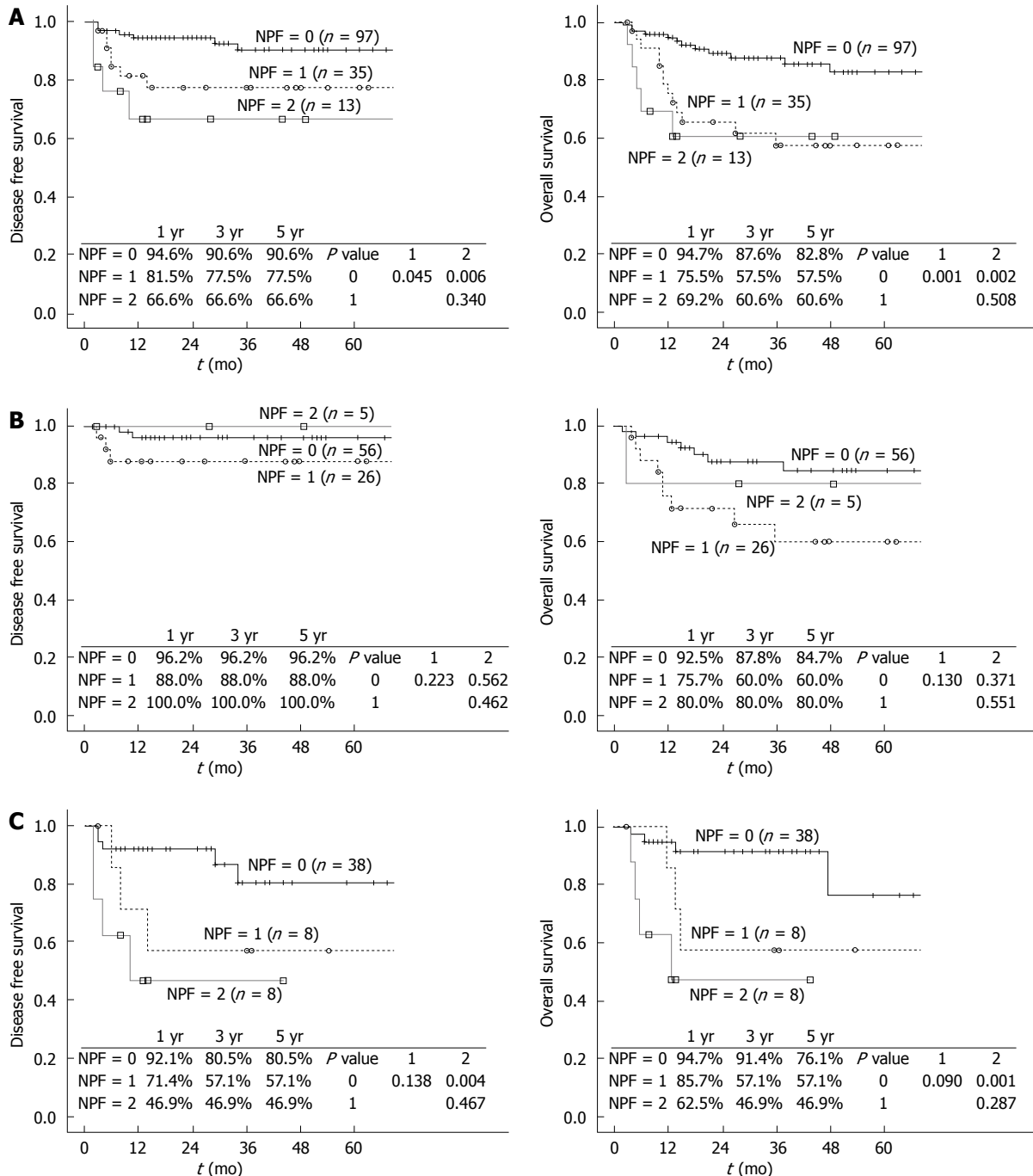


Figure 1 Disease free survival and overall survival according to the new prognostic factor score model. Based on the results for neutrophil-lymphocyte ratio and C-reactive protein; each factor (neutrophil-lymphocyte ratio level ≥ 6.0 or CRP level ≥ 1.0) was given a score of 1. A: Whole population; B: Within the Milan criteria; C: Beyond the Milan criteria. NPF: New prognostic factor.

of the Milan criteria by Mazzaferro *et al.*^[3] in 1996, the disease-free survival and overall survival after LT for patients with HCC meeting the Milan criteria have been equivalent to those of non-HCC patients. LT has become the treatment of choice for cirrhotic patients with HCC. However, there are some limitations in the Milan criteria that must be adopted in the standard selection criteria for HCC patients. First, the Milan criteria depend exclusively on tumor size and number of pretransplant radiologic evaluations. Many reports have demonstrated that tumor biology such as microvascular invasion and tumor dif-

ferentiation^[10,11] and pretransplant tumor markers such as AFP and PIVKA-II^[12,13] affect HCC recurrence after LT. Second, because the Milan criteria are so strict, many patients who may benefit from LT will be rejected. Many centers have reported good results despite expansion of the selection criteria^[14,15]. Therefore, it is important to establish more appropriate selection criteria for LT candidates with HCC. The aim of the present study was to identify new factors related to DFS and OS after LT in order to expand the selection criteria in patients with HCC.

Inflammation is a critical component of tumor progression^[6,16]. Many cancers generate from sites of infection, chronic irritation and inflammation. This process is thought to be related to upregulation of cytokines and inflammatory mediators. Inflammation promotes the proliferation and survival of tumor cells and angiogenesis and provides a good microenvironment for tumor growth^[17]. It is reported that proinflammatory cytokines such as TNF, IL-1, and IL-6 as well as tumor associated macrophages and IL-17 are involved in this inflammatory process^[18,19]. However, it is difficult to measure these cytokines and factors routinely before transplantation. NLR and CRP have been suggested as surrogate markers which can be easily measured preoperatively. An elevated NLR level is associated with poor outcomes in patients with several types of malignant tumors, including colorectal cancer^[20], pancreatic cancer^[21], intrahepatic cholangiocarcinoma^[22], and HCC^[7]. Furthermore, an elevated NLR is related to a poor prognosis in patients undergoing LT for HCC^[23,24]. Also, a high CRP level has been shown to be significantly correlated with a poor outcome in patients undergoing hepatic resection for HCC^[25,26].

In the present study, the pretransplant NLR and CRP level were predictive of disease-free survival and overall survival after LT in patients with HCC. These factors correlated with hepatic functional parameters such as the MELD score and Child-Pugh score rather than tumor size and number, and were more relevant to overall survival than disease-free survival. Also, the subgroup analysis using the Milan criteria showed that there were more significant differences in disease-free survival and overall survival of patients with HCC beyond the Milan criteria. Because the Milan criteria that adopted the standard selection criteria for HCC patients are so strict, many patients are excluded from LT who could actually benefit greatly from the transplantation. However, the new score model used in this study may help to identify patients with HCC who could benefit most from LT, beyond the limitations imposed by the Milan criteria. The present study is the first report to analyze NLR and CRP levels together and to compare both factors. A new score model using these factors may help to predict disease-free survival and overall survival after LDLT in patients with HCC. Our findings showed that the CRP level was more powerful than the NLR level in predicting disease-free survival and overall survival after LDLT in patients with HCC. However, in the present study, the sample size for the CRP level was smaller than that for the NLR level. If the sample size for the CRP level was increased, better results may be obtained.

There were some limitations to this study. First, it was of a retrospective design. The pretransplant CRP level was not measured routinely until 2006. Thus, the sample size available for the pretransplant CRP level was smaller than the sample sizes for the other data. Second, pretransplant NLR and CRP levels may have been affected by various clinical factors (including preoperative sepsis,

recent pretransplant locoregional treatments, and massive alimentary bleeding). We attempted to reduce these confounding factors that can result in falsely elevated NLR and CRP through careful pretransplant examination.

In conclusion, pretransplant NLR and CRP levels are useful biomarkers to predict disease-free survival and overall survival after LDLT in patients with HCC. These factors are more useful in patients with HCC beyond the Milan criteria than in patients with HCC who meet the Milan criteria. Our score model may assist in the selection of patients with HCC who would benefit from LDLT, but would otherwise have been excluded by the Milan criteria.

COMMENTS

Background

Liver transplantation (LT) is an ideal treatment for selected patients with hepatocellular carcinoma (HCC) because it targets not only the tumor but also the underlying liver disease. Although survival rates after LT have improved dramatically, HCC recurrence remains a significant problem. Inflammation is a critical component of tumor progression. Several inflammatory markers, such as the neutrophil-lymphocyte ratio (NLR) and C-reactive protein (CRP), have been suggested as surrogate markers of treatment response and survival in patients with HCC.

Research frontiers

Because the Milan criteria that adopted the standard selection criteria for HCC patients are so strict, many patients are excluded from LT who could actually benefit greatly from the transplantation. The aim of the present study was to identify new factors related to disease-free survival and overall survival after LT in order to expand the selection criteria in patients with HCC.

Innovations and breakthroughs

In the present study, the pretransplant NLR and CRP level were predictive of disease-free survival and overall survival after LT in patients with HCC. Also, the subgroup analysis using the Milan criteria showed that there were more significant differences in disease-free survival and overall survival of patients with HCC beyond the Milan criteria.

Applications

The score model of this paper may assist in the selection of patients with HCC who would benefit from living donor liver transplantation, but would otherwise have been excluded by the Milan criteria.

Peer review

This is a retrospective study including 224 living related LTx for HCC with almost 60% meeting the Milan criteria for LTx. The main finding of the study is that by adding some markers of inflammation such as NLR and CRP there is evidence that the Milan criteria may improve. The authors have used statistical methods trying to find cutoffs for NLR and CRP with reliable results. The authors acknowledge the limitations of their study but the results are of particular interest if reproduced in a prospective way.

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Hematologic diseases: High risk of *Clostridium difficile* associated diarrhea

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METHODS: We retrospectively reviewed the medical records of patients who underwent *C. difficile* testing in a tertiary hospital in 2011. The incidence and risk factors for CDAD and its clinical course including recurrence and mortality were assessed in patients with hematologic disease and compared with those in patients with nonhematologic disease.

RESULTS: About 320 patients were diagnosed with CDAD (144 patients with hematologic disease; 176 with nonhematologic disease). The incidence of CDAD in patients with hematologic disease was estimated to be 36.7 cases/10000 patient hospital days, which was higher than the 5.4 cases/10000 patient hospital days in patients with nonhematologic disease. Recurrence of CDAD was more frequent in patients with hematologic disease compared to those with nonhematologic disease (18.8% vs 8.5%, $P < 0.01$), which was associated with higher re-use of causative antibiotics for CDAD. Mortality due to CDAD did not differ between the two groups. Multivariate analysis showed that intravenous immunoglobulin was the only significant factor associated with a lower rate of recurrence of CDAD in patients with hematologic disease.

CONCLUSION: The incidence and recurrence of CDAD was higher in patients with hematologic disease than in those with nonhematologic disease.

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Key words: *Clostridium difficile* associated diarrhea; Incidence; Clinical outcome; Patients with hematologic disease; Intravenous immunoglobulin

Abstract

AIM: To investigate the incidence and clinical outcome of *Clostridium difficile* (*C. difficile*) associated diarrhea (CDAD) in patients with hematologic disease.

Core tip: Our study included a large number of *Clostridium difficile* associated diarrhea (CDAD) patients at a dedicated hematopoietic stem cell transplantation center, which is one of the most renowned centers for

the treatment of hematologic diseases. The incidence and recurrence of CDAD was higher in patients with hematologic disease than in those with nonhematologic disease. This might be related to higher use of antibiotics. Use of intravenous immunoglobulin was associated with a lower CDAD recurrence rate. Based on our data, we suggest that physicians should be more aware of the higher incidence and rate of recurrence of CDAD in patients with hematologic disease.

Gweon TG, Choi MG, Baeg MK, Lim CH, Park JM, Lee IS, Kim SW, Lee DG, Park YJ, Lee JW. Hematologic diseases: High risk of *Clostridium difficile* associated diarrhea. *World J Gastroenterol* 2014; 20(21): 6602-6607 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i21/6602.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6602>

INTRODUCTION

Diarrhea is a common problem in patients with hematologic disease. Major causes of diarrhea include graft-versus-host disease, anticancer chemotherapy, and infections such as *Clostridium difficile* (*C. difficile*), *Escherichia coli* and cytomegalovirus^[1,2]. Patients with hematologic disease are susceptible to *C. difficile* associated diarrhea (CDAD) because of their frequent antibiotic use, prolonged duration of hospital stay, and chemotherapy-induced disruption of the intestinal mucosa^[3-7]. Prophylactic and empirical use of broad spectrum antibiotics is the common treatment for neutropenic fever patients with hematologic disease^[8,9]. It is difficult for most patients with hematologic disease and CDAD to discontinue treatment with broad spectrum antibiotics. From this perspective, the incidence and the clinical outcome of CDAD in hematologic disease might be different from that in nonhematologic disease. The incidence of CDAD in patients with hematologic disease has been reported to be 7.0%-14%^[4,6,7,10]. However, most studies have dealt with a small number of patients and some studies only included patients receiving hematopoietic stem cell transplantation^[4,6,7]. The aims of this study were to evaluate the incidence of CDAD in patients with hematologic disease and to assess factors associated with its clinical outcome.

MATERIALS AND METHODS

Study population

Between January 2011 and December 2011, 53334 patients (hematologic disease patients 2061; nonhematologic disease patients 51273) were admitted to Seoul St. Mary's Hospital, a tertiary university-affiliated hospital in South Korea. Among them, we retrospectively reviewed the medical records of patients who underwent *C. difficile* testing during the same period. Our hospital is one of the most renowned centers for the treatment of hematologic diseases with 360 cases of hematopoietic stem cell

transplantation carried out in 2011, which was the highest number in Asia. CDAD was defined as a combination of toxigenic stool culture (chromID *C. difficile*; bioMérieux, Marcy l'Etoile, France) and the presence of diarrhea of 3 unformed stools in 24 h^[11]. The toxin assay was conducted by either enzyme immunoassay (Wampole Tox A/B Quik Chek; Alere, Orlando, FL, United States) or polymerase chain reaction to detect toxin genes (*tcdA*, *tcdB*, *cdtA*, *cdtB*).

Exclusion criteria were as follows: (1) community-acquired CDAD, defined as onset of diarrhea within 48 h of hospital admission^[11]; (2) patients with loose stools or diarrhea fewer than 3 times a day; and (3) patients with inadequate medical records. The incidence and risk factors for CDAD and its clinical course including recurrence and mortality were assessed in patients with hematologic disease and compared with those in patients with nonhematologic disease. Risk factors for recurrence of CDAD were investigated in patients with hematologic disease. This study protocol was approved by the Institute Review Board of Seoul St. Mary's Hospital.

Methods

Demographic information, risk factors for CDAD, medications, and hospitalization information during the previous 60 d were investigated in patients with CDAD. Blood samples taken within 2 d of testing for *C. difficile* were used in the analysis. Admission days and antibiotic duration were calculated from the sum of hospital days during the 60 d prior to the index *C. difficile* test. Recurrence was defined as presence of diarrhea and positive toxigenic stool culture at least 2 wk after resolution of CDAD. Severe CDAD was defined as the presence of any of the following: (1) leukocytosis with white blood cell (WBC) count $\geq 15000/\text{mm}^3$; (2) acute kidney injury (AKI), serum creatinine $\geq 1.5 \times$ baseline creatinine; and (3) hypoalbuminemia with serum albumin < 2.5 g/dL^[11,12]. Antibiotic use was defined as the use of any antimicrobial agents once or more during the 60 d prior to the index *C. difficile* test. Concomitant medication was defined as the use of such agents for more than 5 d during the 60 d prior to the index *C. difficile* test.

Statistical analysis

The incidence, risk factors, and clinical course of CDAD were compared between the two groups. For this analysis, we used a *t* test for continuous variables and a χ^2 test or *F* test for categorical variables. The incidence of hospital-acquired CDAD was calculated as the total number of CDAD cases per 10000 patient hospital days. In patients with hematologic disease, factors possibly related to recurrence of CDAD were investigated in univariate and multivariate logistic regression models. Odds ratio and 95% confidence intervals were calculated for each risk factor. A *P* value < 0.05 was considered significant. All statistical analyses were conducted using SAS software (SAS Institute, Cary, NC, United States).

Table 1 Demographic characteristics and risk factors for *Clostridium difficile* associated diarrhea of the study subjects *n* (%)

| Characteristics | HD (<i>n</i> = 144) | NHD (<i>n</i> = 176) | <i>P</i> value |
|---|-------------------------|--------------------------|----------------|
| Male | 84 (58.3) | 102 (58.0) | 0.90 |
| Age (mean ± SD) | 47.4 ± 17.2 | 65.2 ± 16.0 | < 0.01 |
| Body mass index (mean ± SD), kg/m ² | 22.4 ± 3.4 | 20.4 ± 7.1 | < 0.01 |
| Charlson score | 2.3 ± 1.0 | 4.0 ± 2.5 | < 0.01 |
| WBC (mean ± SD)/mm ³ | 3093.6 ± 3879.1 | 9160.0 ± 6252.1 | < 0.01 |
| ANC (mean ± SD)/mm ³ | 2014.7 ± 3052.4 | 6843.2 ± 5473.9 | < 0.01 |
| Neutropenia | 67 (46.5) | 4 (2.3) | < 0.01 |
| Total hospital days within 60 d | 22.2 ± 13.9 | 23.1 ± 17.0 | 0.62 |
| Previous anti-cancer chemotherapy | 139 (96.5) | 60 (34.1) | < 0.01 |
| Antibiotics | | | |
| Use of antibiotics | 142 (98.6) | 166 (94.3) | 0.07 |
| Number of antibiotics | 4.0 ± 1.6 | 2.6 ± 1.6 | < 0.01 |
| Days of antibiotics | 27.8 ± 15.7 | 17.3 ± 13.9 | < 0.01 |
| Concomitant medications | | | |
| Anti-fungal agents | 111 (77.1) | 21 (11.9) | < 0.01 |
| Acyclovir, ganciclovir | 44 (31.7) | 7 (4.0) | < 0.01 |
| Proton pump inhibitor | 62 (43.1) | 56 (31.8) | 0.04 |
| H2 antagonist | 71 (51.1) | 91 (45.3) | 0.67 |
| Toxin assay | | | 0.30 |
| Toxin A + B | 111 (77.1) | 132 (75.0) | |
| Toxin B | 26 (18.1) | 40 (22.7) | |
| Toxin A + B + binary toxin | 7 (4.9) | 4 (2.3) | |
| Pseudomembranous colitis | 7/31 (22.6) | 16/32 (50) | 0.02 |

CDAD: *Clostridium difficile* associated diarrhea; HD: Hematologic disease; NHD: Nonhematologic disease.

RESULTS

Incidence of CDAD

In 2011, 2106 patients were tested for *C. difficile*, 408 of whom had toxigenic *C. difficile*. Eighty-eight patients were excluded for the following reasons: 14 with community acquired CDAD, 20 with inadequate medical records and 54 with diarrhea or loose stools fewer than 3 times per day. Three hundred and twenty patients were diagnosed with CDAD, of whom 144 had hematologic disease and 176 nonhematologic disease. Total episodes of CDAD was 174 in the hematologic disease group and 194 in the nonhematologic disease group. The overall incidence of CDAD in our hospital was 9.1 cases/10000 patient hospital days. The incidence of CDAD in patients with hematologic disease was 36.7 cases/10000 patient hospital days, which was higher than that in patients with nonhematologic disease (5.4 cases/10000 patient hospital days).

Demographic characteristics and risk factor for CDAD

Patients with hematologic disease group were comprised as follows: acute myeloid leukemia 62, acute lymphoid leukemia 32, lymphoma 14, multiple myeloma 14, myelodysplastic syndrome 11, others 11. Among them, 56 patients underwent hematopoietic stem cell transplantation. Comorbidities of patients with nonhematologic

Table 2 Antibiotic use of the study subjects *n* (%)

| Antibiotics | HD (<i>n</i> = 144) | NHD (<i>n</i> = 176) | <i>P</i> value |
|--------------------------------|-------------------------|--------------------------|----------------|
| Cephalosporin | 128 (88.9) | 119 (67.6) | < 0.01 |
| Aminoglycoside | 96 (66.7) | 34 (19.3) | < 0.01 |
| Quinolone | 99 (68.9) | 63 (35.8) | < 0.01 |
| Carbapenem | 58 (40.3) | 39 (22.2) | < 0.01 |
| Glycopeptide | 43 (29.9) | 47 (26.7) | 0.53 |
| β lactam/β lactamase inhibitor | 40 (27.8) | 82 (46.6) | < 0.01 |
| TMP/SMX | 30 (20.8) | 9 (5.1) | < 0.01 |
| Macrolide | 16 (11.1) | 19 (10.8) | 0.93 |

HD: Hematologic disease; NHD: Nonhematologic disease; TMP/SMX, Trimethoprim/sulfamethoxazole.

disease were as follows: solid organ cancer 62, infection (pneumonia or acute pyelonephritis or cholecystitis) 42, cardiovascular disease 20, cerebrovascular disease 16, musculoskeletal disease 9, chronic kidney disease or liver cirrhosis 12, abdominal organ surgery 4, others 11. Patients with hematologic disease were younger and had a higher body mass index and lower Charlson comorbidity score than patients with nonhematologic disease (Table 1). WBC counts and absolute neutrophil counts were lower in patients with hematologic disease.

Almost all patients with hematologic disease had received previous anticancer chemotherapy. The percentage of the patients who received antibiotic therapy did not differ significantly between the two groups (98.6% *vs* 94.3%, *P* = 0.07). However, the total number of antibiotics administered and the duration of antibiotic treatment were higher in patients with hematologic disease than in patients with nonhematologic disease (*P* < 0.01). Cephalosporin, quinolone, and carbapenem were used in 88.9%, 68.9% and 40.3%, respectively, of patients with hematologic disease, which was significantly higher than the rate in patients with nonhematologic disease (Table 2). Concomitant use of an antifungal agent and antiviral agents (acyclovir, ganciclovir) was higher in patients with hematologic disease. Use of a proton pump inhibitor (PPI) was higher in patients with hematologic disease, but use of an H2 antagonist was not. The results of the toxin assay did not differ between the two groups.

Clinical course of CDAD

Treatment of CDAD included cessation of causative antibiotics, metronidazole and oral vancomycin. Initial treatments for CDAD did not differ between the two groups (Table 3). The rate of additional use of causative antibiotics was higher in patients with hematologic disease (*P* < 0.01), as was the rate of concomitant use of intravenous immunoglobulin (*P* < 0.01). Severe CDAD was less common in patients with hematologic disease. Overall mortality (16.0% *vs* 16.5%, *P* = 0.90) and mortality attributable to CDAD (0.7% *vs* 0.6%, *P* = 0.89) within 1 mo did not differ between the two groups. The rate of recurrence of CDAD in patients with hematologic disease was 18.8%, which was higher than that in patients with nonhematologic disease (8.5%).

Table 3 Treatment and clinical course of the study subjects *n* (%)

| Characteristics | HD (<i>n</i> = 144) | NHD (<i>n</i> = 176) | <i>P</i> value |
|--|-------------------------|--------------------------|----------------|
| Treatment | | | 0.82 |
| Cessation of causative antibiotics for CDAD | 42 (29.2) | 57 (32.4) | |
| Metronidazole | 95 (66.0) | 111 (63.1) | |
| Oral vancomycin | 7 (4.9) | 8 (4.5) | |
| Additional use of causative antibiotics for CDAD | 109 (75.7) | 79 (44.9) | < 0.01 |
| Continuous use | 67 (46.5) | 22 (12.5) | |
| Re-use | 42 (29.2) | 57 (32.4) | |
| Concomitant use of IVIG | 80 (55.6) | 11 (6.3) | < 0.01 |
| Severe CDAD | 11 (7.6) | 43 (24.4) | < 0.01 |
| Leukocytosis | 3 (2.1) | 18 (10.2) | < 0.01 |
| Hypoalbuminemia | 5 (3.5) | 31 (17.6) | < 0.01 |
| AKI | 3 (2.1) | 11 (6.3) | 0.10 |
| Clinical outcome | | | |
| Overall mortality within 1 mo | 23 (16.0) | 29 (16.5) | 0.90 |
| Mortality due to CDAD within 1 mo | 1 (0.7) | 1 (0.6) | 0.89 |
| Recurrence | 27 (18.8) | 15 (8.5) | < 0.01 |

HD: Hematologic disease; NHD: Nonhematologic disease; CDAD: CDAD: *Clostridium difficile* associated diarrhea; IVIG: Intravenous immunoglobulin; AKI: Acute kidney injury.

Factors projecting recurrence of CDAD in patients with hematologic disease

Univariate analysis showed that a low WBC count, neutropenia, toxin A + B, toxin B, and additional use of causative antibiotics were significantly associated with recurrence. Use of intravenous immunoglobulin was higher in patients with nonrecurrence. Multivariate analysis demonstrated that intravenous immunoglobulin was the only significant factor associated with reduced recurrence of CDAD (Table 4).

DISCUSSION

The annual incidence of CDAD at our hospital was 9.0 cases/10000 patient hospital days, which was comparable to a previous report from a single tertiary hospital in South Korea (7.2 cases/10000 patient hospital days)^[13]. The incidence of CDAD in Korea seems to be lower than in Western countries. One study from Canada reported the incidence of hospital-acquired CDAD as 28.1 cases/10000 patient hospital days^[14]. The higher incidence of CDAD in Western countries might be associated with a higher prevalence of the hypervirulent strain B1/NAP1/027^[3,15,16], which comprises up to 60% of hospital-acquired CDAD in Western countries^[14,16] compared with 2.1% of CDAD reported in South Korea^[13].

At a single tertiary center in Korea, the incidence of CDAD in patients with hematologic disease was estimated to be 36.7 cases/10000 patient hospital days, which was higher than the 5.4 cases/10000 patient hospital days in patients with nonhematologic disease. CDAD recurrence was more frequent in patients with hematologic disease than in patients with nonhematologic disease. Higher recurrence of CDAD in patients with hemato-

logic disease was associated with higher additional use of causative antibiotics for CDAD^[17]. Multivariate analysis revealed that intravenous immunoglobulin was the only significant preventative factor for recurrence of CDAD in patients with hematologic disease.

Well-known risk factors for CDAD are older age, use of PPI, prolonged duration of hospital stay, comorbidity, and antibiotics^[18]. Despite the younger age and lower Charlson comorbidity score of the patients with hematologic disease in our study, their incidence of CDAD was higher than that in patients with nonhematologic disease. This might be related to their previous anticancer chemotherapy and higher number of antibiotics with longer treatment duration. Febrile neutropenia is a common complication of chemotherapy and hematopoietic stem cell transplantation in patients with hematologic disease^[19]. Broad spectrum antibiotics are routinely prescribed to prevent and to treat neutropenic fever^[2,8,9]. In South Korea, fourth generation cephalosporin and aminoglycoside are also commonly used for empirical therapy in neutropenic fever^[20]. Cumulative exposure to antibiotics increases the risk of CDAD^[21]. Cephalosporin, quinolone, and carbapenem were the antibiotics most frequently associated with CDAD^[18,22,23], and were used in 88.9%, 68.9% and 40.3%, respectively, of the patients in this study with hematologic disease, a significantly higher rate than in patients with nonhematologic disease. Use of PPI was higher in patients with hematologic disease, which might increase the risk of CDAD^[24,25].

In our study, risk factors associated with the recurrence of CDAD in patients with hematologic disease were low WBC count, number of neutropenia, toxin A + B, continuous use of causative antibiotics for CDAD, and lower use of intravenous immunoglobulin in univariate analysis. Interestingly, use of intravenous immunoglobulin was the only factor associated with fewer recurrences of CDAD in multivariate analysis. Intravenous immunoglobulin had been used as adjuvant therapy for infection, and is routinely administered in hematopoietic stem cell transplantation^[2,26,27]. Several studies have reported that intravenous immunoglobulin is a promising adjuvant therapy for CDAD. However, its therapeutic efficacy against CDAD remains controversial, although monoclonal antibodies targeting *C. difficile* toxin were effective for prevention of CDAD recurrence^[28-31]. Given our data showing a favorable effect of intravenous immunoglobulin, further study is needed to investigate its use in treatment of CDAD in patients with hematologic disease. Mortality due to CDAD did not differ between the two groups. Mortality due to CDAD is less common in Korea than in Western countries, which might be explained by the fact that there has been no outbreak of the hypervirulent B1/NAP1/027 strain in South Korea^[13,32].

Due to the retrospective design, some patients might have been omitted from *C. difficile* testing which is a limitation of our study.

This study was a large-scale single-center study comparing CDAD in patients with hematologic disease with

Table 4 Comparison of recurrent *vs* non-recurrent *Clostridium difficile* associated diarrhea in the hematologic disease group *n* (%)

| Characteristics | Recurrent | Single episode | Univariate analysis | Multivariate analysis | |
|--|---------------------|---------------------|---------------------|-----------------------|--------------------|
| | <i>n</i> = 27 | <i>n</i> = 117 | <i>P</i> value | <i>P</i> value | Odds ratio (95%CI) |
| Male | 13 (48.1) | 71 (60.7) | 0.23 | | |
| Age (mean \pm SD) | 47.5 \pm 16.0 | 47.3 \pm 17.6 | 0.93 | | |
| Body mass index (mean \pm SD), kg/m ² | 22.3 \pm 3.6 | 22.4 \pm 3.4 | 0.87 | | |
| Total hospital days within 60 d | 24.3 \pm 12.9 | 21.8 \pm 14.1 | 0.89 | | |
| Charlson score | 2.5 \pm 1.7 | 2.2 \pm 0.8 | 0.13 | | |
| Antibiotics | | | | | |
| Use of antibiotics | 27 (100) | 115 (98.3) | 0.49 | | |
| Number of antibiotics | 4.2 \pm 1.4 | 4.0 \pm 1.7 | 0.57 | | |
| Duration of antibiotics | 27.8 \pm 13.3 | 27.8 \pm 16.2 | 1.0 | | |
| WBC (mean \pm SD)/mm ³ | 1326.7 \pm 1813.7 | 3501.4 \pm 4113.1 | < 0.01 | 0.50 | 1.0 (0.998-1.003) |
| ANC (mean \pm SD)/mm ³ | 751.1 \pm 1224.5 | 2306.2 \pm 3269.9 | 0.02 | 0.83 | 1.0 (0.997-1.002) |
| Neutropenia | 17 (63.0) | 50 (42.7) | 0.04 | 0.87 | 1.13 (0.37-4.74) |
| Severe CDAD | 2 (7.4) | 9 (7.7) | 1.0 | | |
| Toxin assay | | | | | |
| Toxin A + B | 26 (96.2) | 85 (72.6) | < 0.01 | 0.87 | 0.82 (0.8-8.8) |
| Toxin B | 0 (0.0) | 26 (22.2) | < 0.01 | 1.00 | 0 |
| Toxin A + B + binary toxin | 1 (3.7) | 6 (5.1) | 1.0 | | |
| Treatment for CDAD | | | 0.68 | | |
| Metronidazole or vancomycin | 20 (74.1) | 82 (70.1) | | | |
| Discontinuation of causative antibiotics | 7 (25.9) | 35 (29.9) | | | |
| IVIG | 8 (29.6) | 72 (61.5) | < 0.01 | < 0.01 | 0.24 (0.09-0.65) |
| Additional use of causative antibiotics for CDAD | 27 (100.0) | 82 (70.1) | < 0.01 | 1.00 | 0 |
| Continuous use | 20 (74.1) | 47 (40.2) | | | |
| Re-use | 7 (25.9) | 35 (29.9) | | | |

CDAD: *Clostridium difficile* associated diarrhea; WBC: White blood cell; ANC: Absolute neutrophil count; IVIG: Intravenous immunoglobulin.

that in patients with nonhematologic disease. Our study showed that the incidence of hospital-acquired CDAD in patients with hematologic disease was about six times higher than that in patients with nonhematologic disease, and that CDAD recurrence was more frequent in patients with hematologic disease. Use of intravenous immunoglobulin was associated with a lower CDAD recurrence rate. Based on our data, we suggest that physicians should be more aware of the higher incidence and rate of recurrence of CDAD in patients with hematologic disease.

COMMENTS

Background

Patients with hematologic disease are susceptible to *Clostridium difficile* associated diarrhea (CDAD) because of their frequent antibiotic use. The aims of this study were to evaluate the incidence of CDAD in patients with hematologic disease and to assess factors associated with its clinical outcome.

Research frontiers

Treatment failure and recurrence of CDAD is increasing, especially in Western countries. Some drugs for CDAD and fecal microbiota transplantation for refractory CDAD are hot research topics.

Innovations and breakthroughs

Patients with hematologic disease are susceptible to CDAD because of frequent antibiotic use and immunocompromised status. However comprehensive clinical studies regarding this issue are rare. A large number of patients was included in this study. The authors revealed higher incidence and recurrence rate of CDAD in patients with hematologic disease compared with those in patients with nonhematologic disease. However, mortality due to CDAD was not different between the two groups.

Applications

Physicians should be more aware of the higher incidence and rate of recurrence of CDAD in patients with hematologic disease. Metronidazole was a good treatment option for the treatment of CDAD in patients with hematologic

disease.

Peer review

The authors have retrospectively investigated the incidence and clinical outcome of CDAD in patients with hematologic disease and compared them with those in patients with nonhematologic disease in a large-scale single center setting. The data are interesting and provide some reference in clinical practice. The increased incidence of CDAD in hematologic diseases was shown and the multivariate analysis revealed that intravenous immunoglobulin-injected patients showed less frequent recurrence of CDAD in hematologic disease patients.

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Value of ^{18}F -FDG PET-CT in surveillance of postoperative colorectal cancer patients with various carcinoembryonic antigen concentrations

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follow-up over at least six months.

RESULTS: The sensitivity, specificity, and accuracy of FDG PET/CT were 95.2%, 82.6%, and 92.5%, and 94.8%, 81.4% and 92.8%, respectively, in the case- and lesion-based analyses. The sensitivity and accuracy of FDG PET/CT significantly differed from CT in both analyses ($\chi^2 = 8.186$, $P = 0.004$; $\chi^2 = 6.201$, $P = 0.013$; $\chi^2 = 13.445$, $P = 0.000$; $\chi^2 = 11.194$, $P = 0.001$). In the lesion-based analysis, the sensitivity, specificity, and accuracy of FDG PET/CT in the abnormal CEA group were 97.8%, 82.6%, and 95.6%, compared with 81.3%, 80%, and 80.6% for patients with normal CEA levels. In case-based analysis, the sensitivity, specificity, and accuracy of FDG PET/CT were 97.2%, 77.8%, and 95% in abnormal CEA group. Only in lesion-based analysis, the sensitivity and accuracy of FDG PET/CT in the abnormal CEA group were significantly superior to those in the normal CEA group ($\chi^2 = 6.432$, $P = 0.011$; $\chi^2 = 7.837$, $P = 0.005$). FDG PET/CT changed the management in 45.8% of patients with positive scans.

CONCLUSION: FDG PET/CT showed superior diagnostic value and is an advisable option in surveillance of postoperative CRC patients with a vague diagnosis.

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Abstract

AIM: To evaluate the value of positron emission tomography (PET)/computerized tomography (CT) in surveillance of colorectal cancer (CRC) patients with different carcinoembryonic antigen (CEA) concentrations.

METHODS: One hundred and six postoperative CRC patients who had suspected recurrence or metastasis and received fluorodeoxyglucose (FDG) PET/CT within one week were included in this study. The final diagnosis was confirmed by histological examination or clinical

Key words: Colorectal cancer; Carcinoembryonic antigen; Fluorodeoxyglucose positron emission tomography/computed tomography; Recurrence; Metastasis

Core tip: In this paper, fluorodeoxyglucose (FDG) positron emission tomography (PET)/computerized tomography (CT) showed an excellent diagnostic performance and its sensitivity and accuracy were significantly superior to those of CT. FDG PET/CT changed the management in some metastatic patients who might obtain the chance for a second remission. The study also showed

that FDG PET/CT was effective similarly in the patients with normal and abnormal carcinoembryonic antigen (CEA) levels but had a tendency to increase with the CEA level. FDG PET/CT was an advisable option for surveillance of postoperative colorectal cancer (CRC) patients with a vague diagnosis and should be recommended in surveillance of post-operative CRC patients even with normal CEA.

Zhang Y, Feng B, Zhang GL, Hu M, Fu Z, Zhao F, Zhang XL, Kong L, Yu JM. Value of ^{18}F -FDG PET-CT in surveillance of postoperative colorectal cancer patients with various carcinoembryonic antigen concentrations. *World J Gastroenterol* 2014; 20(21): 6608-6614 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6608.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6608>

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancer entities worldwide^[1]. Despite the fact that 70% of the patients have a chance of radical operation, 30%-50% of them will develop metastasis or local recurrence within two years after operation^[2,3]. For CRC patients, both local recurrence and metastasis can be addressed by reoperation. Only 10%-30% of recurrent patients can be treated by salvage surgery^[4,5], but curative-intent surgeries are associated with a 5-year survival rate of 30%-40% in selected patient populations with single organ metastasis^[6]. Thus, the surveillance of postoperative colorectal cancer should enhance the proportion of resectable cases by early detection of the recurrence and metastasis in order to improve the survival of CRC patients.

The surveillance is usually performed by a regular physical examination, determination of the serum carcinoembryonic antigen (CEA) level, colonoscopy, and conventional imaging techniques such as ultrasound of the liver, contrast-enhanced computerized tomography (ceCT) and magnetic resonance imaging (MRI). It remains unverified what strategy can provide significant survival benefits in routine follow-up of CRC patients^[7,8]. Serum CEA is generally used as a tumor marker and CEA elevation predicts a high risk of recurrence and poor survival for CRC^[9]. Elevated CEA values can not provide any accurate information regarding the sites of recurrence and complementary imaging techniques should be provided for the diagnosis of CRC recurrence. Conventional imaging techniques primarily offer morphologic data based on anatomical information. It is difficult to identify recurrent disease from nonmalignant changes, such as scars, inflammation lesions and radiation necrosis by this morphological imaging tool. Fluorine-18 (^{18}F) fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT is an integrated imaging modality of anatomic and functional imaging and can show metabolic changes before morphological ones. In the literature, ^{18}F -FDG PET/CT was considered to be superior to the conventional imaging in

Table 1 Characteristics of patients in the study

| Characteristic | n |
|--|------------|
| Male/female | 71/35 |
| Age (yr, median) | 27-75 (56) |
| Primary site | |
| Colon | 42 |
| Rectum | 64 |
| Pathological type | |
| Adenocarcinoma | 94 |
| Adenosquamous carcinoma | 2 |
| Squamous cell carcinoma | 1 |
| Mucinous adenocarcinoma | 9 |
| Therapy | |
| Operation | 21 |
| Operation and chemotherapy | 13 |
| Operation and radiotherapy | 37 |
| Operation, chemotherapy and radiotherapy | 35 |

the early detection of recurrence and metastases in CRC patients with elevated CEA^[2,10]. Recently, it has been reported that PET/CT showed a high-positive predictive value for metastases in postoperative colorectal cancer patients with normal CEA levels^[6,11]. However, due to some limitations in previous studies, such as small number of patients and unsatisfactory statistic analysis, the clinical value and efficacy of FDG PET/CT in surveillance are not yet fully established.

In this retrospective study, we aimed to evaluate the diagnostic performance of ^{18}F -FDG PET/CT in surveillance of postoperative CRC patients as compared with CT and to investigate the role of FDG PET/CT in patients with different CEA concentrations.

MATERIALS AND METHODS

Patients

A total of 106 postoperative CRC patients who underwent FDG PET/CT examinations at our institution from January 2008 to April 2012 were included in this study. The inclusion criteria were as follows: (1) histopathologic confirmation of primary CRC; (2) undergoing complete treatment including curative resection with or without chemoradiation therapy; (3) regular clinical examination every three or six months, including physical examinations, determination of serum CEA concentration, and chest and abdomen ceCT; (4) the patients suspected with recurrence or metastasis by routine examination received FDG PET/CT within one week; and (5) at least six months of clinical follow-up. Patient's characteristics and other preoperative information are summarized in Table 1. This study was approved by the Institutional Review Board at our institution. Informed consent was waived due to the retrospective design of the study.

PET/CT scanning

The ^{18}F -FDG PET/CT was performed using an integrated PET/CT system (GE Discovery LS, GE Healthcare). All patients fasted for at least 6 h before the injection of 5 MBq/kg of ^{18}F -FDG. Images were obtained ap-

Table 2 Intersections of positron emission tomography/computerized tomography and computerized tomography diagnoses

| CT (n = 106) | PET/CT (n = 106) | | | |
|--------------|------------------|------------|-------------|------------|
| | TP (n = 79) | FP (n = 4) | TN (n = 19) | FN (n = 4) |
| TP (n = 67) | 67 | | | |
| FP (n = 6) | | 4 | 2 | |
| TN (n = 17) | | | 17 | |
| FN (n = 16) | 12 | | | 4 |

PET: Positron emission tomography; CT: Computerized tomography; TP: True positive; FP: False positive; TN: True negative; FN: False negative.

proximately 1 h after an intravenous injection of FDG. The PET/CT system was used for 4-slice helical CT acquisition, followed by a full-ring dedicated PET scan of the same axial range. PET scans were performed in the whole-body mode from top to the middle thigh for 4 min per field of view, each covering 14.5 cm, at an axial sampling thickness of 4.25 mm/slice. PET images were reconstructed with CT-derived attenuation correction using ordered-subset expectation maximization software. The attenuation-corrected PET images, CT images, and fused PET/CT images were available for review in axial, coronal, sagittal planes, and a cine display of maximum intensity projections of the PET data, using the manufacturer's review station (Xeleris; GE Healthcare).

FDG PET/CT interpretation

The attenuation-corrected PET images, CT images, and fused PET/CT images displayed as coronal, sagittal, and transaxial slices were viewed on a Xeleris workstation. Two experienced nuclear medicine physicians, who were aware of the patient's clinical history and recent radiographic data, interpreted the PET/CT images side-by-side using visual observation and semi-quantity analysis. It was considered positive when the maximum standard uptake value (SUV_{max}) of the region of interest (ROI) exceeded 2.5.

CEA examination

Serum CEA concentration was determined by electrochemiluminescence immunoassay with normal reference ranging from 0 to 3.4 ng/mL. It was categorized as abnormal when it exceeded 3.4 ng/mL. The patients were divided into four groups according to the CEA levels: group 1 (CEA \leq 3.4 ng/mL), group 2 (CEA 3.4-10 ng/mL), group 3 (CEA 10-30 ng/mL), and group 4 (CEA > 30 ng/mL).

Statistical analysis

The final diagnosis of recurrence or metastasis was confirmed by gold standard (histopathological or cytological confirmation or at least six months of clinical follow-up). PET/CT findings and ceCT findings were classified as true positive (TP), false positive (FP), true negative (TN), and false negative (FN), as compared to those of the gold standard. The sensitivity, specificity, and accuracy

of 18 F-FDG PET/CT and ceCT were calculated using standard statistical formula in the case-based and lesion-based analyses.

The SPSS version 17.0 (SPSS Inc, Chicago, IL, United States) was used for statistical analyses. The Chi-square test was used to compare the differences between the two imaging modalities.

RESULTS

Overall diagnostic performance of FDG PET/CT

In the group, 51 patients were confirmed by histocytology while 55 patients by follow-up. In the case-based analysis, 83 patients had positive findings in PET/CT and 79 patients were finally diagnosed as TP. Nineteen patients were identified as TN, including four patients with benign diseases (one thyroid adenoma and three enteric polyps). Among the four FP patients, two were suspected to have recurrence at the anastomotic site and finally confirmed to have inflammatory changes by colonoscopy. In two FN patients, tiny peritoneum and lung metastases were revealed with a diameter less than 0.5 cm, which were not visualized by PET/CT but confirmed by ceCT imaging several months later when the diameter was larger. With little FDG uptake, two mucinous adenocarcinoma patients showed negative PET/CT scans and were determined as FN by histological confirmation. In all patients, 67 TP patients and 17 TN patients were diagnosed by ceCT and there were intersections of PET/CT and ceCT (Table 2).

In the lesion-based analysis, out of the 152 positive lesions determined by 18 F-FDG PET/CT, 146 were confirmed as TP lesions and six as FN lesions by gold standard. Thirty five lesions were confirmed as TN scans and eight lesions were identified as FP scans. The locations of the foci included anastomotic site, intraperitoneal and thoracic lymph nodes, pelvic, bone, liver, and lungs. Three FP lesions showed nonspecific FDG uptake, which were confirmed as inflammatory changes in the wall of the bowel or around operation site, and five lesions had hypermetabolic lesions in the liver, lung, pelvic, and bone. Three FN lesions were mucinous adenocarcinoma and the others were mediastinal lymph nodes and small nodules of the lung and peritoneum. The sensitivity and accuracy of FDG PET/CT significantly differed from those of ceCT in both case- and lesion-based analyses ($P < 0.05$, Table 3). As for the specificity, the PET/CT results did not show a significant difference from those of ceCT in both analyses.

Impact of CEA concentrations on diagnostic accuracy of FDG PET/CT

We detected serum CEA concentration in all of the patients. In case-based analysis, the sensitivity, specificity, and accuracy of PET/CT were 97.2%, 77.8%, and 95%, respectively, in the group with abnormal CEA levels and did not significantly differ from those in the group with normal CEA levels. In lesion-based analysis, the sensitiv-

Table 3 Diagnostic performance comparisons in case-based and lesion-based analyses

| | PET/CT and CT in all patients | | PET/CT in patients with different CEA levels | |
|-----------------------|-------------------------------|-----------------------------|--|-----------------------------|
| | PET/CT | CT | PET/CT (CEA ≤ 3.4 ng/mL) | PET/CT (CEA > 3.4 ng/mL) |
| Case-based analysis | | | | |
| Sensitivity (%) | 95.2 (79/83) ¹ | 80.7 (67/83) ¹ | 83.3 (10/12) | 97.2 (69/71) |
| Specificity (%) | 82.6 (19/23) | 73.9 (17/23) | 85.7 (12/14) | 77.8 (7/9) |
| Accuracy (%) | 92.5 (98/106) ² | 79.3 (84/106) ² | 84.6 (22/26) | 95 (76/80) |
| Lesion-based analysis | | | | |
| Sensitivity (%) | 96.1 (146/152) ³ | 83.1 (118/142) ³ | 81.3 (13/16) ⁵ | 97.8 (133/136) ⁵ |
| Specificity (%) | 81.4 (35/43) | 73.5 (25/34) | 80 (16/20) | 82.6 (19/23) |
| Accuracy (%) | 92.8 (181/195) ⁴ | 81.3 (143/176) ⁴ | 80.6 (29/36) ⁶ | 95.6 (152/159) ⁶ |

¹ $\chi^2 = 8.186$, $P = 0.004$; ² $\chi^2 = 6.201$, $P = 0.013$; ³ $\chi^2 = 13.445$, $P = 0.000$; ⁴ $\chi^2 = 11.194$, $P = 0.001$; ⁵ $\chi^2 = 6.432$, $P = 0.011$; ⁶ $\chi^2 = 7.837$, $P = 0.005$. PET: Positron emission tomography; CT: Computerized tomography; CRC: Colorectal cancer.

Table 4 Diagnostic performance of positron emission tomography/computerized tomography and computerized tomography in patients with various carcinoembryonic antigen levels

| CEA level (ng/mL) | CT (%) | | | PET/CT (%) | | |
|---------------------|--------------|-------------|--------------|--------------|--------------|--------------|
| | Sensitivity | Specificity | Accuracy | Sensitivity | Specificity | Accuracy |
| ≤ 3.4 ($n = 26$) | 57.1 (8/14) | 75 (9/12) | 65.4 (17/26) | 83.3 (10/12) | 85.7 (12/14) | 84.6 (22/26) |
| 3.4-10 ($n = 29$) | 78.3 (18/23) | 66.7 (4/6) | 75.9 (22/29) | 96 (24/25) | 75 (3/4) | 93.1 (27/29) |
| 10-30 ($n = 34$) | 86.7 (26/30) | 75 (3/4) | 85.3 (29/34) | 96.7 (29/30) | 75 (3/4) | 94.1 (32/34) |
| > 30 ($n = 17$) | 93.8 (15/16) | 100 (1/1) | 94.1 (16/17) | 100 (16/16) | 100 (1/1) | 100 (17/17) |

PET: Positron emission tomography; CT: Computerized tomography; CRC: Colorectal cancer; CEA: Carcinoembryonic antigen.

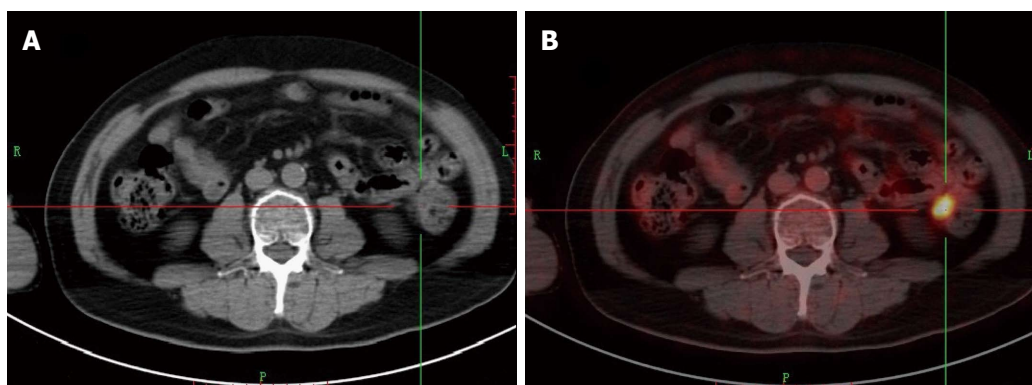


Figure 1 Positron emission tomography/computerized tomography and computerized tomography images of a 60-year-old man with rising carcinoembryonic antigen level of 32.3 ng/mL who had undergone rectal cancer resection and chemoradiotherapy 3 years ago. ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography (¹⁸F-FDG PET/CT) image showed a FDG-avid metastasis lesion, which is not typical in computerized tomography (CT) image. A: CT; B: ¹⁸F-FDG PET/CT.

ity and accuracy of PET/CT in the group with abnormal CEA levels were significantly superior to those in the group with normal CEA levels ($P < 0.05$, Table 3). The diagnostic performance of FDG PET/CT and ceCT was calculated in each group with various CEA levels. The sensitivity, specificity, and accuracy of both imaging techniques were increased with the CEA level, but there was no significant statistical difference among the groups of patients with different CEA levels due to the small quantity of sample in each group (Table 4).

FDG PET/CT scan and change of management

Among all the patients, 69 showed positive scans in both FDG PET/CT and ceCT, but 18 showed extra lesions in FDG PET/CT. In 12 patients, FDG PET/CT showed

positive scans, while CT was negative (Figures 1 and 2). Of the patients with recurrence and metastasis, 57 received radiotherapy or/and chemotherapy. Ten patients received secondary surgery for a single metastatic lesion and eight patients had canceled operative scheme due to the observation of extra lesions by FDG PET/CT. Of the total 83 patients with positive scans, FDG PET/CT changed the management in 38 (45.8%) patients (Table 5).

DISCUSSION

In surveillance of postoperative CRC, CT is the preferred imaging technique for local recurrence detection^[6] and MRI is regarded as the most sensitive conventional imaging tool for liver metastases^[12,13]. However, the early

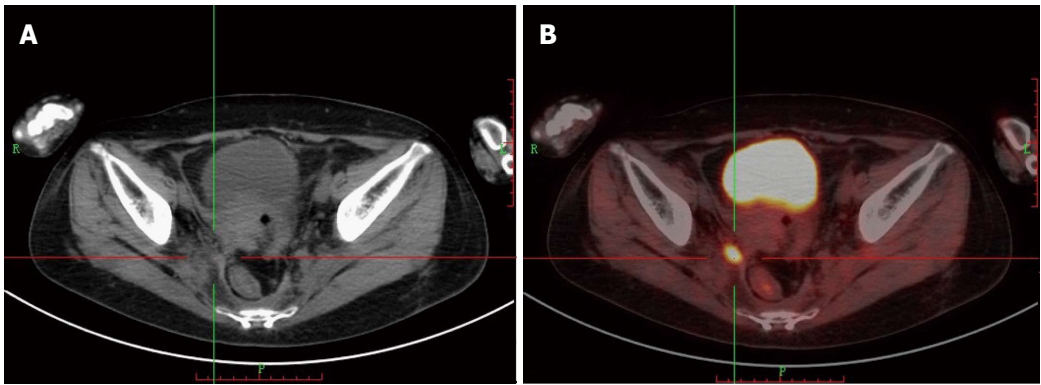


Figure 2 A 38-year-old woman revealed normal carcinoembryonic antigen level of 2.8 ng/mL who underwent rectal cancer resection 22 mo ago. ¹⁸F-fluorodeoxyglucose positron emission tomography/computed tomography (¹⁸F-FDG PET/CT) image displayed a lymph node but computerized tomography (CT) did not show it clearly. A: CT; B: ¹⁸F-FDG PET/CT.

| Table 5 Positron emission tomography/computerized tomography scan and change of management | | | | |
|--|---------|--------------|--------------|-------------------|
| | Surgery | Radiotherapy | Chemotherapy | Chemoradiotherapy |
| Added cases (n = 23) | 6 | 3 | 9 | 5 |
| Reduced cases (n = 15) | 8 | 2 | 3 | 2 |
| Total (n = 38) | 14 | 5 | 12 | 7 |

PET: Positron emission tomography; CT: Computerized tomography.

diagnosis of recurrence is still difficult through conventional surveillance strategies. It has been reported that PET/CT showed better sensitivity and specificity (87%-100% and 90%-98%, respectively) for detection of hepatic and extra-hepatic metastasis than CT^[14-16]. In some studies, the sensitivity of PET/CT for hepatic lesions was 91%-100%, which was similar to that of MRI^[17,18]. In our study, ¹⁸F-FDG PET/CT showed an excellent diagnostic performance in the detection of CRC recurrence and metastasis and its sensitivity and accuracy were significantly superior to those of ceCT. We found that many positive lesions in FDG PET/CT, which were once classified as negative lesions by CT (Table 5), were finally confirmed as true positives by gold standard. This showed that it would be possible that some CRC patients with recurrences and metastasis might obtain the chance for a second remission and improve the prognosis through PET/CT findings.

CEA is used as the early indicator for the recurrent disease and is elevated in approximately 60%-70% of patients with recurrence^[10,19]. It has been observed that some potentially curable recurrent tumors were detected by routine imaging techniques, while CEA levels were still normal. Therefore, CEA measurements had only a marginal effect on survival^[11]. Moreover, for postoperative CRC patients who have a suspicion of recurrence based on the rise in the CEA level, there is controversy on the

most accurate imaging technique. Ozkan *et al*^[2] reported that the sensitivity and specificity of ¹⁸F-FDG PET/CT in the detection of disease recurrence in postoperative CRC patients were 97% and 61%, while they were 51% and 60% for CT, respectively. Mittal *et al*^[20] reported that PET/CT showed recurrences in 71% of CRC patients and the positive rate increased with the CEA level. Our data also showed coincidentally that PET/CT was superior to CT in terms of sensitivity, specificity, and accuracy in all groups of patients and had a tendency to increase with the CEA level (Table 4). Lee *et al*^[21] evaluated a group of CRC patients with normal CEA levels and reported that the sensitivity, specificity, and accuracy for FDG PET/CT were 95%, 76.6%, and 88.8%, respectively. In our study, the sensitivity, specificity, and accuracy of PET/CT in patients with normal CEA was similar to the data. In addition, the sensitivity and accuracy of FDG PET/CT in patients with abnormal CEA levels significantly differed from those with normal CEA levels in the lesion-based analysis. But in the case-based analysis, the FDG PET/CT diagnostic performance did not show a significant difference between these two groups. It may be related to the small quantity of patients with normal CEA levels, which could influence the statistical analysis. However, the data still suggested that FDG PET/CT was effective similarly in patients with normal and abnormal CEA levels. For patients with a vague diagnosis of recurrence or metastasis based on a routine examination, FDG PET/CT might provide much benefit to patients by increasing the diagnostic accuracy. Based upon these results, FDG PET/CT is an advisable option for CRC patients with an indefinable diagnosis and should be recommended in surveillance of post-operative CRC patients even with normal CEA.

In this study, the specificity of FDG PET/CT did not significantly differ from that of ceCT in both case- and lesion-based analyses. FDG PET/CT scans showed false-positive results which mainly included the inflammatory lesion and single hypermetabolic lesion in the organs. Our data demonstrated that inflammatory processes can result in hypermetabolism and consequently false-

positive results, for example, the inflammatory processes of colitis in the anastomotic site. In addition, for a single hypermetabolic lesion of the bone, metastasis should be differentiated from the injuries and restorations correlated to other imaging systems. For lung nodules, metastasis should be differentiated from inflammatory lesions and tuberculosis.

It has been reported that mucinous carcinoma usually showed little uptake of the FDG and the sensitivity of FDG-PET imaging for detection of mucinous carcinoma was significantly lower than that for nonmucinous carcinomas^[3]. In our results, two quarters of FN cases and three sixths of FN lesions were mucinous adenocarcinoma and it demonstrated that mucinous carcinoma was the main factor responsible for FN scans. Therefore, it was suggested that for mucinous adenocarcinoma patients with negative FDG-PET imaging results, other imaging modalities should be recommended for further diagnosis. Otherwise, nonvisualization of FDG PET/CT detection should be attributed to the little FDG uptake for lymph nodes and peritoneal micrometastases, consistent with the literature report. Moreover, our FDG PET/CT scanning data were acquired in a 2D mode with 4.25 mm spatial resolution and a 256 x 256 matrix. Given better spatial and temporal resolution (3D mode with 2 mm spatial resolution, 400 x 400 matrix and continuous table movement), more tiny lymph nodes and metastases can be revealed precisely. With the decrease of false diagnosis, the further accuracy elevation can be expected.

In terms of the superiority of FDG PET/CT over CT in detection of recurrence and metastasis, FDG PET/CT might provide chances to select suitable patients for surgical resection or other local treatments (radiotherapy, embolization, and radio-frequency ablation). Meanwhile, some unnecessary operations might be avoided. Many studies demonstrated some disease management change as a result of PET/CT usage in 30%-56% of patients with a suspected or confirmed recurrence of CRC^[22-24]. It is possible to benefit from this strategy in terms of patient survival with early detection and treatment of tumor recurrence by FDG PET/CT^[6,25]. In our studies, 45.8% of FDG positive patients had a changed disease management and received suitable treatments. Second operation was performed only in 12% of retreatment candidate patients, because most patients of the groups had already developed multi-organ metastases when they received the FDG PET/CT scan. It suggested that the early detection due to the use of PET/CT in surveillance of CRC could correct the disease management strategy and improve the treatment efficacy by reoperation for potential curative patients.

Our study had two potential limitations. Due to the retrospective nature of the study, we were unable to obtain unified clinical data for the patients and there was an inter-observer variation for imaging interpretation that might have had some influence on the sequences. The second was that histopathological confirmation was only performed in some of the patients in this study.

In conclusion, FDG PET/CT showed a superior diagnostic performance in surveillance of postoperative CRC patients. For patients with suspicious recurrence or metastasis based on a routine examination, our data suggest that PET/CT is an excellent option to replace CT in the follow-up of CRC patients even when CEA is normal.

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COMMENTS

Background

Recurrent or metastatic colorectal cancer (CRC) patients will have a chance of remission by early detection in postoperative surveillance and improve the survival. It remains unverified which strategy can provide significant survival benefits in routine surveillance of CRC patients. In the literature, fluorodeoxyglucose (FDG) positron emission tomography (PET)/computerized tomography (CT) was considered to be superior to conventional imaging in early detection of recurrence and metastasis in CRC patients. However, due to insufficient evidence, the clinical value of FDG PET/CT in surveillance is not definite.

Research frontiers

Recently, as an integrated imaging modality of anatomic and functional imaging diagnosis, FDG PET/CT showed enormous potential in area of diagnosis, staging and monitoring and response evaluation of CRC. In the surveillance of postoperative CRC patients, the hotspots are whether PET/CT is more effective than conventional imaging such as CT and what kind of patients should be suitable candidates for PET/CT.

Innovations and breakthroughs

Previous studies usually compared the diagnostic performance of imaging modes in all patients regardless the carcinoembryonic antigen (CEA) level. In the current literature, the diagnostic performances of FDG PET/CT were evaluated only in patients with normal or abnormal CEA levels. In this retrospective study, the authors evaluated the diagnostic performance of Fluorine-18 (¹⁸F)-FDG PET/CT in surveillance of postoperative CRC patients as compared with CT and to investigate the role of FDG PET/CT in patients with different CEA levels. The data showed that PET/CT was superior to CT in terms of sensitivity, specificity, and accuracy in all groups of patients. The data also suggested that FDG PET/CT was effective in patients with normal or abnormal CEA levels.

Applications

The study suggested that PET/CT was an excellent option to replace CT in surveillance of CRC patients who were suspected to have recurrence or metastasis by routine examinations even when CEA is normal.

Terminology

¹⁸F-FDG PET/CT is an integrated imaging modality of anatomic and functional imaging and can show metabolic changes before morphological ones. It depicts the spatial distribution of metabolic or biochemical activity in the body and has excellent diagnostic performance in various tumors.

Peer review

This is a good retrospective study in which the authors compared the diagnostic performance of ¹⁸F-FDG PET/CT with CT in surveillance of postoperative CRC patients and assessed the value of FDG PET/CT in patients with normal or abnormal CEA levels.

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Telomerase and hTERT: Can they serve as markers for gastric cancer diagnosis?

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Abstract

AIM: To investigate telomerase activity and human telomerase reverse transcriptase (hTERT) expression in normal human gastric mucosal epithelial cells (nhGMECs) and fibroblasts (nhGMFs).

METHODS: nhGMECs and nhGMFs were isolated and cultured from specimens obtained during routine surgery for bleeding peptic ulcer. Telomerase activity in nhGMFs, nhGMECs, and the tumor cell lines BGC-823, SGC-7901 and MKN-28 cells was analyzed using the telomeric repeat amplification protocol assay. hTERT protein was determined in nhGMECs, nhGMFs, BGC-823, SGC-7901 and MKN-28 cells by indirect immunofluorescence.

RESULTS: A similar level of telomerase activity was ob-

served in nhGMECs, nhGMFs and BGC-823, SGC-7901, MKN-28 cell lines. Positive hTERT immunostaining was detected in nhGMECs, nhGMFs, BGC-823, SGC-7901 and MKN-28 cell lines.

CONCLUSION: The use of telomerase or hTERT as diagnostic markers for gastric cancer may require further studies.

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Key words: Gastric cancer; Telomerase; Human telomerase reverse transcriptase; Normal human gastric mucosal epithelial cell; Normal human gastric mucosal fibroblast

Core tip: Telomerase activity and human telomerase reverse transcriptase (hTERT) protein expression were detected in normal human gastric mucosal epithelial cells isolated from human gastric tissues, and were similar to those found in cell lines from human gastric adenocarcinoma. The results of this study suggest that the use of telomerase or hTERT as diagnostic markers for gastric cancer may require further studies.

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INTRODUCTION

Despite its decreasing frequency worldwide, gastric cancer remains one of the major causes of cancer-related deaths^[1,2]. This is due to the fact that most cases are in the advanced stages of disease when diagnosed. While the

5-year survival of patients with advanced gastric cancer is approximately 20%, early tumor resection can achieve a 5-year survival rate of around 90%^[3]. Therefore, early diagnosis is an important measure to improve the prognosis of patients with gastric cancer. Researchers have been looking for early diagnostic markers for gastric cancer for more than ten years^[4-8].

Telomerase is a specialized reverse transcriptase that adds telomeric repeats to the ends of eukaryotic chromosomes, and is responsible for continuous cell growth. Human telomerase reverse transcriptase (hTERT) is the major subunit of the telomerase enzyme complex and plays a critical role in the regulation of telomerase activity^[1,9]. They are observed in 80%-90% of human tumors including gastric cancer and nearly all cancer-derived cell lines^[4,5,10], and are not observed in the majority of normal tissues and somatic cells, therefore could be considered useful markers for the early diagnosis of human gastric cancer. However, hTERT expression was also found in normal gastric tissues; a full-length hTERT mRNA was present in 43% of normal gastric specimens and hTERT protein was expressed at all the proliferation zones in crypts^[11]. Therefore, the use of hTERT and subsequently telomerase as gastric cancer markers is unclear.

In the present study, we determined the expression of telomerase and hTERT in primary cultured cells from normal human gastric mucosal epithelium, and evaluated whether they could be used as cytological markers for the diagnosis of gastric cancer.

MATERIALS AND METHODS

Cell culture

After the study protocol was approved by the university and hospital ethical committees and informed consent was obtained from the patients, normal human gastric mucosal epithelial cells (nhGMECs) were isolated from specimens obtained during routine surgery for bleeding peptic ulcer using a method previously developed by us^[12]. Cell viability was estimated by methyl thiazolyl tetrazolium assay to examine the general growth process. Periodic acid-Schiff (PAS) staining was used to identify mucinogen granules in epithelial cells and cytokeratin (CK)-18 staining was used to identify epithelial cells. Light microscopy and transmission electron microscopy were used to observe the morphological structures of cells. Toluidine blue (0.5%) staining was used to observe the nucleus of nhGMECs and SGC-7901 cells. Normal human gastric mucosal fibroblasts (nhGMFs) were also isolated from the same specimens. Male or female patients aged 40-71 years provided the gastric samples. BGC-823, SGC-7901 and MKN-28 cell lines maintained in our laboratory were used as controls. All cells were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum without antibiotics.

Telomerase activity assay

Telomerase activity was determined using the telomeric

repeat amplification protocol (TRAP) assay and a telomerase detection kit (Dingguo, Beijing, China). nhGMFs, nhGMECs, BGC-823, SGC-7901 and MKN-28 cells were analyzed according to the manufacturer's protocol. Protein was extracted from 3×10^6 cells in each group. After 35 polymerase chain reaction cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, the products were electrophoresed on 12.5% polyacrylamide gels.

hTERT protein detection

hTERT protein was determined in nhGMECs, nhGMFs, BGC-823, SGC-7901 and MKN-28 cells by indirect immunofluorescence. Cells were grown on slides coated with polylysine, fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.5% Triton X-100 for 10 min at room temperature. An hTERT antibody (Santa Cruz Biotechnology, CA, United States) was added to the slides and incubated for 2 h at 37 °C. After washing with phosphate-buffered saline, the cells were further incubated with TrITC conjugated secondary antibodies for 50 min at 37 °C. Finally, the cell nuclei were stained with diaminidino-phenyl-indole (DAPI) (Vector Laboratories, United States) and observed under a Leica TCS SP5 laser scanning confocal microscope. Phosphate-buffered saline replaced the primary antibody in the controls.

RESULTS

Primary culture of nhGMECs

nhGMECs were dissociated and cultured. The viability of these cells showed a maximal increase between the 2nd and 3rd day, reached a peak on the 4th day, and then declined gradually. As shown in Figure 1, cultured cells were PAS-positive and CK-18 positive. On the 2nd day of inoculation, the cells grew in clumps and proliferated rapidly, then gradually ceased to grow after the 4th day and began to detach and die on the 5th day. Transmission electron microscopy revealed microvilli and secretory granules in gastric mucosal epithelial cells. Toluidine blue staining was weakly positive in nhGMECs and strongly positive in SGC-7901 cells. nhGMFs were also isolated and cultured.

Telomerase activity

Telomerase activity was detected in all cultured cells using TRAP assay. Amplified telomeric repeats (160 bp) in nhGMECs and nhGMFs were equal to those in BGC-823, SGC-7901 and MKN-28 tumor cell lines (Figure 2). These results suggested that a similar level of telomerase expression was seen in nhGMECs, nhGMFs and the tumor cell lines.

hTERT protein expression

In situ detection of hTERT showed that positive hTERT immunostaining was detected in nhGMECs, nhGMFs, BGC-823, SGC-7901 and MKN-28 cells (Figure 3). Both cellular cytoplasm and nuclear compartments were stained with the hTERT antibody. There was little difference in hTERT expression among nhGMECs, nhGMFs

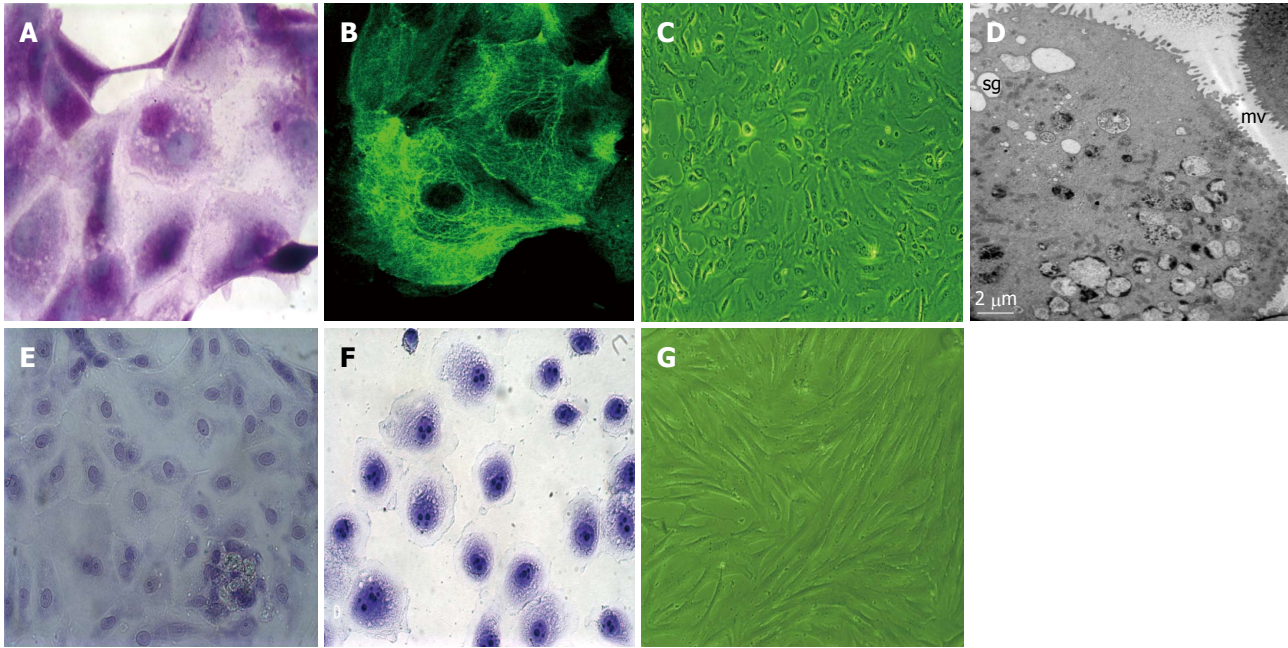


Figure 1 Periodic acid-Schiff, cytokeratin-18 and toluidine blue staining and cell morphology. A: Cytoplasm of normal human gastric mucosal epithelial cells (nhGMECs) was stained purple with periodic acid-Schiff (PAS), and contained neutral mucin granules, magnification $\times 1000$; B: nhGMEC network-structure staining with an antibody against cytokeratin (CK)-18 demonstrates the presence of CK-18; C: Phase-contrast micrograph of nhGMECs after 4 d of culture, magnification $\times 100$; D: Transmission electron microscopy revealed the presence of microvilli (mv) and secretory granules (sg) in nhGMECs, magnification $\times 1000$; E: nhGMECs were detected by toluidine blue staining, and nuclei (light color) were observed, magnification $\times 400$; F: SGC-7901 cells were detected by toluidine blue staining, and nuclei with multiple nucleoli (deep color) were observed, magnification $\times 400$; G: Phase-contrast micrograph of nhGMFs after 13 d of culture, magnification $\times 100$.

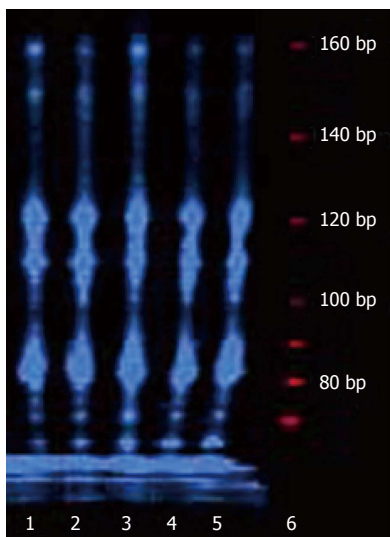


Figure 2 Telomerase activity in normal human gastric mucosal epithelial cells, normal human gastric mucosal fibroblasts and tumor cell lines. Lane 1: Normal human gastric mucosal fibroblasts; lane 2: Normal human gastric mucosal epithelial cells; lane 3: BGC-823 cells; lane 4: SGC-7901 cells; lane 5: MKN-28 cells; lane 6: Marker.

and the tumor cell lines.

DISCUSSION

Increased telomerase activation and hTERT expression are generally considered early events in carcinogenesis^[10,13]. Their assessment as diagnostic markers in various types of cancers has been carried out for more than ten

years. However, some researchers have recently found similar expression of hTERT in both normal and cancerous gastric specimens^[11], which challenges the widespread concept that hTERT and telomerase are repressed in normal tissues. Many highly proliferative normal human cells such as lymphocytes, hematopoietic progenitor cells and basal epidermal cells have been shown to express telomerase and hTERT^[14,15]. Consistent with this, telomerase activity and hTERT protein were detected in primary cultured nhGMECs in our study. Telomerase activity and hTERT protein expression in nhGMECs were very similar to those in the three human gastric adenocarcinoma cell lines, BGC-823, SGC-7901 and MKN-28. In general, primary cultured nhGMECs are from the proliferation zones of crypts in gastric glands and have good proliferative ability. The presence of telomerase and hTERT in these cells could affect the feasibility of the diagnostic markers in the neoplastic process.

Fibroblasts are widely found in various tissues, both in benign and malignant tissues. They were previously believed to lack telomerase activity and hTERT expression^[16]. However, several studies have confirmed the presence of telomerase and hTERT in human fibroblasts in recent years^[15,17]. In the present study, telomerase activity and hTERT expression were similarly detected in primary cultured nhGMFs isolated from human gastric tissues, which will interfere with their use as gastric cancer markers.

Novel molecular biology techniques have generated some new ideas for therapeutics such as gene therapy in gastric cancer. Telomerase is one of therapeutic tar-

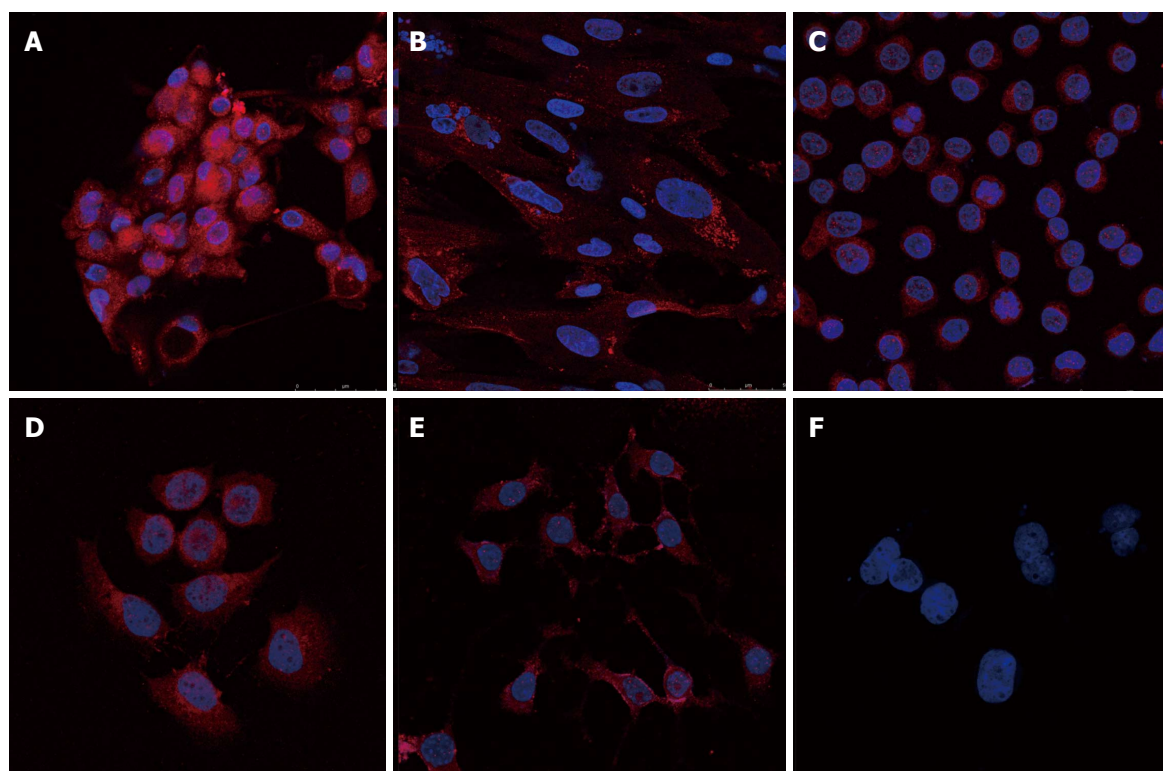


Figure 3 Expression of human telomerase reverse transcriptase in cultured cells, stained with diamidino-phenyl-indole, human telomerase reverse transcriptase antibody, and rhodamine labeled human telomerase reverse transcriptase second antibody. A: Normal human gastric mucosal epithelial cells; B: Normal human gastric mucosal fibroblasts; C: BGC-823 cells; D: SGC-7901 cells; E: MKN-28 cells; F: Negative control.

gets^[17,18]. Its presence in nhGMECs and nhGMFs isolated from gastric tissues suggests that anti-telomerase treatment may trigger undesired toxicity in normal gastric cells, which will have an influence on the therapeutic outcome.

In conclusion, the present study demonstrated the expression of telomerase and hTERT in primary cultured nhGMECs and nhGMFs isolated from gastric tissues. Combined with the observation that hTERT expression occurs in normal human gastric tissues^[6], from a cytological and histological point of view, the use of telomerase and hTERT as diagnostic markers for gastric cancer may require further investigation.

COMMENTS

Background

Gastric cancer remains one of the major causes of cancer-related deaths. This is due to the fact that most cases are in the advanced stages of disease when diagnosed. Therefore, early diagnosis is important in improving the prognosis of patients with gastric cancer. Researchers have been looking for early diagnostic markers for gastric cancer for more than ten years, including telomerase and human telomerase reverse transcriptase (hTERT).

Research frontiers

The assessment of telomerase and hTERT as diagnostic markers in various types of cancers has been carried out for more than ten years. However, the use of hTERT and subsequently telomerase as gastric cancer markers is still unclear. In this study, the authors detected telomerase activity and hTERT expression in primary cultured normal human gastric mucosal epithelial cells (nhGMECs) and normal human gastric mucosal fibroblasts (nhGMFs), which challenges the widespread concept that hTERT and telomerase are repressed in normal tissues.

Innovations and breakthroughs

Telomerase and hTERT are observed in 80%-90% of human tumors including gastric cancer and nearly all cancer-derived cell lines, and are not observed in the majority of normal tissues and somatic cells. However, some researchers have recently found that hTERT mRNA and protein were expressed in normal gastric tissues. In this study, the authors observed that both telomerase and hTERT were expressed in primary cultured normal human gastric mucosal cells including nhGMECs and nhGMFs.

Applications

The present study demonstrated the expression of telomerase and hTERT in primary cultured nhGMECs and nhGMFs isolated from gastric tissues, which suggested from the cytological point of view that using telomerase and hTERT as useful markers for early diagnosis and promising targets for gastric cancer treatment may need further investigation.

Terminology

Telomerase is a specialized reverse transcriptase that adds telomeric repeats to the ends of eukaryotic chromosomes, and is responsible for continuous cell growth. hTERT is the major subunit of the telomerase enzyme complex and plays a critical role in the regulation of telomerase activity. Increased telomerase activation and hTERT expression are generally considered the early events in carcinogenesis.

Peer review

The authors observed telomerase activity and hTERT expression in normal human gastric mucosal epithelial cells and fibroblasts. The results told us again that the use of hTERT and subsequently telomerase as gastric cancer markers is unclear. At the same time, if telomerase or hTERT are used as gastric cancer therapeutic targets, the adverse effects on normal somatic cells should be noted.

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Liver resection in hepatitis B related-hepatocellular carcinoma: Clinical outcomes and safety in elderly patients

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Abstract

AIM: To compare the morbidity and mortality in young and elderly hepatocellular carcinoma (HCC) patients undergoing liver resection.

METHODS: We retrospectively enrolled 1543 consecutive hepatitis B (HBV)-related HCC patients undergoing elective hepatic resection in our cohort, including 207 elderly patients (≥ 65 years) and 1336 younger patients (< 65 years). Patient characteristics and clinical outcomes after liver resection were compared between the two groups.

RESULTS: Elderly patients had more preoperative comorbidities and lower alanine aminotransferase and aspartate aminotransferase levels. Positive rates for hepatitis B surface antigen ($P < 0.001$), hepatitis B e antigen ($P < 0.001$) and HBV DNA ($P = 0.017$) were more common in younger patients. Overall complications and their severity classified using the Clavien system were similar

in the two groups (33.3% vs 29.6%, $P = 0.271$). Elderly patients had a higher rate of postoperative cardiovascular complications (3.9% vs 0.6%, $P = 0.001$), neurological complications (2.9% vs 0.4%, $P < 0.001$) and mortality (3.4% vs 1.2%, $P = 0.035$), and had more hospital stay requirement (13 d vs 12 d, $P < 0.001$) and more intensive care unit stay (36.7% vs 27.8%, $P = 0.008$) compared with younger patients. However, postoperative hepatic insufficiency was more common in the younger group (7.7% vs 3.4%, $P = 0.024$).

CONCLUSION: Hepatectomy can be safely performed in elderly patients. Age should not be regarded as a contraindication to liver resection with expected higher complication and mortality rates.

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Key words: Elderly; Hepatocellular carcinoma; Hepatectomy; Complication; Hepatitis B

Core tip: Elderly patients are regarded as unsuitable for liver resection due to the presence of comorbidities. Our study found that elderly patients did have more comorbidities than younger patients, but also had better liver function and reduced hepatitis B infection. Elderly patients had similar overall morbidity and higher mortality compared with younger patients. Older patients also had more cardiovascular complications, neurological complications and a longer hospital stay, but less hepatic insufficiency. Our study suggested that liver resection can be safely performed in carefully selected elderly patients with accepted higher complication and mortality rates.

Wang HQ, Yang J, Yan LN, Zhang XW, Yang JY. Liver resection in hepatitis B related-hepatocellular carcinoma: Clinical outcomes and safety in elderly patients. *World J Gastroenterol* 2014; 20(21): 6620-6625 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6620.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6620>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common global cause of cancer-related deaths^[1]. Fifty to fifty-five percent of HCC cases are attributed to chronic hepatitis B virus infection worldwide, and up to 80% in China^[2]. There will be more elderly patients as people live longer, especially in China, which has the world's largest population. Moreover, aging itself is a risk factor for HCC carcinogenesis and development^[3]. Therefore, the number of elderly HCC patients will increase^[3], which may also result in social problems. The elderly tend to be considered clinically "fragile" due to comorbidity and a poorer performance status, which make them less amenable and tolerant to resection^[4]. Liver resection is the treatment of choice in HCC patients, however, elderly HCC patients with comorbidity may have an increased surgical risk and may have higher morbidity and mortality^[5]. These age-related contraindications have prevented elderly HCC patients from receiving optimal surgical treatment. With the refinement of surgical techniques and perioperative management in liver surgery during the last few decades, liver resection in elderly HCC patients has become safer^[3].

Many studies have reported the safety of liver resection in elderly HCC patients, but have drawn inconsistent conclusions. The aim of our study was to evaluate the safety of liver resection in a large sample of elderly HCC patients, by comparing the outcome of liver resection performed in patients younger and older than 65 years.

MATERIALS AND METHODS

Population and study design

We carried out a retrospective study. Between January 2009 and March 2013, 1543 consecutive hepatitis B virus (HBV)-related HCC patients undergoing elective hepatic resection were included in this study. All included patients were diagnosed with HCC by histology and with current or a history of HBV infection. All patients underwent surgery only when the Child-Turcotte-Pugh (CTP) class was A. Patient data on pre-, intra-, and postoperative parameters were collected prospectively from the West China Hospital of Sichuan University HCC database (HCCWCHSU System). The protocol was approved by the West China Hospital Ethics Committee and written informed consent was obtained from all patients before inclusion. Based on the age distribution, the patients were divided into the elderly group (≥ 65 years) and the younger group (< 65 years). The primary outcomes were preoperative mortality and postoperative complications in the elderly group compared with the younger group.

Perioperative management

All the included patients were managed by the same surgical team. All patients underwent a thorough history enquiry, physical examination and routine preoperative laboratory measurements. Echocardiography, chest radiography or computed tomography, pulmonary function

test and coronary angiography were carried out if necessary. Routine preoperative imaging examinations to evaluate the tumor included contrast computed tomography or magnetic resonance imaging of the abdomen. American Society of Anesthesiologists (ASA) category was used for anesthetic assessment. Patients were explored through an extended right subcostal incision and intraoperative ultrasonography was performed routinely. Hemihepatic vascular inflow occlusion^[6] or the Pringle maneuver^[7] were used according to the surgeon's preference in most patients. Liver parenchymal transection was performed using the Hooking ligation technique or an ultrasonic dissector with coagulator^[6]. Based on preoperative and intraoperative conditions, patients were transferred to the intensive care unit for treatment if necessary.

Definition of the parameters used

Mortality was defined as death within 30 d after surgery or death before discharge involving a hospital stay of more than 30 d. The Clavien-Dindo complications classification system^[8] was used to grade postoperative complications. Liver resection of more than 3 segments was defined as major resection, and liver resection of less than 3 segments was defined as minor resection^[5]. Portal hypertension was defined as esophageal varices detected by endoscopy or splenomegaly (major diameter > 12 cm) with a platelet count $< 100000/\text{mm}^3$ according to the Barcelona Clinic Liver Cancer Group criteria^[9]. For individual pre-existing disease, we used the Charlson index^[10,11] to quantify comorbidities. The 50-50 criteria^[12] defined as prothrombin time $< 50\%$ and serum bilirubin level $> 50 \mu\text{mol/L}$ on day 5 after liver resection, was defined as liver failure. Hepatic insufficiency was defined as serum bilirubin $> 60 \mu\text{mol/L}$ on postoperative day 5. Extrahepatic procedures included all other operations, except liver resection, such as bowel resection, adrenalectomy, diaphragm resection, biliary tract exploration and adhesion separation due to reoperation.

Statistical analysis

Statistical analysis was performed using SPSS Version 17 statistical analysis software and significance was set at $P < 0.05$. The Student *t* and Mann-Whitney *U* tests were used to compare continuous variables when appropriate. The χ^2 test and Fisher exact test were used to compare categorical variables.

RESULTS

Patient clinical characteristics

Clinical characteristics of the elderly and younger groups are shown in Table 1. All 1543 patients were diagnosed with HBV-related HCC. Of these patients, 13.4% were elderly, with a median age of 68 years (interquartile range: 66-73 years), and the median age of the younger group was 47 years (interquartile range: 40-56 years). A similar distribution in gender and portal hypertension was seen in both groups. No significant differences were found for platelets, white blood cells and body mass index.

Table 1 Clinical characteristics in elderly and younger hepatitis B virus-related hepatocellular carcinoma patients *n* (%)

| Clinical characteristics | Elderly (≥ 65 yr) (<i>n</i> = 207) | Younger (< 65 yr) (<i>n</i> = 1336) | <i>P</i> value |
|--|--|---|----------------|
| Age (yr), median (IQR) | 68 (66-73) | 47 (40-56) | < 0.001 |
| Male | 166 (80.2) | 1128 (84.4) | 0.123 |
| Body mass index (kg/m ²), mean (SD) | 22.8 (2.9) | 22.9 (2.9) | 0.899 |
| HBsAg(+) | 123 (59.4) | 1127 (84.4) | < 0.001 |
| HBeAg(+) | 10 (4.8) | 236 (17.7) | < 0.001 |
| HBV DNA (+) | 50 (24.2) | 433 (32.4) | 0.017 |
| AST (U/L), median (IQR) | 35 (25-55) | 40 (29-64) | 0.001 |
| ALT (U/L), median (IQR) | 34 (24-49) | 40 (29-60) | < 0.001 |
| White Blood Cells (10 ⁹ /L), median (IQR) | 5.34 (4.18-6.67) | 5.37 (4.25-6.63) | 0.656 |
| Hemoglobin (g/L), median (IQR) | 134 (122-144) | 143 (130-153) | < 0.001 |
| Platelets (10 ⁹ /L), median (IQR) | 136 (92-185) | 130 (92-183) | 0.999 |
| Portal hypertension | 52 (25.1) | 376 (28.1) | 0.366 |

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV-DNA: Positive indicated by hepatitis B virus DNA > 2000U/mL; IQR: Interquartile range; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 2 Comorbidity in elderly and younger patients *n* (%)

| Comorbidity | Elderly (≥ 65 yr) (<i>n</i> = 207) | Younger (< 65 yr) (<i>n</i> = 1336) | <i>P</i> value |
|------------------------------|--|---|----------------|
| ASA grade (III + IV) | 133 (64.3) | 95 (7.1) | < 0.001 |
| Charlson index, median (IQR) | 2 (1-3) | 1 (1-3) | < 0.001 |
| Charlson index > 3 | 37 (17.9) | 114 (8.5) | < 0.001 |
| Comorbidity | 122 (58.9) | 326 (24.4) | < 0.001 |
| Hypertension | 89 (43) | 188 (14.1) | < 0.001 |
| Cardiovascular disease | 14 (6.8) | 28 (2.1) | < 0.001 |
| Pulmonary disease | 22 (10.6) | 25 (1.9) | < 0.001 |
| Diabetes mellitus | 39 (18.8) | 78 (5.8) | < 0.001 |
| Renal-related disease | 6 (2.9) | 21 (1.6) | 0.285 |

Cardiovascular diseases included coronary heart disease, previous coronary revascularization, cerebral arterial occlusive disease, and/or peripheral vascular occlusive disease. Pulmonary diseases indicated chronic obstructive pulmonary disease, asthma, chronic bronchitis and tuberculosis. Renal disease indicated chronic glomerulonephritis, renal insufficiency, hydronephrosis, lithangiuria, and diabetic nephropathy. ASA: American Society of Anesthesiologists category; IQR: Interquartile range.

Compared with younger patients, elderly patients had a significantly lower positive rate of hepatitis B surface antigen (HBsAg) (59.4% *vs* 84.4%, *P* < 0.001), hepatitis B e antigen (HBeAg) (4.8% *vs* 17.7%, *P* < 0.001), HBV DNA (24.2% *vs* 32.4%, *P* = 0.017) and lower hemoglobin concentration (*P* < 0.001). Although liver resections were performed for CTP grade A, elderly patients had better liver function, with lower aspartate aminotransferase (AST) (*P* = 0.001) and alanine aminotransferase (ALT) levels (*P* < 0.05).

Table 3 Intraoperative parameters in elderly and younger patients *n* (%)

| Intraoperative parameters | Elderly (≥ 65 yr) (<i>n</i> = 207) | Younger (< 65 yr) (<i>n</i> = 1336) | <i>P</i> value |
|--------------------------------|--|---|----------------|
| Major resection | 77 (37.2) | 497 (37.2) | 1.000 |
| Extrahepatic procedures, | 38 (18.4) | 266 (19.9) | 0.601 |
| Inflow occlusion | 82 (39.6) | 560 (41.9) | 0.532 |
| Liver resection with | | | |
| Hooking | | | |
| with ligation | 78 (37.7) | 460 (34.4) | 0.361 |
| Liver resection with | | | |
| ultrasonic dissector | 129 (62.3) | 876 (65.6) | 0.361 |
| Laparoscopic | 4 (1.9) | 15 (1.1) | 0.519 |
| hepatectomy | | | |
| Blood loss (mL), mean \pm SD | 432 \pm 327 | 496 \pm 528 | 0.092 |
| Blood transfusion | 38 (18.4) | 250 (18.7) | 0.903 |
| Anatomic resection | 76 (36.7) | 481 (36) | 0.843 |

With regard to comorbidities (Table 2), 122 of 207 (58.9%) elderly patients had more than one comorbidity, compared with 326 of 1336 (24.4%) in the younger group (*P* < 0.001). In addition, elderly patients had a significantly higher Charlson index, a higher proportion of Charlson index > 3 (17.9% *vs* 8.5%, *P* < 0.001) and ASA grade III–VI (64.3% *vs* 7.1%, *P* < 0.001). In the elderly group, the most common comorbid conditions were hypertension, diabetes mellitus, pulmonary disease, cardiovascular disease and renal-related disease, which were all significantly higher, with the exception of renal-related disease, than those in the younger patients.

Intraoperative data

The same proportion (37.2%) of patients undergoing major liver resection was found in both groups (Table 3). For the elderly and younger groups, respectively, 38 (18.4%) and 266 (19.9%) patients underwent a simultaneous non-hepatic procedure, most commonly adhesiolysis, portal vein tumor thrombus resection, biliary tract exploration, diaphragm resection, splenectomy and bowel resection. There were no significant differences between the two groups with regard to the parameters analyzed (Table 3). Information on the Ishak score was available in only 713 patients and there was no significant difference in the rate of cirrhosis (Ishak score ≥ 5) between the elderly and younger groups (*P* = 0.404).

Postoperative outcome

Postoperative complications and their severity are shown in Table 4. The elderly group had similar morbidity and levels of complications (from grade I to VI) to those in the younger group. However, the elderly patients had a higher mortality than the younger group (3.4% *vs* 1.2%, *P* = 0.035). Cardiovascular complications and neurological complications were more frequent in the elderly patients (*P* = 0.001). In addition, the incidence of hepatic insufficiency was higher in younger patients (*P* = 0.024). The most common complications in elderly patients were pul-

Table 4 Postoperative outcomes in elderly and younger patients *n* (%)

| Postoperative outcomes | Elderly (≥ 65 yr) (<i>n</i> = 207) | Younger (< 65 yr) (<i>n</i> = 1336) | <i>P</i> value |
|---------------------------------|--|---|----------------|
| Total complications | 69 (33.3) | 395 (29.6) | 0.271 |
| Grade I | 18 (8.7) | 128 (9.6) | 0.686 |
| Grade II | 30 (14.5) | 163 (12.2) | 0.354 |
| Grade III | 7 (3.4) | 64 (4.8) | 0.368 |
| Grade IV | 7 (3.4) | 24 (1.8) | 0.213 |
| Mortality (grade V) | 7 (3.4) | 16 (1.2) | 0.035 |
| Bile Leakage | 1 (0.5) | 20 (1.5) | 0.396 |
| Liver failure | 2 (1.0) | 25 (1.9) | 0.523 |
| Hepatic insufficiency | 7 (3.4) | 103 (7.7) | 0.024 |
| Cardiovascular complications | 8 (3.9) | 8 (0.6) | < 0.001 |
| Pulmonary complications | 19 (9.2) | 80 (6.0) | 0.081 |
| Infectious complications | 14 (6.8) | 67 (5.0) | 0.294 |
| Bleeding | 3 (1.4) | 17 (1.3) | 1.000 |
| Neurological complications | 6 (2.9) | 6 (0.4) | 0.001 |
| Ascites | 7 (3.4) | 61 (4.6) | 0.440 |
| Gastrointestinal complications | 5 (2.4) | 32 (2.4) | 1.000 |
| ICU stay | 76 (36.7) | 371 (27.8) | 0.008 |
| ICU stay (d), median (IQR) | 0 (0-1) | 0 (0-1) | 0.006 |
| Hospital stay (d), median (IQR) | 13 (11-17) | 12 (10-15) | < 0.001 |

Neurological complications indicated confusion and epilepsy. Cardiovascular complications included myocardial infarction, arrhythmia, heart failure and sudden cardiac arrest. Pulmonary complications indicated pulmonary infection, respiratory failure, hydrothorax and hydrothorax. ICU: Intensive care unit; IQR: Interquartile range.

monary complications, followed by infectious complications, cardiovascular disease, hepatic insufficiency, ascites, and neurological complications.

DISCUSSION

The incidence rate of HCC among the elderly is progressively increasing^[3], however, only a minority undergo curative procedures^[13]. Historically, there were biases against cancer treatment for the elderly as life expectancy of elderly patients will be determined by medical comorbidities and not malignancy^[14]. Moreover, aging leads to a number of structural and functional changes in the liver, including a decline in liver volume, a reduction in the mass of functional hepatocytes, and alterations in hepatic microcirculation, which may make liver resection less tolerable^[15]. With the refinement of surgical techniques and perioperative management in liver surgery during the last few decades, some studies have suggested that liver resection is a safe and effective treatment in elderly patients, even for elderly patients over 80 years old^[16]. However, there is still controversy as to whether age influences the postoperative outcome of HCC patients.

Several studies^[13,16-19], which included elderly patients aged 70 to 80 years, showed that there were no differences

in morbidity and mortality, however, other studies^[20-22] found that elderly patients had more complications. The present study assessed the safety of liver resection in a large sample of elderly patients enrolled in a retrospective cohort. Our elderly and younger HCC patients differed with regard to several features. Elderly patients had a higher rate of comorbidity, lower AST and ALT levels, and lower positive rates for HBsAg, HBeAg and HBV DNA, but higher cardiovascular and neurological complications. The overall morbidity was similar in the two groups, but elderly patients had high mortality and longer hospital and more intensive care unit (ICU) stay requirement.

The cut-off age for elderly HCC patients varies widely in the literature from 65 to 80 years^[16,23-25]. However, most studies^[13,17,20-21,24,26-28] used 70 years as the cut-off age and these studies included HCC due to various etiologies, such as HBV, hepatitis C virus (HCV) and nonalcoholic fatty liver disease. The average age at onset of HBV-related HCC was reported to be 10 years younger than that of HCV-related HCC^[3]. In our cohort, HCC was related to HBV infection and we defined elderly patients as aged more than 65 years. That was because the mean age at HCC diagnosis was found to be 55-59 years in China^[29], and in South Korea^[2], however, the mean age at HCC diagnosis was 63-65 years in Europe and North America and 75 or older in low-risk populations^[29]. Thus, 65 years is a more suitable cut-off for elderly patients in China.

Based on the cut-off age of 65 years, 13.4% of the patients in our cohort were elderly, which was less than that in other studies^[13-14,17,20,23-24]. This may be because elderly patients were highly selected for liver resection based on preoperative general condition and assessment of hepatic reserve in our center.

In agreement with previous reports^[17,20], elderly patients showed a higher rate of comorbidities, ASA grade \geq III and higher quantitative comorbidity (Charlson index). The higher prevalence of hypertension and cardiovascular disease may be the reason for the higher rate of postoperative cardiovascular complications in elderly patients. The function of most organs usually deteriorates with age^[16] and this could explain why elderly patients had more neurological complications. Cho *et al.*^[17] reported that confusion after liver resection was far more common in the elderly than in younger patients.

Although both elderly and younger patients had preserved liver function with CTP class A, the AST and ALT levels were significantly higher in younger patients. Approximately 80% of HBV-related HCC cases occur in patients with cirrhosis^[1], and cirrhosis severity influences liver function and postoperative complications. The rate of cirrhosis was not different between the two groups in our cohort. We compared the rate of portal hypertension, which caused the underlying liver damage, but no difference was observed between the two groups. The positive rates of HBsAg, HBeAg and HBV DNA were significantly lower in the elderly group, this meant that the younger patients had worse underlying liver damage resulting from HBV infection. This study revealed an

age-related difference in HBV infection status (current or previous infection) and more elderly patients had a history of HBV infection. This difference may also indicate that inflammation of the liver due to HBV infection was less active in elderly patients as younger patients had higher AST and ALT levels. The same phenomenon was observed in the study by Oishi *et al.*^[30] in which elderly patients > 75 years with HCC had better liver function than younger patients as assessed by prothrombin time, AST and ALT. Several studies have also found^[23-24,30] better preoperative liver function in elderly patients. In addition, postoperative hepatic insufficiency was found to be more common in younger patients and Yau *et al.*^[31] also found that young patients had a significantly higher rate of liver derangement after TACE than elderly patients. Several reasons could explain this result. Firstly, the preoperative AST and ALT levels and HBV infection status may influence postoperative liver function. Secondly, HCC is less frequently associated with cirrhosis in elderly patients^[32]. It is possible that patients with cirrhosis and HCC died before reaching elderly status and the surviving patients had well preserved hepatic function^[32]. In addition, this result may be due to elderly patients being highly selected for liver resection based on the assessment of hepatic reserve in our center. Thus, considering postoperative liver-related complications, age is not a contraindication to liver resection, although aging may lead to a number of structural and functional changes in the liver.

Compared with younger patients, overall complications and their severity, classified using the Clavien system, were similar in elderly patients, but these patients had higher mortality (3.4%). Despite higher mortality in elderly patients, a mortality rate of 3.4% suggested that liver resection was relatively safe in the elderly, compared with mortality of 3.15% in a meta-analysis which included 35000 hepatic resections^[33]. Many studies^[13-14,17-19] have also drawn the same conclusion in that there were no significant differences in postoperative complications. These data suggest that hepatic resections can be safely performed in elderly patients. However, elderly patients had a longer hospital stay and more ICU stay requirement. Therefore, although the elderly were not predisposed to postoperative complications, recovery in these patients may be slower due to less physiologic reserve compared with younger patients^[14]. Therefore, advanced age is not the major determinant in the incidence and severity of postoperative complications.

The results of our study should be interpreted cautiously, as our analysis was restricted to patients with HBV-related HCC, and may not be appropriate for other etiologies. Moreover, it is important to point out that the elderly patients in our study were highly selected for surgical safety.

In conclusion, liver resection can be safely performed in carefully selected elderly patients. Although elderly patients had more cardiovascular and neurological complications, age should not be regarded as a contraindication to liver resection.

COMMENTS

Background

There will be more elderly patients in the future and this may cause social problems as elderly patients have more comorbidity and a poorer performance status. Hepatocellular carcinoma is a common cancer and usually occurs in older patients. The safety of liver resection in elderly patients is still a concern.

Research frontiers

Aging not only results in more and severe comorbidities, but usually leads to a number of structural and functional changes in the liver, including a decline in liver volume, a reduction in the mass of functional hepatocytes, and alterations in hepatic microcirculation. These may make liver resection less tolerable. The research hotspot is to evaluate the safety of liver resection in elderly patients.

Innovations and breakthroughs

The authors' study found that elderly patients had more preoperative comorbidities compared with younger patients. However, elderly patients did not only have better liver function, but also had less hepatitis B infection. Overall complications were similar in the two groups. However, elderly patients had more postoperative cardiovascular complications, mortality and less hepatic insufficiency.

Applications

In general, hepatectomy can be safely performed in elderly patients with expected higher complications and mortality rates. Aging should not be regarded as a contraindication to liver resection when surgeons make decisions before surgery.

Peer review

The authors present a series of 1543 liver resections in patients diagnosed with hepatitis B virus related hepatocellular carcinoma. There were 1336 young patients and 207 elderly patients. It is a series collected in a period of four years. The article is well redacted and its conclusions are very interesting for the international literature.

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Controlled attenuation parameter for evaluating liver steatosis in chronic viral hepatitis

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Abstract

AIM: To assess the performance of controlled attenuation parameter (CAP) in patients with chronic viral hepatitis.

METHODS: CAP is a new technique that measures the attenuation in the liver of an ultrasound beam, which is directly related to lipid accumulation. Consecutive patients undergoing liver biopsy for chronic viral hepatitis were studied using the M probe of FibroScan device (Echosens, Paris, France). The device estimates liver steatosis in decibel per meter (dB/m). An expert operator performed all measurements. Steatosis was graded according to Kleiner's classification. Pearson or Spearman rank coefficient was used to test correlation between two study variables. Linear regression was used for multivariate model to assess the association between CAP and other variables. Receiver operating characteristic curve analysis was performed to calculate area under the curve (AUROC) for S0 vs S1-S3 and S0-S1 vs S2-S3.

RESULTS: 115 subjects (85 males and 30 females) were prospectively studied. The mean values of CAP were 227.1 ± 43.1 for S0; 254.6 ± 38.9 for S1; 297.8 ± 49.4 dB/m for S2-S3. In univariate analysis CAP showed a significant correlation with age, body mass index (BMI), degree of steatosis, and cholesterol. Multivariate regression analysis confirmed the correlation with the degree of steatosis [coefficient, 1.2 (0.60-1.83); $P < 10^{-5}$] and BMI [coefficient, 4.1 (0.5-7.8); $P = 0.03$] but not with all other variables. Optimal cutoff values for $S \geq 1$ and $S \geq 2$ were 219 dB/m [AUROC, 0.76 (0.67-0.84); sensitivity, 91.1% (78.8-97.5); specificity, 51.6% (38.7-64.2); positive predictive value, 56.9% (44.7-68.6); negative predictive value, 89.2% (74.3-97.0); positive likelihood ratio, 1.88 (1.4-2.5); negative likelihood ratio, 0.17 (0.07-0.5)] and 296 dB/m [AUROC, 0.82 (0.74-0.89); sensitivity, 60.0% (32.3-83.7); specificity, 91.5% (83.9-96.3); positive predictive value, 52.9% (27.8-77.0); negative predictive value, 93.5% (86.3-97.6); positive likelihood ratio, 7.05 (3.2-15.4); negative likelihood ratio, 0.44 (0.2-0.8)], respectively.

CONCLUSION: Controlled attenuation parameter could be a useful tool in the clinical management of patients with chronic viral hepatitis for detecting liver steatosis.

Key words: Liver steatosis; Noninvasive techniques; Controlled attenuation parameter; Transient elastography; Chronic liver disease

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Core tip: A number of factors may affect response to treatment of patients with chronic viral hepatitis and it is well known that patients with liver steatosis are less responsive to antiviral drugs. On the other hand, early stages of liver steatosis are usually reversible with appropriate intervention. Controlled attenuation parameter (CAP) is a new method for non-invasive quantification of liver steatosis. The results of our study show that CAP is highly and significantly correlated with the extent of liver fat accumulation and it could be a useful tool in the clinical setting to diagnose the presence/absence of liver steatosis.

Ferraioli G, Tinelli C, Lissandrin R, Zicchetti M, Dal Bello B, Filice G, Filice C. Controlled attenuation parameter for evaluating liver steatosis in chronic viral hepatitis. *World J Gastroenterol* 2014; 20(21): 6626-6631 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i21/6626.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6626>

INTRODUCTION

In developed countries liver steatosis is a major health problem since 20%-30% of the population is affected with nonalcoholic fatty liver disease^[1,2]. Moreover, steatosis, oxidative stress, and insulin resistance have been proposed as important factors in hepatitis C virus (HCV) infection and are reported to be closely interconnected and responsible of accelerating the progression of fibrosis^[3,4]. In patients with chronic hepatitis C the presence of liver steatosis affects the response to treatment and can predict the occurrence of hepatocellular carcinoma independently of fibrosis stage^[5-7]. Steatosis is a relatively common finding in hepatitis B virus (HBV)-infected patients and metabolic host factors rather than viral factors are responsible for this finding in these patients^[8].

Liver histology is the reference standard for grading steatosis even though sampling variability due to the uneven distribution of lipid accumulation throughout the liver parenchyma does exist^[9]. On the other hand, liver biopsy (LB) is an invasive procedure which has some risk of morbidity and mortality, and it is not the ideal procedure to follow up patients^[10]. Recently, a novel controlled attenuation parameter (CAP) for the assessment of liver steatosis has been developed^[11]. CAP is based on the properties of ultrasound signal acquired by transient elastography (FibroScan®, Echosens, Paris) using the postulate that fat affects ultrasound propagation^[12]. Transient elastography is a technique that noninvasively estimates the elasticity of liver parenchyma, which is directly related to the amount of fibrosis. Several studies have shown significant positive correlation between liver stiffness measurements (LSM) and the stage of liver fibrosis^[13-22]. CAP is evaluated using the same radio-frequency data, and the same region of interest used to assess LSM^[23].

The aim of this study was to assess the performance

of CAP in detecting liver steatosis in patients with chronic viral hepatitis undergoing liver biopsy in the same day.

MATERIALS AND METHODS

Subjects

This was a single center cross-sectional study. The performance of CAP was prospectively estimated in a cohort of consecutive patients undergoing LB for chronic viral hepatitis. Inclusion criteria were the presence of serum markers of infection with HBV/HCV, or HCV/HIV coinfection and serum alanine aminotransferase (ALT) levels > 1.5 the upper normal limit, either persistently or intermittently. Exclusion criteria was decompensated liver cirrhosis. As a rule, patients with clinically overt cirrhosis were not scheduled for LB.

Subject characteristics, epidemiological data, and biochemical tests were recorded. LB was performed on the same day as CAP and LSM, as day case procedure.

The study protocol was approved by the institutional Ethics Committee and it was in accordance with the Helsinki Declaration of 1975. Participants gave their informed written consent.

Controlled attenuation parameter

CAP and LSM were obtained by using the FibroScan® 502 touch (Echosens, Paris, France). The device estimates liver stiffness in kilopascal (kPa) and liver steatosis in decibel per meter (dB/m). The principles of CAP have been described elsewhere^[11]. As of today, CAP measurement is available only on the M probe of the Fibroscan device, and it is computed only when the associated liver stiffness measurement is valid. Thus, all patients were studied by using the M probe of the Fibroscan device after fasting for at least six hours. All examinations were carried out by the same physician with three years of experience in LSM (M.Z.). As reported in the literature, only LSM with 10 validated measurements and an interquartile range/mean (IQR/M) < 30% for values higher than 7.1 kPa were considered reliable^[24]. CAP examinations with no successful measurements after at least 10 attempts were deemed as failures.

Liver biopsy and histology

Ultrasound-assisted percutaneous LB was performed by three experienced physicians (C.F., G.M., and E.B.) by using an intercostal approach. A disposable 1.4-mm-diameter modified Menghini needle (Hepafix; Braun, Melsungen, Germany) was used. All biopsy specimens were fixed in formalin and embedded in paraffin. The length of each LB specimen (in centimetres) was recorded.

The specimens were interpreted on site by a single expert liver pathologist (B.D.B.), blind to CAP and LSM results, but not to the patient's clinical and biochemical data. Fibrosis and necro-inflammation were evaluated semiquantitatively according to the METAVIR system^[25]. Steatosis was expressed as a percentage of fat in the hepatocytes and graded according to the method of

Table 1 Main clinical and demographic characteristics of the subjects *n* (%)

| Characteristics | Total, <i>n</i> = 115 | S0, <i>n</i> = 66 | S1, <i>n</i> = 33 | S2-S3, <i>n</i> = 16 |
|---|-----------------------|-------------------------------|-------------------------------|-------------------------------|
| Sex, females | 30 (24.1) | 16 (24.2) | 10 (30.3) | 4 (25.0) |
| Age, yr (mean \pm SD) | 43.1 \pm 10.5 | 41.3 \pm 11.0 | 46.8 \pm 8.6 | 43.0 \pm 11.1 |
| BMI, kg/m ² (mean \pm SD) | 24.8 \pm 4.2 | 24.0 \pm 3.5 | 25.6 \pm 5.2 | 26.7 \pm 3.6 |
| BMI \geq 25 kg/m ² | 57 (50.0) | 28 (42.4) | 18 (56.2) | 11 (68.7) |
| AST, IU/L (IQR) | 43 (28-73) | 39 (25-59) | 52 (31-88) | 45 (35-73) |
| ALT, IU/L (IQR) | 63 (38-110) | 54 (33-73) | 70 (38-142) | 98 (63-125) |
| Alkaline phosphatase, IU/L (mean \pm SD) | 72.2 \pm 25.5 | 77.1 \pm 27.8 | 65.4 \pm 16.9 | 62.6 \pm 24.6 |
| GGT, IU/L (IQR) | 48 (26-88) | 39 (20-57) | 58 (32-133) | 73 (40-106) |
| Total bilirubin, μ mol/L (IQR) | 0.69 (0.49-0.92) | 0.70 (0.46-1.06) | 0.64 (0.55-0.92) | 0.70 (0.54-0.86) |
| Platelets count, 10 ³ /mm ³ (mean \pm SD) | 221 \pm 78 | 229 \pm 81 | 204 \pm 66 | 223 \pm 90 |
| Prothrombin time, % (mean \pm SD) | 93.9 \pm 12.6 | 93.4 \pm 11.5 | 94.2 \pm 15.0 | 95.4 \pm 12.2 |
| HCV infection | 82 (71.3) | 44 (66.7) | 26 (78.8) | 12 (75.0) |
| HBV infection | 28 (24.3) | 18 (27.3) | 7 (21.2) | 3 (18.7) |
| HCV/HIV infection | 5 (4.3) | 4 (6.1) | 0 (0) | 1 (6.2) |
| Fibrosis score (Metavir) | | | | |
| F0 | 14 (12.2) | 9 (13.6) | 5 (15.1) | 0 (0) |
| F1 | 42 (36.5) | 29 (43.9) | 7 (21.2) | 6 (37.5) |
| F2 | 31 (27.0) | 17 (25.8) | 10 (30.3) | 4 (25.0) |
| F3 | 18 (15.6) | 9 (13.6) | 7 (21.2) | 2 (12.5) |
| F4 | 10 (8.7) | 2 (3.0) | 4 (12.1) | 4 (25.0) |
| Activity grade (Metavir) | | | | |
| A0 | 12 (10.4) | 8 (12.1) | 3 (9.1) | 1 (6.2) |
| A1 | 66 (57.4) | 43 (65.1) | 14 (42.4) | 9 (52.2) |
| A2 | 19 (16.5) | 7 (10.6) | 9 (27.3) | 3 (18.7) |
| A3 | 18 (15.6) | 8 (12.1) | 7 (21.2) | 3 (18.7) |
| LSM, kPa (IQR) | 6.7 (5.1-9.1) | 6.4 (4.9-8.4) | 6.7 (4.8-9.5) | 8.0 (5.6-11.1) |
| CAP, dB/m (mean \pm SD) | 244.4 \pm 49.1 | 227.1 \pm 43.1 ^a | 254.6 \pm 38.9 ^b | 297.8 \pm 49.4 ^d |

SD values represent mean, and interquartile range (IQR) values represent median. ^a $P < 0.05$ (S0 vs S1); ^b $P < 0.01$ (S1 vs S2-S3); ^d $P < 0.01$ (S0 vs S2-S3). BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; CAP: Controlled attenuation parameter; LSM: Liver stiffness measurement.

Kleiner *et al*^[26] as S0, steatosis in less than 5% of hepatocytes; S1, 5%-33%; S2, 34%-66%; and S3, more than 66%. For the purpose of this study, S2 and S3 grades were grouped in the subsequent statistical analysis.

Statistical analysis

Sample size considerations for performance of CAP: A total sample size of 115—which includes 50 subjects with the disease, *i.e.* a prevalence approximately 45%—achieves 83% power to detect a change in sensitivity and in specificity from 0.80 to 0.90 using a one-sided binomial test. The target significance level is 0.05.

Descriptive statistics were produced for demographic, clinical and laboratory characteristics for this study sample of patients. The Shapiro-Wilk test was used to test the normal distribution of quantitative variables. When quantitative variables were normally distributed, the results were expressed as mean values and SD, otherwise median and interquartile range (IQR; 25th-75th percentile) were reported; qualitative variables were summarized as counts and percentages. One-way ANOVA or Kruskal-Wallis analysis of variance by ranks, with Bonferroni correction, was used to analyze differences among patients undergoing liver biopsy. Pearson or Spearman rank coefficient was used to test correlation between two study variables. Linear regression was used for multivariate model to assess the association between CAP and other variables. The diagnostic performance of CAP was assessed by using receiver operating characteristic (ROC) curves and

the area under the ROC (AUROC) curve analysis.

Data analysis was performed with STATA statistical package (release 11.1, 2010, Stata Corporation, College Station, Texas, United States) and Medcalc (Version 11.2, 2011 MedCalc Software bvba, Be).

RESULTS

From February 2012 to November 2013, 115 subjects were enrolled into the study. For all patients the consumption of alcohol was less than 20 g/d. The characteristics of the study population is shown in Table 1.

In six patients (5.2%) the examination failed with the M probe of the Fibroscan device. No unreliable measurements were obtained. In all patients LB was performed on the same day as CAP and LSM measurements, no complication was observed. The mean length of the LB specimen was 2.2 (0.73) cm. At histology, all specimens contained \geq 10 portal tracts. None of the specimens showed steatohepatitis or siderosis. Sixty-six (57.4%) subjects were S0, 33 (28.7%) S1, 11 (9.6%) S2, and 5 (4.3%) S3.

In univariate analysis CAP showed a significant correlation with age, body mass index (BMI), degree of steatosis, and cholesterol. Corresponding *r* values were 0.29 ($P = 0.002$); 0.55 ($P < 10^{-3}$); 0.55 ($P < 10^{-3}$); 0.35 ($P = 0.04$), respectively. LSM showed a significant correlation with METAVIR stage, METAVIR grade, AST, and platelets. Corresponding *r* values were 0.41 ($P < 10^{-3}$); 0.28 ($P = 0.003$); 0.26 ($P = 0.02$); 0.23 ($P = 0.04$) respectively.

Table 2 Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio of controlled attenuation parameter best cut-off values

| | Sensitivity% (95%CI) | Specificity% (95%CI) | PPV% (95%CI) | NPV% (95%CI) | +LR (95%CI) | -LR (95%CI) |
|------------|----------------------|----------------------|------------------|------------------|-----------------|-----------------|
| S \geq 1 | 91.1 (78.8-97.5) | 51.6 (38.7-64.2) | 56.9 (44.7-68.6) | 89.2 (74.3-97.0) | 1.88 (1.4-2.5) | 0.17 (0.07-0.5) |
| S \geq 2 | 60.0 (32.3-83.7) | 91.5 (83.9-96.3) | 52.9 (27.8-77.0) | 93.5 (86.3-97.6) | 7.05 (3.2-15.4) | 0.44 (0.2-0.8) |

PPV: Positive predictive value; NPV: Negative predictive value; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

Multivariate regression analysis confirmed the correlation with the degree of steatosis [coefficient, 1.2 (95%CI: 0.60-1.83); $P < 10^{-5}$] and BMI [coefficient, 4.1 (95%CI: 0.5-7.8); $P = 0.03$] for CAP, and with METAVIR stage for LSM [3.1 (coefficient, 95%CI: 0.25-5.9); $P = 0.03$] but not with all other variables.

The mean values of CAP were 227.1 ± 43.1 dB/m for S0; 254.6 ± 38.9 dB/m for S1; 297.8 ± 49.4 dB/m for S2-S3. ROC curve analysis showed that optimal cut-off values for the diagnosis of steatosis - S0 *vs* S1-S3 ($S \geq 1$) - and for the assessment of significant steatosis-S0-S1 *vs* S2-S3 ($S \geq 2$)-were 219 dB/m [AUROC, 0.76 (95%CI: 0.67-0.84)] and 296 dB/m [AUROC, 0.82 (95%CI: 0.74-0.89)]. The corresponding sensitivity, specificity, positive and negative predictive value, positive and negative likelihood ratio are reported in Table 2. CAP demonstrated excellent negative predictive value for assessing and grading steatosis. For the diagnosis of liver steatosis 35 of 109 (32.1%) subjects were misclassified, of these 31 were false positive cases and 4 false negative. The 31 false positive cases had a significantly higher BMI compared to the 33 true negative cases [25.6 (3.4) *vs* 22.1 (2.3); $P < 0.0001$].

DISCUSSION

The availability of new and more effective treatment options for patients with chronic hepatitis has questioned the utility of liver biopsy, that is an invasive procedure not free of risks. As a consequence, non-invasive methods for assessing liver fibrosis are becoming widely used^[13-22,24]. They have no complications and can be performed also to monitor progression or improvement of liver fibrosis over time. On the other hand, a number of factors may affect response to treatment of patients with chronic viral hepatitis and it is well known that patients with liver steatosis are less responsive to antiviral drugs^[6]. In patients with chronic hepatitis C liver steatosis is associated with the progression of liver fibrosis^[5]. Thus, there is a need to noninvasively and reliably assess not only fibrosis but also fatty infiltration of the liver. Moreover, early stages of liver steatosis are usually reversible with appropriate intervention. Magnetic resonance imaging shows high accuracy for quantification of liver steatosis, but it has high cost and is too complex to be used to monitor the disease^[27]. Ultrasound is a low cost imaging modality but it lacks sensitivity for detection of mild steatosis. CAP is a new method for quantification of liver steatosis, and it has the advantage of being measured at the same time as liver

stiffness and it is not influenced by fibrosis^[11,12].

The results of our study show that CAP is highly and significantly correlated with the percentage of liver fat accumulation and it could be a useful tool in the clinical setting to diagnose the presence/absence of liver steatosis. In our series, in the detection of liver steatosis CAP misclassified one third of cases. Nonetheless, the technique showed a high sensitivity, thus it was able to confidently identify patients with liver steatosis.

The optimal cut-off value obtained in our series for the diagnosis of steatosis ($S \geq 1$) is similar to that obtained by Sasso *et al*^[12] in a series of patients with chronic hepatitis C and by de Ledinghen *et al*^[28] in a series of patients with chronic hepatitis of mixed etiologies.

We observed that the performance of CAP in detecting and quantifying liver steatosis was moderate compared to what was found in other series which included patients with alcoholic liver disease and metabolic syndrome^[11]. These differences may be due to the low prevalence of steatosis in our series of patients with chronic viral hepatitis.

In our study CAP values were not influenced by fibrosis stage or necro-inflammation but, in addition to the degree of liver steatosis, a correlation between CAP and BMI that persisted in multivariate analysis after correction for confounding variables was found. We would like to underline that in our series the false positive cases for $S=0$ had significantly higher BMI. This finding could be due to the comparison with a histological classification that is fairly subjective for low grades of liver steatosis. Moreover, it should be underlined that we compared a method that gives continuous measurements - such as CAP-to liver histology, which gives a semiquantitative grading of steatosis in a categorical scale. On the other hand, the histological grading of fatty infiltration of the liver is not a perfect gold standard because it examines only a small sample and this could lead to sampling bias especially when fat is heterogeneously distributed throughout the liver as it may happens in mild steatosis^[29]. This difference could reduce the information given by the CAP method. CAP is evaluated in the same region of interest of LSM, which is a volume at least 100 times bigger than a biopsy sample thus more representative of liver parenchyma^[14]. Further studies aimed at comparing CAP also with techniques that give a quantification of the fat in the liver may help understanding whether there is any limitation when using as reference a histological classification of liver steatosis based only in a four-point scale.

The optimal cutoff value to assess significant steatosis

($S \geq 2$) was similar to that obtained in another series and it had only a fair positive predictive value, probably due to the low prevalence of subjects with grade S2 or more in our study population^[30].

This study has limitations. First, even though consecutive subjects were studied, a low prevalence of severe obesity was observed in our cohort. Second, we did not assess the correlation with biochemical markers of liver steatosis because they were available only for some subjects. Nonetheless, BMI could be regarded as a surrogate marker of the metabolic syndrome, thus we believe that this is not a flaw of the study. Third, the accuracy of the method was evaluated in a small number of subjects. Fourth, the patients undergoing liver biopsy had chronic viral hepatitis, thus the applicability of the cutoffs in the general population and in patients with nonalcoholic fatty liver disease could be limited and needs to be further validated to determine the possible influence of etiology.

In conclusion, CAP could be a useful tool in the clinical management of patients with chronic viral hepatitis for detecting liver steatosis. Further studies in larger series are needed to assess the value of CAP in grading liver steatosis.

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COMMENTS

Background

In developed countries liver steatosis is a major health problem since 20%-30% of the population is affected with nonalcoholic fatty liver disease. Moreover, steatosis, oxidative stress, and insulin resistance have been proposed as important factors in hepatitis C virus infection and are reported to be closely interconnected and responsible of accelerating the progression of fibrosis.

Research frontiers

Liver histology is the reference standard for grading steatosis even though sampling variability due to the uneven distribution of lipid accumulation throughout the liver parenchyma does exist. On the other hand, liver biopsy is an invasive

procedure which has some risk of morbidity and mortality, and it is not the ideal procedure to follow up patients. Recently, a novel controlled attenuation parameter (CAP) for the noninvasive assessment of liver steatosis has been developed.

Innovations and breakthroughs

A number of factors may affect response to treatment of patients with chronic viral hepatitis and it is well known that patients with liver steatosis are less responsive to antiviral drugs. Moreover, early stages of liver steatosis are usually reversible with appropriate intervention. Magnetic resonance imaging shows high accuracy for quantification of liver steatosis, but it has high cost and is too complex to be used to monitor the disease. Ultrasound is a low cost imaging modality but it lacks sensitivity for detection of mild steatosis. CAP is a new method for quantification of liver steatosis, and it has the advantage of being measured at the same time as liver stiffness and it is not influenced by fibrosis.

Applications

CAP could be a useful tool in the clinical management of patients with chronic viral hepatitis for detecting liver steatosis. Further studies in larger series are needed to assess the value of CAP in grading liver steatosis.

Terminology

CAP is a measure of the ultrasound attenuation which corresponds to the decrease in amplitude of ultrasound waves as they propagate through the liver. The unit of measure is decibel per meter.

Peer review

The manuscript is well documented and interesting. The performance of methodology and the statistical analysis are very well established. The study is in agreement with previous ones that have shown the correlation of CAP with liver steatosis. He also believes that the development and standardization of CAP will be a useful tool in the future in detecting and measuring steatosis. Concerning the limitations of the study He agrees with the authors that CAP needs to be further validated in a larger number of patients with respect to the etiology of steatosis (viral hepatitis, non-alcoholic fatty liver disease), but the efforts the authors have done still remains reliable.

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Potential role of human papilloma virus in the pathogenesis of gastric cancer

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Abstract

AIM: To demonstrate the presence and biological activity of human papilloma virus (HPV) in gastric cancer (GAC) tissues.

METHODS: The study involved 84 surgically treated patients with gastric adenocarcinoma, regardless of the clinical stage of the disease. The presence of HPV DNA of high oncogenic risk types in formalin-fixed, paraffin-embedded tumor samples was determined using quantitative polymerase chain reaction analysis. A stringent

protocol of prevention of cross- and environmental contamination was applied during DNA isolation, and amplification, as well as confirmation of the biological activity of the virus in tumor cells, was implemented. The study utilized the Real-time High Risk HPV test, which detects the DNA of 14 HPV subtypes that are considered to have high oncogenic potential. The over-expression of the p16^{INK4a} protein assessed immunohistochemically was considered confirmation of the HPV infection.

RESULTS: Among the 89 patients initially included in the study group, diagnostic results were obtained for 84 individuals. In five cases, either the histopathological material was too scant to isolate the necessary amount of DNA, or the isolated DNA was significantly degraded, resulting in the failure of internal control amplification within the predefined number of 35 cycles. Those patients were excluded from further analysis. The amplification of HPV DNA was demonstrated in none of the 84 tissue samples; thus, all cases were considered to have a negative DNA status of highly oncogenic HPV subtypes. Immunohistochemical staining provided diagnostic results for all of the examined tissue samples, and excluded the accumulation of the p16^{INK4a} protein in tumor cells, thus confirming the lack of active HPV infection in all of the individuals.

CONCLUSION: The study does not confirm the presence or biological activity of HPV in tumor tissues. Thus, the relationship between GAC and HPV infection, in the Central European population seems doubtful.

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Key words: Gastric cancer; Human papilloma virus; Quantitative polymerase chain reaction; P16^{INK4a} expression

Core tip: The study aimed to demonstrate the presence and biological activity of human papilloma virus (HPV)

in gastric cancer tissues. The genomes of 14 HPV subtypes of high oncogenic potential were assessed using quantitative polymerase chain reaction in 84 tumor samples. A stringent protocol for preventing sample contamination, and confirming the biological activity of the virus in the tumor cells, was applied. The study did not confirm either the presence of the HPV genome or viral activity in the examined tumor tissues.

Snietura M, Waniczek D, Piglowski W, Kopec A, Nowakowska-Zajdel E, Lorenc Z, Muc-Wierzgon M. Potential role of human papilloma virus in the pathogenesis of gastric cancer. *World J Gastroenterol* 2014; 20(21): 6632-6637 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6632.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6632>

INTRODUCTION

Alimentary tract carcinoma is a group of cancers characterized by a high risk of occurrence and a relatively high mortality rate. Recently, significant progress has been made in understanding the epidemiology, pathology and pathogenesis of alimentary tract carcinomas. Gastric cancer is the third leading cause of cancer death in both sexes worldwide (723000 deaths, 8.8% of the total)^[1]. The greater proportion of the disease occurs within the male population in developing countries - mostly East Asia, South America and Eastern Europe. A downward trend has also been observed in the incidence of this cancer^[1,2]. Among the etiological factors, the most significant is improper eating habits, particularly a diet rich in cured and smoked meat products, with low antioxidant content. Additionally tobacco smoking and alcohol consumption have a significant effect on the gastric cancer development. An important role is also played by chronic *Helicobacter pylori* (*H. pylori*) or Epstein-Barr virus (EBV) infection^[2-5]. Neoplastic transformation is usually a long, highly complex and multi-stage process. that is caused by various genetic and epigenetic changes. human papilloma virus (HPV) is a suspected risk factor of neoplastic transformation. Abundant evidence has demonstrated the oncogenic properties of HPV in studies on anal^[6,7], oral^[8,9] and pharyngeal cancers^[8,10], suggesting a role for the virus in the pathogenesis of cancer of other sections of the alimentary tract. Only a few studies have addressed the role of HPV in the epidemiology and development of gastric cancer (GAC), and they are limited mainly to South and East Asia. However, the results obtained seem divergent, and the conclusions drawn are controversial.

The present study was aimed to evaluate the presence of HPV DNA in GAC tissues using quantitative polymerase chain reaction (PCR) method and to indirectly confirm active infection through a demonstration of the p16^{INK4a} protein overexpression using the immunohistochemical method.

Table 1 Clinical and histopathological characteristics of the study group

| Feature | Value | |
|--|------------------|----------------------------------|
| Gender | Female | 38 |
| | Male | 46 |
| | Total | 84 |
| Age | Median | 64 yr |
| | Range | 18-85 yr |
| Ethnicity | Caucasian | 84 |
| | Other | 0 |
| Histopathological type | Adenocarcinoma | 84 |
| | Intestinal type | 13 |
| | Mucous type | 17 |
| | Signet ring type | 5 |
| Histopathological grading | G1 | 3 |
| | G2 | 35 |
| | G3 | 46 |
| Clinical staging according to the pTNM scale | T | T1-7, T2-16, T3-51, T4-10 |
| | N | N0-23, N1-28, N2-19, N3-11, Nx-3 |
| | M | M0-62, M1-20, Mx-2 |

MATERIALS AND METHODS

The study involved 89 consecutive patients treated surgically for GAC from 2007 to 2013, regardless of the clinical stage of the disease. The inclusion criteria were as follows: age > 18 years and confirmed gastric adenocarcinoma. The exclusion criteria included the following: previous diagnosis of malignant carcinoma and previous anti-cancer therapy (radiotherapy, chemotherapy) for GAC. Paraffin blocks of the tumor tissue, histopathological and clinical documentation allowing for the determination of the primary location of the lesion, histological type of the tumor, histopathological grading and clinical staging using the pTNM scale were studied. Clinical and histopathological characteristics of the patients who were included in the study are presented in Table 1.

Isolation of the genomic DNA aseptically from the paraffin tissue blocks was confirmed using a Maxwell AS2000 instrument and the Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega Corporation, Madison, WI, United States). The purity of the obtained isolates and DNA concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, United States). The presence of HPV DNA of high oncogenic risk types was confirmed using a quantitative PCR (Q-PCR) analysis. The study utilized the RealTime High Risk HPV test (Abbott Laboratories, Abbott Park, IL, United States) to detect the DNA of 14 HPV subtypes that are considered to have high oncogenic risk. The usefulness of the test in the assessment of HPV status in formalin-fixed, paraffin-embedded tissue samples has been demonstrated elsewhere^[8,10].

The reaction was a multiplex PCR using 5 (3 forward and 2 reverse) consensual starters for conservative sequences in the L1 gene of the virus, allowing simultaneous DNA amplification of several subtypes of the virus^[11]. The detection of amplification products was

completed using a set of fluorescence marked hybridization probes, specific for individual subtypes of the virus. That method allows for the separate detection of HPV for types 16 and 18 and for one or more of the other less common types: 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. This configuration of the test covers over 93% of the high oncogenic risk subtypes reported for the European population^[12]. The entire amplification and reaction product detection process was realized using the RT7500 Fast platform (Life Technologies Inc., Carlsbad, CA, United States). Although the test uses quantitative PCR, its result is of qualitative character - confirming or excluding the presence of an individual type of the virus. The results with a threshold cycle less than or equal to 32 ($C_T \leq 32$) were considered positive. An internal control in the form of human β -globin was included in each sample. Amplification of the β -globin gene confirmed the isolation of a sufficient amount of DNA of satisfactory quality and excluded the possibility of false-negative results.

Commercially available negative and positive controls (RealTime High Risk HPV Control Kit, Abbott Laboratories, Abbott Park, IL, United States) were also included in each run to verify that the sample processing, amplification and detection steps were performed correctly. The negative control was formulated with DNA containing the β -globin sequence and poly-dA:dT as carrier DNA. The positive control contained HPV16, HPV18, HPV58 and β -globin sequences and carrier DNA.

A possible biological activity of the virus was confirmed immunohistochemically based on the accumulation of the p16^{INK4a} protein caused by inhibition of the retinoblastoma gene (*pRB*) by the viral oncoprotein E7.

Determination of the p16^{INK4a} protein overexpression was completed on 3- μ m-thick paraffin sections, following their dewaxing and rehydration. The antigen retrieval procedure was carried out in Antigen Retrieval Solution (Mtm Laboratories Inc., Heidelberg, Germany) (100 mmol/L Tris, 10 mmol/L EDTA (pH 9.0), 15 mmol/L sodium azide). The presence of the p16^{INK4a} protein in the examined tissues was detected using mouse anti-human p16^{INK4a} monoclonal antibody, clone E6H4 (Mtm Laboratories Inc., Heidelberg, Germany) at a ready-to-use concentration and was visualized using the HRP/DAB+ system (Dako Denmark A/S, Glostrup, Denmark). Contrast staining was completed using hematoxylin according to Meyer. Each batch was supplemented with a positive control in the form of a section of squamous cell carcinoma of a tonsil with a known, positive HPV status (demonstrating a strong and uniform color reaction) and negative controls in which the primary antibody was replaced by TBS buffer.

The intensity of the cytoplasmic immunohistochemical reaction was evaluated under a light microscope BX41 (Olympus Corporation, Tokyo, Japan) at magnifications of 100 x and 200 x using a semiquantitative three-grade scale: total lack of p16^{INK4a} expression in the tumor tissue; focal staining in separated cells; and moderate or

strong color reaction involving most of the cancer cells. Only a moderate or strong color reaction was considered a positive result.

Ethical consideration

The present study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from all of the patients.

RESULTS

Among the 89 patients initially included in the study, diagnostic results were obtained for 84 samples. In five cases, either the histopathological material was insufficient to isolate the necessary DNA amount or the isolated DNA was significantly degraded, resulting in the failure of internal control amplification within the predefined number of 35 cycles. Those patients were excluded from further analysis. The amplification of high risk HPV DNA was demonstrated in none of the 84 tissue samples; thus, all of the cases were considered negative (Figure 1).

Immunohistochemical staining provided diagnostic results for all of the examined samples, and excluded the accumulation of the p16^{INK4a} protein, confirming the lack of active HPV infection in all individuals. In 80 cases the complete absence of staining with the specific anti-p16^{INK4a} antibody was observed. In the other four cases, a focal, weakly expressed reaction within the neighboring epithelium, in a single tumor or in stromal cells was observed; (all of these expression patterns were classified as negative results) (Figure 2).

DISCUSSION

In GAC, dietary and lifestyle factors primarily contribute to the risk of developing cancer, in addition to infections with *H. pylori* or EBV^[1-4]. It was demonstrated that approximately 9% of GAC display EBV in the tumor cells, although its effect on carcinogenesis and the development of GAC remains unclear^[5,13,14]. Some authors believe that there may be a correlation between HPV infection and the development of GAC similar to that found for EBV. However, the role of HPV in GAC has not been yet extensively studied. Therefore, some recent papers have aimed at demonstrating of a correlation between HPV and GAC and other alimentary tract carcinomas. Unfortunately, those papers provided contradictory data. In studies from various authors, the incidence of HPV in patients with alimentary tract carcinomas is highly variable, depending on the selected detection technique. Currently, PCR is most often used due to its high sensitivity, but its disadvantage is its low specificity^[15-18]. The data variability may be a result of using not only various HPV detection methods but also various study material collection procedures and sites, various methods of specimen protection against viral contamination, geographical differences and various selected subtypes of high

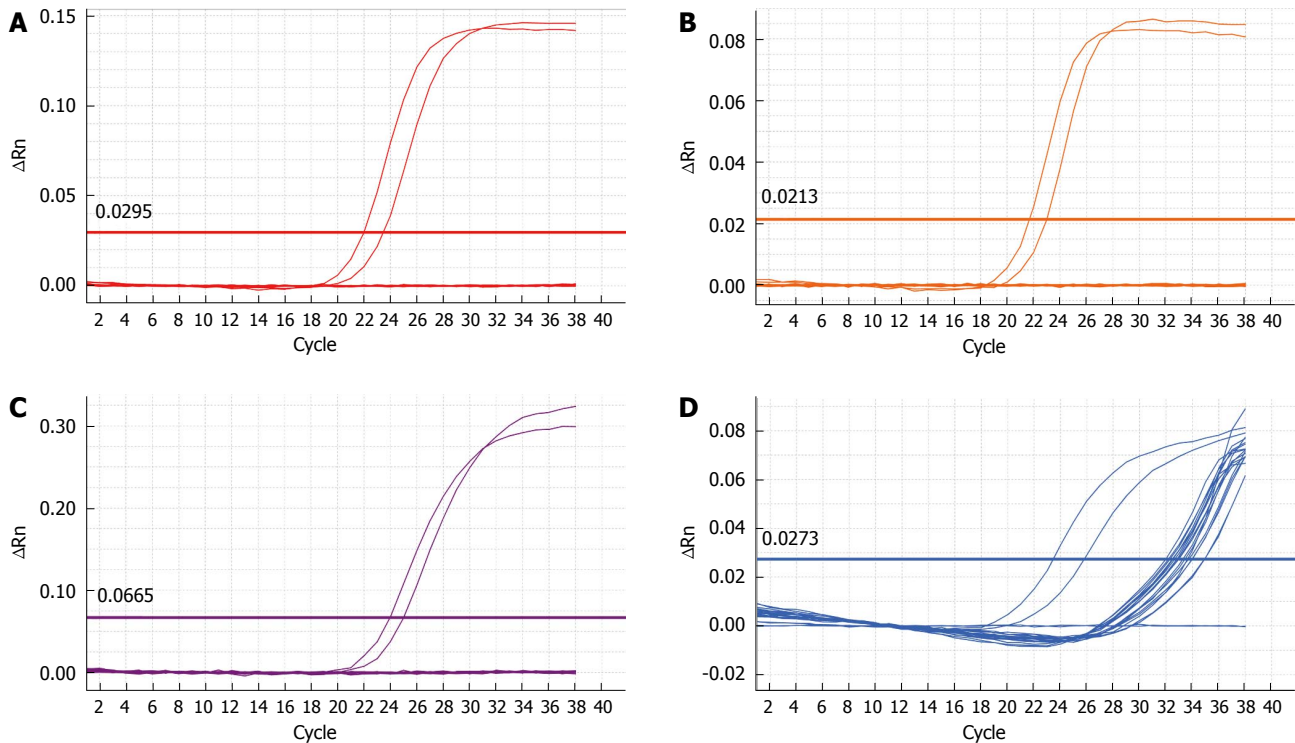


Figure 1 Representative subset of quantitative polymerase chain reaction amplification plots for 55 patients including non-informative cases. A: Human papilloma virus (HPV) type 16; B: HPV type 18; C: One or more subtypes from the group of 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68; D: Internal control (β -globin). The two leftmost curves correspond to the positive controls, horizontal line corresponds to ΔRn threshold. ΔRn : The magnitude of the generated fluorescent signal.

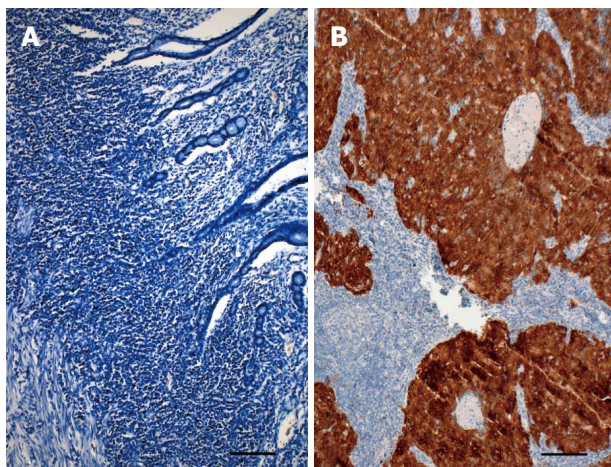


Figure 2 Representative results of immunostaining for p16INK4a protein. A: Negative immunostaining in gastric adenocarcinoma G3; B: Human papilloma virus-positive squamous cell carcinoma of the oropharynx as the positive control. Scale bar: 200 μm .

oncogenic risk HPV (mostly HPV16 and HPV18). Ma *et al*^[19] using liquid and *in situ* PCR found HPV16E6 genes in 37.5% of GAC (15/40) and stated that the gastric adenoid epithelium may be a target of HPV-dependent carcinogenesis. Xu *et al*^[20] using the *in situ* hybridization technique, found HPV in 68% of the examined GAC samples and even in 20% (10/50) of the normal gastric mucosa tissue samples. Among 23 cases of concurrent esophageal squamous cell carcinoma (ESCC) and GCA (gastric cardia cancer), Ding *et al*^[21] using PCR, found

HPV DNA in 29% of GCA and 47% of ESCC cases. Although HPV occurred less commonly in GCA than it did in ESCC, higher p16^{INK4a} expression was observed in GCA than in ESCC (75% *vs* 25%, respectively; $P < 0.05$). In some older studies on EBV, some authors demonstrated the presence of EBV or HPV (less commonly) in GAC; in some cases, the simultaneous presence of both viruses was revealed^[22,23]. In contrast, Koshiol *et al*^[24] found no HPV16/18 in any of the tissues collected from 144 cardia cancers. They used a standardized protocol to minimize the potential for environmental HPV to contaminate the tissues. Control β -globin, and therefore the DNA quality, was adequate in 75% of the cases (108/144). Among the 108 cases, all were negative for HPV DNA based on Linear Array and E6/E7-based PCR. Yuan *et al*^[25] investigated the relationship between GAC and HPV. They performed PCR analyses of tissue samples from 98 patients with gastroduodenal diseases, including GAC, in a region presenting a high incidence rate of GAC. HPV genotypes were detected using the HPV GenoArray test kit (HybriBio Ltd, Hong Kong). HPV DNA was not detected in any of the patients' tissues, including: GAC cells, adjacent dysplastic epithelium, surrounding lymphocytes, and paired normal gastric mucosa. The results of Kamangar *et al*^[26]'s prospective, seroepidemiological study in a high-risk region of GAC in China did not support a major role for HPV 16, HPV 18 and HPV 73 in GAC etiology. Lagergren *et al*^[27] performed a population-based, case-control study and found no association between HPV16 and an inverse association between HPV 18, and

adenocarcinomas of the esophagus or gastroesophageal junction (OR = 0.2; 95%CI: 0.1-0.7). Other studies have also ruled out the presence of HPV in GAC or have found that it has low significance^[28,29].

In the present study, we used the unique combination of viral DNA detection and confirmation of transcriptional activity of the virus, by demonstrating of p16^{INK4a} accumulation in target cells. This algorithm based on p16 immunostaining followed by GP5+/6+ PCR in the p16-positive cases proposed by Smeets *et al.*^[30] is claimed to be 100% sensitive and specific. Moreover, it should be noted that the expression of p16^{INK4a} is a sensitive surrogate marker of HPV infection but is not limited only to HPV-positive tumors, and the use of this marker alone as an indicator of biologically relevant HPV infections inevitably entails the risk of including some HPV-negative p16^{INK4a}-positive results^[31].

Another distinguishing attribute of the present study is the application of quantitative PCR, which covers more than 93% of the subtypes of high-oncogenic-risk viruses, including HPV16, HPV18 and twelve less abundant subtypes. The analytical parameters of this assay were precisely described^[12] and the usefulness of HPV detection in the formalin-fixed, paraffin-embedded tissue samples was demonstrated in our previous studies^[8,10] and by independent investigators^[32]. The results obtained in the study failed to detect the presence of the HPV genome in GAC, suggesting that the incidence of high-oncogenic-risk HPV in GAC tissue is very low; therefore, the potential participation of the virus in GAC development is highly doubtful.

In conclusion, infectious agents such as HPV are suspected to play causal roles in various human malignancies. However, the present study failed to confirm the presence of the HPV genome as well as any viral biological activity in GAC tissues. Therefore, any role of the virus in the pathogenesis of GAC, at least in the Caucasian population of Middle end Eastern Europe, is doubtful.

COMMENTS

Background

Despite the significant progress made in the understanding of the epidemiology, pathology and pathogenesis of alimentary tract carcinomas, gastric cancer remains the third leading cause of cancer death in both sexes worldwide. The highest mortality rates are observed in the developing countries of Eastern Asia and of Central and Eastern Europe. Among the factors that may lead to the development of gastric cancer, the most significant are as follows: a diet rich in cured and smoked meat products with low antioxidant content, tobacco smoking and alcohol consumption and long-lasting infection by the bacterium called *Helicobacter pylori*.

Research frontiers

Some types of human papilloma virus (HPV) are proven risk factors of neoplastic transformation in cervical, anal, oral or pharyngeal cancers, suggesting a role for the virus in the pathogenesis of cancer of other sections of the alimentary tract, including gastric cancer.

Innovations and breakthroughs

Previous publications using different techniques of HPV detection showed contradictory results and were restricted mainly to the Asian population. In the present study, we used the unique combination of viral DNA detection using quantitative polymerase chain reaction (PCR) that can detect 14 oncogenic sub-

types of the virus, and confirmation of tumor cell infection by demonstrating the changes in cellular metabolism caused by HPV. Using this combined approach, we could eliminate the false-positive results emerging from HPV presence in the alimentary tract without infecting its tissues. This study is the first from Middle and Eastern Europe characterized by one of the highest gastric cancer incidence and mortality rates. Obtained results confirmed neither the presence of virus nor its biological activity in the tested tissues of gastric cancer.

Applications

The potential participation of HPV in the development of gastric cancer, at least in the population of the Middle and Eastern Europe, is highly doubtful.

Terminology

HPVs represent a large group of relatively small viruses that contain DNA as genetic material and can infect epithelial tissues of mammals, including humans. Most HPVs are benign and cause skin or genital warts, but a subgroup, known as high oncogenic subtypes, are responsible for the development of several cancers, including cervical, anal and pharyngeal cancer.

Peer review

In this study, the authors explored the presence of high-risk HPVs and the expression of p16^{INK4a} in a cohort of 84 gastric cancer tissues using RT-PCR and IHC analysis. The author failed to detect HPVs and p16^{INK4a} expression in all samples. Therefore, they claimed that the presence and role of high-risk HPVs in the pathogenesis of gastric adenocarcinoma in a European population are not evident. This study is interesting and within the scope of the journal.

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Protocol liver biopsy is the only examination that can detect mid-term graft fibrosis after pediatric liver transplantation

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Abstract

AIM: To assessed the clinical significance of protocol liver biopsy (PLB) in pediatric liver transplantation (LT).

METHODS: Between July 2008 and August 2012, 89 and 55 PLBs were performed in pediatric patients at two and five years after LT, respectively. We assessed the histopathological findings using the Metavir scoring system, including activity (A) and fibrosis (F), and we identified factors associated with scores of $\geq A1$ and $\geq F1$. Our results clarified the timing and effectiveness of PLB.

RESULTS: The incidences of scores of $\geq A1$ and $\geq F1$ were 24.7% and 24.7%, respectively, at two years after LT and 42.3% and 34.5%, respectively, at five years. Independent risk factors in a multivariate analysis of a score of $\geq A1$ at two years included ≥ 2 h of

cold ischemic time, no acute cellular rejection and an alanine amino transaminase (ALT) level of ≥ 20 IU/L ($P = 0.028$, $P = 0.033$ and $P = 0.012$, respectively); however, no risk factors were identified for a score of $\geq F1$. Furthermore, no independent risk factors associated with scores of $\geq A1$ and $\geq F1$ at five years were identified using multivariate analysis. A ROC curve analysis of ALT at two years for a score of $\geq A1$ demonstrated the recommended cutoff value for diagnosing $\geq A1$ histology to be 20 IU/L. The incidence of scores of $\geq A2$ or $\geq F2$ at two years after LT was 3.4% (three cases), and all patients had an absolute score of $\geq A2$. In contrast to that observed for PLBs at five years after LT, the incidence of scores of $\geq A2$ or $\geq F2$ was 20.0% (11 cases), and all patients had an absolute score of $\geq F2$. In all cases, the dose of immunosuppressants was increased after the PLB, and all ten patients who underwent a follow-up liver biopsy improved to scores of $\leq A1$ or $F1$.

CONCLUSION: PLB at two years after LT is an unnecessary examination, because the serum ALT level reflects portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect this finding.

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Key words: Protocol liver biopsy; Graft fibrosis; Immunosuppression; Liver function test; Pediatric liver transplantation

Core tip: Few studies have investigated the impact of the timing and effectiveness of post-transplant protocol liver biopsy (PLB). We assessed the histopathological findings of these biopsies using the Metavir scoring system, and our results clarified the timing and effective-

ness of PLB. PLB at two years after pediatric liver transplantation is an unnecessary examination, because the serum alanine amino transaminase (ALT) level reflects portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect this finding.

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INTRODUCTION

Liver transplantation (LT) is an established curative treatment for pediatric patients with end-stage liver disease or acute liver failure^[1-3]. Graft fibrosis and/or chronic rejection can still occasionally lead to graft failure or even death despite improvements and innovations in immunosuppressive therapy, and the histopathological assessments performed after LT remain insufficient. It is therefore necessary to further improve the prognosis by maintaining the function of the liver graft using a minimum degree of immunosuppression to achieve an optimal balance between the effectiveness and side effects of individual immunosuppressants.

The development of liver graft fibrosis after pediatric LT has been reported to occur in 69%-97% of cases, including cases of mild fibrosis^[4-8]. Graft dysfunction does not occur unless the fibrosis becomes advanced, and the occurrence of graft fibrosis or portal inflammation cannot be predicted using the standard liver function test (LFT) alone. Therefore, histopathological assessments using protocol liver biopsy (PLB) have recently been reported to be important^[4-9]. However, the significance of mild to severe fibrosis is unclear, and the indications for the treatment of abnormal PLB findings are controversial. In addition, few studies have investigated the impact of the timing and effectiveness of PLB. This retrospective study assessed the clinical significance of the timing and effectiveness of PLB after pediatric living donor liver transplantation (LDLT).

MATERIALS AND METHODS

Patients

Between July 2008 and August 2012, 144 PLBs were performed in pediatric patients at two and five years after LDLT at the Department of Transplant Surgery, Jichi Medical University, Japan (Table 1). The observation pe-

riod was between six and 55 mo.

Immunosuppressive therapy

Tacrolimus (Tac) and methylprednisolone (MP) were used as the standard postoperative immunosuppressive regimen. The target trough levels of Tac were 15-20 ng/mL during the first week, 8-12 ng/mL during the first month, 5-8 ng/mL during the first six months, 3-5 ng/mL during the first year and 2-4 ng/mL thereafter. MP was administered at an initial dose of 20 mg/kg intravenously on the morning of the operation and before graft reperfusion. The MP dose was thereafter decreased gradually to 3 mg/kg per day on postoperative day (POD) 1, 0.5 mg/kg per day on POD 7 and 0.25 mg/kg per day at one month after LDLT and was then discontinued within one year except in patients in whom immunosuppression could not be maintained at the lowest dose. Mycophenolate mofetil (MMF) was used when more potent immunosuppression was required, such as in ABO-incompatible recipients older than five years, patients with steroid-resistant acute rejection episodes and patients with liver dysfunction following the cessation of MP therapy.

Diagnosis of acute cellular rejection

All episodes of acute cellular rejection were diagnosed based on the histopathological findings of a liver biopsy. In all specimens, the diagnosis of acute cellular rejection was evaluated by highly experienced pathologists and graded into four classes according to the Banff scheme^[10]. The degrees of portal infiltration by lymphocytes (P0-3), bile duct inflammation or damage (B0-3) and venous endothelial inflammation (V0-3) in the Banff scheme were evaluated. A liver biopsy was indicated when all liver function data (aspartate amino transferase, alanine amino transferase (ALT), gamma-glutamyl transpeptidase, and total bilirubin) were elevated compared with the previous data.

PLB procedure and timing

We began to perform PLBs in pediatric patients at two and five years after LT in July 2008 because we experienced cases of normal LFTs coexisting with histopathological portal inflammation and fibrosis, including cases 4 and 5, which are discussed later. In those cases in which the dose of immunosuppressants was increased after the PLB, we generally performed a follow-up liver biopsy between six months and one year after the PLB. In addition to a PLB, an episode biopsy was performed when a recipient with a high serum level of ALT or hyaluronic acid was refractory to an increase in immunosuppressants.

The PLB necessitated an overnight stay at our hospital. A percutaneous transhepatic liver biopsy was performed under analgesia and sedation using ultrasonographically-guided 14 G Monopty (C.R.Bard, Inc. United States). Manual compressive hemostasis was conducted for 20 min, after which compressive bandage hemostasis was performed until the following day. Preventive cefoperazone and sulbactam were also administered on that day.

Table 1 Demographic characteristics of recipients and grafts undergoing protocol liver biopsy at two and five years after living donor liver transplantation

| | PLB at two years after LDLT (<i>n</i> = 89) | PLB at five years after LDLT (<i>n</i> = 55) |
|--|---|---|
| Recipient characteristics at LDLT | | |
| Gender | Male 37, female 52 | Male 20, female 35 |
| Age (mo) | 22 (0-234) | 19 (7-198) |
| Body weight (kg) | 10.7 (2.6-58.5) | 9.7 (5.9-64.9) |
| Original disease | BA 63, OTCD 9, AS 4, FHF 4, CEPS 3, graft failure 2, WD 1, PSC 1, CPS1D 1, LC 1 | BA 43, OTCD 3, AS 2, WD 2, FHF 1, HB 1, CF 1, CEPS 1, graft failure 1 |
| PELD or MELD | 7.4 (-9.7-39.4) | 8.6 (-8.9-39.4) |
| Operation time | 13 h 25 min (7 h 33 min-30 h 28 min) | 17h 19 min (11 h 11 min-30 h 28 min) |
| Cold ischemic time | 2 h 17 min (36 min-8 h 6 min) | 2 h 06 min (25 min-16 h 19 min) |
| Warm ischemic time | 45 min (30 min-2 h 2 min) | 1 h 00 min (30 min-4 h 27 min) |
| Blood loss volume (mL/kg) | 77.0 (3.1-585.1) | 45.5 (6.7-776.2) |
| Transfusion volume (mL/kg) | 91.3 (0.0-597.7) | 68.1 (0.0-670.7) |
| Donor and graft characteristics at LDLT | | |
| Gender | Father; 45, mother; 44 | Father; 30, mother; 25 |
| Age (yr) | 33 (23-57) | 33 (23-53) |
| ABO compatibility | Identical; 55, compatible; 20, incompatible 14 | Identical; 40, compatible; 8, incompatible 7 |
| GV/SLV (%) | 68.0 (33.0-120.9) | 75.8 (35.7-121.2) |
| Graft type | Lateral segment; 57, left lobe; 23, S2 monosegment; 5, left lobe + caudate; 4 | Lateral segment; 43, left lobe; 10, left lobe + caudate; 2 |
| Recipient and graft characteristics at PLB | | |
| Age (mo) | 48 (24-259) | 81 (68-257) |
| Body weight (kg) | 15.6 (7.3-64.6) | 21.4 (14.4-71.6) |
| Total bilirubin (mg/dL) | 0.63 (0.25-3.25) | 0.68 (0.26-2.55) |
| AST (IU/L) | 30 (14-61) | 27 (10-251) |
| ALT (IU/L) | 17 (9-54) | 17 (8-260) |
| γ -GTP (IU/L) | 17 (6-440) | 16 (9-510) |
| Hyaluronic acid (ng/mL) | 21 (9-239) | 17 (9-216) |
| IgG (mg/dL) | 927 (440-2063) | 1148 (475-2961) |
| GV/SLV (%) | 90.6 (70.2-126.9) | 93.0 (58.8-157.0) |
| Spleen volume (mL) | 125 (0-892) | 145 (0-692) |
| Trough of tacrolimus (ng/mL) | 3.4 (0-10.1) | 2.3 (0-15.5) |

PLB: Protocol liver biopsy; LDLT: Living donor liver transplantation; BA: Biliary atresia; OTCD: Ornithine transcarbamylase deficiency; AD: Alagille syndrome; FHF: Fulminant hepatic failure; CEPS: Congenital extrahepatic portsystemic shunt; WD: Wilson disease; PSC: Primary sclerosing cholangitis; CPS1D: Carbamoyl-phosphate synthase 1 deficiency; LC: Liver cirrhosis; HB: Hepatoblastoma; CF: Cystic fibrosis; PELD: Pediatric end-stage liver disease; MELD: Model for end-stage liver disease; GV/SLV: Ratio of graft volume to standard liver volume; AST: Aspartate amino transferase; ALT: Alanine amino transferase; IgG: Immunoglobulin G.

Assessment of the PLB findings

We assessed the histopathological features of the PLB samples using the Metavir scoring system^[11], which grades the activity (A), *i.e.*, the amount of inflammation (specifically, the intensity of necro-inflammatory lesions), on a four-point scale from A0 to A3. Fibrosis (F) was graded on a five-point scale from F0 to F4.

Strategy of increasing the dose of immunosuppressants after LDLT

When the serum level of ALT or hyaluronic acid was found to be high in outpatients, we increased the dose of immunosuppressants if the suspected causes of the elevation of these levels was an immune response. When the serum level of ALT or hyaluronic acid was maintained at a normal level for a few months in the early period or for six months in the late period after LDLT, we gradually decreased the dose of immunosuppressants.

When the PLB score was \geq A2 or \geq F2, we increased the dose of immunosuppressants to provide the early treatment of portal inflammation or fibrosis. When the PLB grade was A0 and F0, we gradually decreased

the dose of immunosuppressants.

Statistical analysis

The significance of the differences between two groups was evaluated using the chi-squared test. Associations between the recipient, donor or graft variables and abnormal histopathological findings were evaluated using univariate and backward selection multivariate Cox regression methods. A ROC curve analysis was performed to identify the cutoff value for the correlation between the ALT level and abnormal histopathological findings. All statistical analyses were performed using the Stat-View software package (SAS Institute, Cary, NC) and EZR (Saitama Medical Center, Jichi Medical University, Japan). Differences of $P < 0.05$ were considered to be significant.

RESULTS

Results of PLB at two years after LDLT

The incidence of scores of \geq A1 and \geq F1 at two years after LDLT was 24.7% and 24.7%, respectively. The ac-

Table 2 Risk factors for \geq A1 and \geq F1 of protocol liver biopsy at two years after living donor liver transplantation: univariate analysis

| Variables | Incidence of \geq A1 (%) | P value | Incidence of \geq F1 (%) | P value |
|--|----------------------------|---------|----------------------------|---------|
| Recipient age at LDLT | | | | |
| < 12 mo (<i>n</i> = 30) <i>vs</i> \geq 12 mo (<i>n</i> = 59) | 26.7 <i>vs</i> 23.7 | 0.762 | 36.7 <i>vs</i> 18.6 | 0.062 |
| Recipient body weight at LDLT | | | | |
| < 10 kg (<i>n</i> = 43) <i>vs</i> \geq 10 kg (<i>n</i> = 46) | 23.3 <i>vs</i> 26.1 | 0.757 | 27.9 <i>vs</i> 21.7 | 0.500 |
| Original disease | | | | |
| Cholestatic diseases (<i>n</i> = 69) <i>vs</i> others (<i>n</i> = 20) | 33.3 <i>vs</i> 38.1 | 0.637 | 33.3 <i>vs</i> 38.1 | 0.637 |
| PELD or MELD | | | | |
| \geq 20 (<i>n</i> = 22) <i>vs</i> < 20 (<i>n</i> = 67) | 22.7 <i>vs</i> 25.4 | 0.803 | 31.8 <i>vs</i> 22.4 | 0.374 |
| Donor age | | | | |
| \geq 35 yr (<i>n</i> = 39) <i>vs</i> < 35 yr (<i>n</i> = 50) | 23.1 <i>vs</i> 26.0 | 0.751 | 25.6 <i>vs</i> 24.0 | 0.858 |
| Gender combinations between donor and recipient | | | | |
| Mismatch (<i>n</i> = 50) <i>vs</i> match (<i>n</i> = 39) | 24.0 <i>vs</i> 25.6 | 0.858 | 22.0 <i>vs</i> 28.2 | 0.501 |
| ABO compatibility | | | | |
| Incompatible (<i>n</i> = 14) <i>vs</i> others (<i>n</i> = 75) | 21.4 <i>vs</i> 25.3 | 0.755 | 14.3 <i>vs</i> 26.7 | 0.324 |
| HLA-A | | | | |
| Mismatch (<i>n</i> = 65) <i>vs</i> match (<i>n</i> = 24) | 30.8 <i>vs</i> 8.3 | 0.029 | 27.7 <i>vs</i> 16.7 | 0.285 |
| HLA-B | | | | |
| Mismatch (<i>n</i> = 84) <i>vs</i> match (<i>n</i> = 5) | 26.2 <i>vs</i> 0.0 | 0.187 | 25.0 <i>vs</i> 20.0 | 0.802 |
| HLA-DRB ¹ | | | | |
| Mismatch (<i>n</i> = 76) <i>vs</i> match (<i>n</i> = 13) | 26.3 <i>vs</i> 15.4 | 0.398 | 26.3 <i>vs</i> 15.4 | 0.398 |
| Lymphocyte cross-matching | | | | |
| \geq 4 \times (<i>n</i> = 7) <i>vs</i> negative (<i>n</i> = 82) | 0.0 <i>vs</i> 26.8 | 0.114 | 28.6 <i>vs</i> 24.4 | 0.805 |
| GV/SLV | | | | |
| < 40 % (<i>n</i> = 6) <i>vs</i> \geq 40 % (<i>n</i> = 83) | 33.3 <i>vs</i> 24.1 | 0.612 | 16.7 <i>vs</i> 25.3 | 0.636 |
| Graft type | | | | |
| Lateral segment graft (<i>n</i> = 57) <i>vs</i> others (<i>n</i> = 32) | 21.1 <i>vs</i> 31.3 | 0.285 | 29.8 <i>vs</i> 15.6 | 0.136 |
| Operation time | | | | |
| \geq 20 h (<i>n</i> = 12) <i>vs</i> < 20 h (<i>n</i> = 77) | 16.7 <i>vs</i> 26.0 | 0.113 | 25.0 <i>vs</i> 24.7 | 0.975 |
| Cold ischemic time | | | | |
| \geq 2 h (<i>n</i> = 49) <i>vs</i> < 2 h (<i>n</i> = 40) | 32.7 <i>vs</i> 15.0 | 0.055 | 28.6 <i>vs</i> 20.0 | 0.351 |
| Warm ischemic time | | | | |
| \geq 45 min (<i>n</i> = 45) <i>vs</i> < 45 min (<i>n</i> = 44) | 20.0 <i>vs</i> 29.5 | 0.297 | 26.7 <i>vs</i> 22.7 | 0.666 |
| Blood loss volume | | | | |
| \geq 100 mL/kg (<i>n</i> = 30) <i>vs</i> < 100 mL/kg (<i>n</i> = 59) | 16.7 <i>vs</i> 28.8 | 0.209 | 26.7 <i>vs</i> 23.7 | 0.762 |
| Transfusion volume | | | | |
| \geq 100 mL/kg (<i>n</i> = 41) <i>vs</i> < 100 mL/kg (<i>n</i> = 48) | 22.0 <i>vs</i> 27.1 | 0.576 | 22.0 <i>vs</i> 27.1 | 0.576 |
| Splenectomy | | | | |
| Yes (<i>n</i> = 7) <i>vs</i> No (<i>n</i> = 82) | 42.9 <i>vs</i> 23.2 | 0.247 | 28.6 <i>vs</i> 24.4 | 0.805 |
| Portal vein complications | | | | |
| Yes (<i>n</i> = 11) <i>vs</i> No (<i>n</i> = 78) | 9.1 <i>vs</i> 26.9 | 0.199 | 27.3 <i>vs</i> 24.4 | 0.834 |
| Hepatic arterial complications | | | | |
| Yes (<i>n</i> = 6) <i>vs</i> No (<i>n</i> = 83) | 16.7 <i>vs</i> 25.3 | 0.636 | 33.3 <i>vs</i> 24.1 | 0.509 |
| Hepaticojejunostomic anastomotic stricture | | | | |
| Yes (<i>n</i> = 14) <i>vs</i> No (<i>n</i> = 75) | 21.4 <i>vs</i> 25.3 | 0.755 | 28.6 <i>vs</i> 24.0 | 0.716 |
| Cytomegalovirus infection | | | | |
| Yes (<i>n</i> = 29) <i>vs</i> No (<i>n</i> = 60) | 31.0 <i>vs</i> 21.7 | 0.337 | 27.6 <i>vs</i> 23.3 | 0.663 |
| Acute cellular rejection | | | | |
| Yes (<i>n</i> = 29) <i>vs</i> No (<i>n</i> = 60) | 10.3 <i>vs</i> 31.7 | 0.029 | 17.2 <i>vs</i> 28.3 | 0.255 |
| Total bilirubin at PLB | | | | |
| \geq 0.7 mg/dL (<i>n</i> = 29) <i>vs</i> < 0.7 mg/dL (<i>n</i> = 60) | 17.2 <i>vs</i> 28.3 | 0.255 | 24.1 <i>vs</i> 25.0 | 0.929 |
| AST at PLB | | | | |
| \geq 30 IU/L (<i>n</i> = 49) <i>vs</i> < 30 IU/L (<i>n</i> = 40) | 24.5 <i>vs</i> 25.0 | 0.956 | 34.7 <i>vs</i> 12.5 | 0.016 |
| ALT at PLB | | | | |
| \geq 20 IU/L (<i>n</i> = 27) <i>vs</i> < 20 IU/L (<i>n</i> = 62) | 40.7 <i>vs</i> 17.7 | 0.021 | 37.0 <i>vs</i> 19.4 | 0.075 |
| γ -GTP at PLB | | | | |
| \geq 20 IU/L (<i>n</i> = 34) <i>vs</i> < 20 IU/L (<i>n</i> = 55) | 32.4 <i>vs</i> 20.0 | 0.189 | 29.4 <i>vs</i> 21.8 | 0.420 |
| Hyaluronic acid at PLB | | | | |
| \geq 20 ng/mL (<i>n</i> = 52) <i>vs</i> < 20 ng/mL (<i>n</i> = 37) | 32.7 <i>vs</i> 13.5 | 0.039 | 23.1 <i>vs</i> 27.0 | 0.671 |
| IgG at PLB | | | | |
| \geq 1200 mg/dL (<i>n</i> = 18) <i>vs</i> < 1200 mg/dL (<i>n</i> = 71) | 27.8 <i>vs</i> 23.9 | 0.737 | 33.3 <i>vs</i> 22.5 | 0.343 |
| ANA at PLB | | | | |
| \geq 20 \times (<i>n</i> = 8) <i>vs</i> < 20 \times (<i>n</i> = 81) | 12.5 <i>vs</i> 25.7 | 0.401 | 12.5 <i>vs</i> 25.9 | 0.401 |
| ASMA at PLB | | | | |
| \geq 20 \times (<i>n</i> = 21) <i>vs</i> < 20 \times (<i>n</i> = 68) | 23.8 <i>vs</i> 25.0 | 0.913 | 28.6 <i>vs</i> 23.5 | 0.640 |

Trough of tacrolimus at PLB

 ≥ 3.0 ng/mL ($n = 54$) *vs* < 3.0 ng/mL ($n = 32$)¹25.9 *vs* 25.0

0.924

24.1 *vs* 25.0

0.924

¹Three cases which were used a cyclosporine were removed. LDLT: Living donor liver transplantation; PELD: Pediatric end-stage liver disease; MELD: Model for end-stage liver disease; GV/SLV: Ratio of graft volume to standard liver volume; PLB: Protocol liver biopsy; AST: Aspartate amino transferase; ALT: Alanine amino transferase; IgG: Immunoglobulin G; ANA: Antinuclear antibody; ASMA: Antismooth nuclear antibody.

Table 3 Risk factors for \geq A1 and \geq F1 of protocol liver biopsy at two and five years after living donor liver transplantation: multivariate analysis

| Variables | OR | 95%CI | P value |
|--|------|--------------|---------|
| Risk factors for \geq A1 of PLB at two years after LDLT | | | |
| HLA-A mismatch | | | |
| Mismatch <i>vs</i> match | 0.46 | 0.145-1.479 | 0.194 |
| Cold ischemic time | | | |
| ≥ 2 h <i>vs</i> < 2 h | 4.15 | 1.164-14.789 | 0.028 |
| Acute cellular rejection | | | |
| Yes <i>vs</i> No | 0.20 | 0.046-0.878 | 0.033 |
| ALT | | | |
| ≥ 20 IU/L <i>vs</i> < 20 IU/L | 4.64 | 1.409-15.306 | 0.012 |
| Hyaluronic acid | | | |
| ≥ 20 ng/mL <i>vs</i> < 20 ng/mL | 3.30 | 0.982-11.076 | 0.054 |
| Risk factors for \geq F1 of PLB at two years after LDLT | | | |
| Recipient age | | | |
| < 1 yr <i>vs</i> ≥ 1 yr | 1.54 | 0.506-4.706 | 0.446 |
| AST | | | |
| ≥ 30 IU/L <i>vs</i> < 30 IU/L | 2.68 | 0.775-9.238 | 0.120 |
| ALT | | | |
| ≥ 20 IU/L <i>vs</i> < 20 IU/L | 1.86 | 0.646-5.335 | 0.251 |
| Risk factors for \geq A1 of PLB at five years after LDLT | | | |
| Cold ischemic time | | | |
| ≥ 2 h <i>vs</i> < 2 h | 2.94 | 0.778-11.140 | 0.112 |
| Acute cellular rejection | | | |
| Yes <i>vs</i> No | 2.26 | 0.728-7.035 | 0.158 |
| Risk factor for \geq F1 of PLB at five years after LDLT | | | |
| Acute cellular rejection | | | |
| Yes <i>vs</i> No | 2.75 | 0.876-8.637 | 0.083 |

PLB: Protocol liver biopsy; LDLT: Living donor liver transplantation; ALT: Alanine amino transferase; AST: Aspartate amino transferase.

tivity score was A0 in 67 patients, A1 in 19 patients and A2 in three patients, and the fibrosis score was F0 in 67 patients, F1 in 21 patients and F2 in one patient.

The impact of various recipient and graft variables on scores of \geq A1 and \geq F1 was assessed, and the results are summarized in Table 2. A univariate analysis revealed the following variables to be risk factors for a score of \geq A1 at two years after LDLT: HLA-A mismatch, no acute cellular rejection, ALT level of ≥ 20 IU/L, and hyaluronic acid level of ≥ 20 ng/mL ($P = 0.029$, $P = 0.029$, $P = 0.021$ and $P = 0.039$, respectively). The only variable with $P < 0.1000$ was ≥ 2 h of cold ischemic time ($P = 0.055$). A multivariate analysis including these variables identified ≥ 2 h of cold ischemic time, no acute cellular rejection and ALT level of ≥ 20 IU/L to be independent risk factors for a score of \geq A1 at two years after LDLT ($P = 0.028$, $P = 0.033$ and $P = 0.012$, respectively) (Table 3). The ROC curve analysis of the ALT level at two years after LDLT in the patients with a score of \geq A1, the recommended cutoff value for diagnosing a score of \geq A1 was 20 IU/L (sensitivity: 50.0%, specificity: 76.1%, area

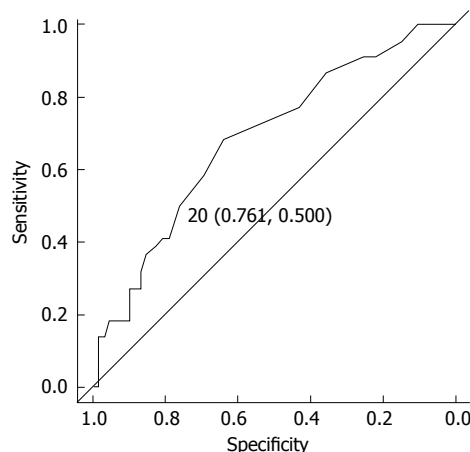


Figure 1 Receiver operating characteristic curve analysis of the alanine amino transferase level at two years after living donor liver transplantation in the patients with a score of \geq A1. The recommended cutoff value for diagnosing a score of \geq A1 was set at 20 IU/L (sensitivity: 50.0%, specificity: 76.1%, area under the curve: 0.685 and 95%CI: 0.557-0.813).

under the curve: 0.685 and 95%CI: 0.557-0.813) (Figure 1). Univariate analysis identified the risk factor for a score of \geq F1 at two years after LDLT to be the aspartate amino transferase level ($P = 0.016$). The variables with $P < 0.100$ included a recipient age of < 12 mo and an ALT level of ≥ 20 IU/L ($P = 0.062$ and $P = 0.075$, respectively). A multivariate analysis of these variables found none to be independent risk factors for a score of \geq F1 at two years after LDLT (Table 3).

The incidence of scores of \geq A2 or \geq F2 at two years after LDLT was 3.4% (three cases), and all patients had a score of \geq A2 (Table 4). In all cases, the dose of immunosuppressants was increased after the PLB, and two patients who underwent a follow-up liver biopsy improved to scores of \leq A1 and F1.

Results of PLB at five years after LDLT

The incidence of scores of \geq A1 and \geq F1 at five years after LDLT was 42.3% and 34.5%, respectively. The activity score was A0 in 29 patients, A1 in 23 patients and A2 in three patients, and the fibrosis score was F0 in 36 patients, F1 in 12 patients and F2 in seven patients.

The impact of various recipient and graft variables on the scores of \geq A1 and \geq F1 was assessed, and the results are summarized in Table 5. A univariate analysis identified no risk factors for scores of \geq A1 at five years after LDLT. The variables with $P < 0.100$ included ≥ 2 h of cold ischemic time and acute cellular rejection ($P = 0.061$ and $P = 0.087$, respectively). Multivariate analysis of these variables found none to be independent risk factors for a score of \geq A1 at five years after LDLT (Table

Table 4 Clinical and histopathological findings of cases with \geq A2 or \geq F2 of protocol liver biopsy at two or five years after living donor liver transplantation

| Case | Original disease | Age at PLB/sex | Previous ACR | Post-transplant complications | IS at PLB | Tac trough at PLB | ALT/HA at PLB | A/F at PLB | IS at follow-up biopsy | A/F at follow-up biopsy |
|------------------------------|------------------|----------------|--------------|-------------------------------|-------------------------------|-------------------|---------------|------------|-------------------------------|-------------------------|
| PLB at two years after LDLT | | | | | | | | | | |
| 1 | OTCD | 71/female | - | - | Tac (3.0) | 2.5 | 12/35 | 2/1 | Tac (1.0)/MMF (400) | 1/1 |
| 2 | OTCD | 164/female | - | BDS | Tac (2.0)/MMF (1000) | 5.2 | 34/13 | 2/1 | Tac (2.0)/MMF (1000) | 1/0 |
| 3 | OTCD | 44/male | - | - | Tac (0.8)/MMF (250) | 2 | 25/< 9 | 2/2 | Tac (0.8)/MMF (500) | N.E. |
| PLB at five years after LDLT | | | | | | | | | | |
| 4 | BA | 70/female | + | Bowel perforation | Tac (0.6) | 1.1 | 22/13 | 2/2 | Tac (2.0)/MMF (1000) | 0/0 |
| 5 | BA | 118/female | - | - | Tac (1.0) | 2.3 | 20/24 | 2/2 | Tac (2.0)/MMF (1000) | 1/0 |
| 6 | BA | 70/female | + | HAT/IHBDS | Tac (0.8)/MMF(500) | 3.6 | 32/28 | 1/2 | Tac (2.0)/MMF (500) | 1/1 |
| 7 | BA | 71/female | - | CMV-I | Tac (0.4) | 0 | 16/< 9 | 2/2 | Tac (1.6) | N.E. |
| 8 | FHF | 83/female | - | - | Tac (2.0)/MMF (500) | 2.2 | 26/< 9 | 1/2 | Tac (2.8)/MMF (500) | N.E. |
| 9 | BA | 77/female | - | CMV-I | Tac (0.4) | 2.6 | 14/29 | 2/3 | Tac (0.8) | 0/1 |
| 10 | BA | 84/female | + | Fungal infection | Tac (0.4) | 2.1 | 26/11 | 2/2 | Tac (0.4),MMF (500) | 1/1 |
| 11 | BA | 89/male | + | PVS | Tac (1.6)/MP (4.0)/MMF (1500) | 2.2 | 12/17 | 2/2 | Tac (1.6)/MP (2.0)/MMF (1500) | 1/1 |
| 12 | BA | 174/male | - | BDS | Tac (3.0) | 2.3 | 16/20 | 1/2 | Tac (4.0) | 0/1 |
| 13 | BA | 69/female | + | CMV-I | Tac (1.6) | 2.8 | 18/< 9 | 1/2 | Tac (2.0)/MMF (1000) | 0/1 |
| 14 | BA | 84/male | - | HVS | Tac (2.0)/MP (1.0)/MMF (1000) | 5.6 | 12/23 | 1/2 | Tac (2.0)/MP (1.0)/MMF (1000) | N.E. |

PLB: Protocol liver biopsy; LDLT: Living donor liver transplantation; ACR: Acute cellular rejection; IS: Immunosuppressants; Tac: Tacrolimus; ALT: Alanine amino transferase; HA: Hyaluronic acid; A: Activity; F: Fibrosis; OTCD: Ornithine transcarbamylase deficiency; BA: Biliary atresia; FHF: Fulminant hepatic failure; BDS: Biliary duct anastomotic stenosis; HAT: Hepatic artery thrombosis; IHBDS: Intrahepatic biliary duct stenosis; CMV-I: Cytomegalovirus infection; PVS: Portal vein stenosis; HVS: Hepatic vein stenosis; MMF: Mycophenolate mofetil; MP: Methylprednisolone.

3). Univariate analysis identified no risk factors for a score of \geq F1 at five years after LDLT. The variable with $P < 0.100$ included acute cellular rejection ($P = 0.079$). Multivariate analysis of these variables found none to be independent risk factors for a score of \geq F1 at five years after LDLT (Table 3).

The incidence of scores of \geq A2 or \geq F2 at five years after LDLT was 20.0% (11 cases), and all patients had a score of \geq F2 (Table 4). In all cases, the dose of immunosuppressants was increased after the PLB, and all eight patients who underwent a follow-up liver biopsy improved to scores of \leq A1 and F1.

Clinical and histopathological findings in the patients who underwent PLB at both two and five years after LDLT

PLBs were performed at both two and five years after LDLT in 21 cases; the results are summarized in Table 6. The activity and fibrosis scores at two years after LDLT were A0 and F0 in 14 patients, A1 or F1 in six patients and \geq A2 or \geq F2 in one patient. Seven patients with scores of A0 and F0 at two years after LDLT maintained scores of A0 and F0 at five years; however, the remaining patients exhibited worse scores of \geq A1 or \geq F1. Three patients with a score of A1 or F1 at two years after LDLT maintained a score of A1 or F1 at five years; how-

ever, the remaining patients exhibited worse a score of \geq A2 or \geq F2.

Complications of PLB

Complications related to the PLB occurred in only one patient (0.7%) who developed acute cholangitis. This complication resolved following the administration of antibiotics for three days.

Case reports

We described two representative liver transplant recipients with abnormal histopathological findings and normal LFT results in whom the dose of immunosuppressants was increased, which led to improvements in the histopathological findings (Table 4).

Case 4: A seven-month-old female infant with biliary atresia underwent ABO-identical LDLT using a left lateral segment graft. Tac and MP were administered as the standard postoperative immunosuppressive regimen. The patient's postoperative course included an episode of small intestine perforation requiring surgical repair and acute cellular rejection requiring steroid pulse treatment; however, she was discharged from the hospital on POD 28 after LDLT. MP was withdrawn at 18 mo after LDLT, and thereafter, only Tac was administered for immuno-

Table 5 Risk factors for \geq A1 and \geq F1 of protocol liver biopsy at five years after living donor liver transplantation: univariable analysis

| Variables | Incidence of \geq A1 (%) | P-value | Incidence of \geq F1 (%) | P-value |
|--|----------------------------|---------|----------------------------|---------|
| Recipient age at LDLT | | | | |
| < 12 mo (<i>n</i> = 18) <i>vs</i> \geq 12 mo (<i>n</i> = 37) | 38.9 <i>vs</i> 51.4 | 0.385 | 38.9 <i>vs</i> 32.4 | 0.637 |
| Recipient body weight at LDLT | | | | |
| < 10 kg (<i>n</i> = 29) <i>vs</i> \geq 10 kg (<i>n</i> = 26) | 41.4 <i>vs</i> 53.8 | 0.355 | 55.0 <i>vs</i> 30.8 | 0.577 |
| Original disease | | | | |
| Cholestatic diseases (<i>n</i> = 45) <i>vs</i> others (<i>n</i> = 10) | 46.7 <i>vs</i> 50.0 | 0.850 | 35.6 <i>vs</i> 33.3 | 0.738 |
| PELD or MELD | | | | |
| \geq 20 (<i>n</i> = 12) <i>vs</i> < 20 (<i>n</i> = 43) | 41.7 <i>vs</i> 48.8 | 0.660 | 41.7 <i>vs</i> 52.6 | 0.558 |
| Donor age | | | | |
| \geq 35 yr (<i>n</i> = 22) <i>vs</i> < 35 yr (<i>n</i> = 33) | 40.9 <i>vs</i> 51.5 | 0.440 | 36.4 <i>vs</i> 33.3 | 0.816 |
| Gender combinations between donor and recipient | | | | |
| Mismatch (<i>n</i> = 30) <i>vs</i> match (<i>n</i> = 25) | 53.3 <i>vs</i> 40.0 | 0.324 | 40.0 <i>vs</i> 28.0 | 0.352 |
| ABO compatibility | | | | |
| incompatible (<i>n</i> = 7) <i>vs</i> others (<i>n</i> = 48) | 42.9 <i>vs</i> 47.9 | 0.802 | 28.6 <i>vs</i> 35.4 | 0.722 |
| HLA-A | | | | |
| Mismatch (<i>n</i> = 41) <i>vs</i> match (<i>n</i> = 14) | 51.2 <i>vs</i> 35.7 | 0.316 | 39.0 <i>vs</i> 21.4 | 0.232 |
| HLA-B | | | | |
| Mismatch (<i>n</i> = 52) <i>vs</i> match (<i>n</i> = 3) | 48.1 <i>vs</i> 33.3 | 0.619 | 32.7 <i>vs</i> 66.7 | 0.229 |
| HLA-DRB ¹ | | | | |
| Mismatch (<i>n</i> = 47) <i>vs</i> match (<i>n</i> = 8) | 51.1 <i>vs</i> 25.0 | 0.172 | 36.2 <i>vs</i> 25.0 | 0.539 |
| Lymphocyte cross-matching | | | | |
| \geq 4 \times (<i>n</i> = 16) <i>vs</i> negative (<i>n</i> = 39) | 31.3 <i>vs</i> 53.8 | 0.127 | 18.8 <i>vs</i> 41.0 | 0.115 |
| GV/SLV | | | | |
| < 40 % (<i>n</i> = 2) <i>vs</i> \geq 40 % (<i>n</i> = 53) | 0.0 <i>vs</i> 49.1 | 0.173 | 0.0 <i>vs</i> 35.8 | 0.295 |
| Graft type | | | | |
| Lateral segment graft (<i>n</i> = 43) <i>vs</i> others (<i>n</i> = 12) | 51.2 <i>vs</i> 33.3 | 0.274 | 39.5 <i>vs</i> 16.7 | 0.141 |
| Operation time | | | | |
| \geq 20 h (<i>n</i> = 16) <i>vs</i> < 20 h (<i>n</i> = 39) | 37.5 <i>vs</i> 51.3 | 0.352 | 31.3 <i>vs</i> 35.9 | 0.742 |
| Cold ischemic time | | | | |
| \geq 2 h (<i>n</i> = 40) <i>vs</i> < 2 h (<i>n</i> = 15) | 55.0 <i>vs</i> 26.7 | 0.061 | 40.0 <i>vs</i> 20.0 | 0.165 |
| Warm ischemic time | | | | |
| \geq 1 h (<i>n</i> = 42) <i>vs</i> < 1 h (<i>n</i> = 13) | 42.9 <i>vs</i> 61.5 | 0.238 | 31.0 <i>vs</i> 46.2 | 0.314 |
| Blood loss volume | | | | |
| \geq 150 mL/kg (<i>n</i> = 11) <i>vs</i> < 150 mL/kg (<i>n</i> = 44) | 27.3 <i>vs</i> 52.3 | 0.137 | 27.3 <i>vs</i> 36.4 | 0.57 |
| Transfusion volume | | | | |
| \geq 100 mL/kg (<i>n</i> = 15) <i>vs</i> < 100 mL/kg (<i>n</i> = 40) | 40.0 <i>vs</i> 50.0 | 0.508 | 40.0 <i>vs</i> 32.5 | 0.603 |
| Splenectomy | | | | |
| Yes (<i>n</i> = 2) <i>vs</i> No (<i>n</i> = 53) | 100.0 <i>vs</i> 45.3 | 0.128 | 0.0 <i>vs</i> 35.8 | 0.295 |
| Portal vein complications | | | | |
| Yes (<i>n</i> = 9) <i>vs</i> No (<i>n</i> = 46) | 44.4 <i>vs</i> 47.8 | 0.852 | 33.3 <i>vs</i> 34.8 | 0.933 |
| Hepatic arterial complications | | | | |
| Yes (<i>n</i> = 4) <i>vs</i> No (<i>n</i> = 51) | 25.0 <i>vs</i> 49.0 | 0.354 | 25.0 <i>vs</i> 35.3 | 0.677 |
| Hepaticojejunostomic anastomotic stricture | | | | |
| Yes (<i>n</i> = 16) <i>vs</i> No (<i>n</i> = 39) | 31.3 <i>vs</i> 53.8 | 0.127 | 25.0 <i>vs</i> 38.5 | 0.340 |
| Cytomegalovirus infection | | | | |
| Yes (<i>n</i> = 17) <i>vs</i> No (<i>n</i> = 38) | 47.1 <i>vs</i> 47.4 | 0.999 | 47.1 <i>vs</i> 28.9 | 0.192 |
| Acute cellular rejection | | | | |
| Yes (<i>n</i> = 23) <i>vs</i> No (<i>n</i> = 32) | 60.9 <i>vs</i> 37.5 | 0.087 | 47.8 <i>vs</i> 25.0 | 0.079 |
| Total bilirubin at PLB | | | | |
| \geq 0.7 mg/dL (<i>n</i> = 25) <i>vs</i> < 0.7 mg/dL (<i>n</i> = 30) | 48.0 <i>vs</i> 46.7 | 0.920 | 36.0 <i>vs</i> 33.3 | 0.836 |
| AST at PLB | | | | |
| \geq 30 IU/L (<i>n</i> = 22) <i>vs</i> < 30 IU/L (<i>n</i> = 33) | 54.5 <i>vs</i> 42.4 | 0.378 | 36.4 <i>vs</i> 33.3 | 0.816 |
| ALT at PLB | | | | |
| \geq 20 IU/L (<i>n</i> = 21) <i>vs</i> < 20 IU/L (<i>n</i> = 34) | 57.1 <i>vs</i> 41.2 | 0.249 | 28.6 <i>vs</i> 38.2 | 0.464 |
| γ -GTP at PLB | | | | |
| \geq 20 IU/L (<i>n</i> = 20) <i>vs</i> < 20 IU/L (<i>n</i> = 35) | 45.0 <i>vs</i> 48.6 | 0.799 | 30.0 <i>vs</i> 37.1 | 0.592 |
| Hyaluronic acid at PLB | | | | |
| \geq 20 ng/mL (<i>n</i> = 22) <i>vs</i> < 20 ng/mL (<i>n</i> = 33) | 50.0 <i>vs</i> 45.5 | 0.741 | 36.4 <i>vs</i> 33.3 | 0.816 |
| IgG at PLB | | | | |
| \geq 1200 mg/dL (<i>n</i> = 24) <i>vs</i> < 1200 mg/dL (<i>n</i> = 31) | 54.2 <i>vs</i> 41.9 | 0.368 | 41.7 <i>vs</i> 29.0 | 0.328 |
| ANA at PLB | | | | |
| \geq 20 \times (<i>n</i> = 14) <i>vs</i> < 20 \times (<i>n</i> = 41) | 35.7 <i>vs</i> 51.2 | 0.316 | 28.6 <i>vs</i> 36.6 | 0.586 |
| ASMA at PLB | | | | |
| \geq 20 \times (<i>n</i> = 10) <i>vs</i> < 20 \times (<i>n</i> = 45) | 70.0 <i>vs</i> 42.2 | 0.111 | 40.0 <i>vs</i> 33.3 | 0.688 |

Trough of tacrolimus at PLB

≥ 3.0 ng/mL (*n* = 19) *vs* < 3.0 ng/mL (*n* = 33)¹52.6 *vs* 42.4

0.477

36.8 *vs* 33.3

0.797

¹Three cases which were used a cyclosporine were removed. LDLT: Living donor liver transplantation; PELD: Pediatric end-stage liver disease; MELD: Model for end-stage liver disease; GV/SLV: Ratio of graft volume to standard liver volume; PLB: Protocol liver biopsy; AST: Aspartate amino transferase; ALT: Alanine amino transferase; IgG: Immunoglobulin G; ANA: Antinuclear antibody; ASMA: Antismooth nuclear antibody.

Table 6 Clinical and histopathological findings of cases who performed protocol liver biopsy at both two and five years after living donor liver transplantation

| Case | Original disease | Age at LT/sex | Previous ACR | Post-transplant complications | IS at two years PLB | Tac trough at PLB | ALT/HA at PLB | A/F at PLB | IS at five years PLB | Tac trough at PLB | ALT/HA at PLB | A/F at PLB |
|------|------------------|---------------|--------------|-------------------------------|------------------------------|-------------------|---------------|------------|-------------------------------|-------------------|---------------|------------|
| 1 | OTCD | 46/female | - | - | Tac (3.0) | 2.5 | 12/35 | 2/1 | Tac (1.0)/MMF (400) | 0.5 | 11/52 | 1/1 |
| 11 | BA | 26/male | + | PVS | Tac (0.8)/MP (4.0)/MMF (500) | 3.2 | 20/11 | 0/1 | Tac (1.6)/MP (4.0)/MMF (1500) | 2.2 | 12/17 | 2/2 |
| 12 | BA | 114/male | - | BDS | Tac (2.0) | 2.6 | 14/21 | 1/0 | Tac (3.0) | 2.3 | 16/20 | 1/2 |
| 13 | BA | 10/female | + | CMV-I | Tac (0.4) | 3.8 | 19/11 | 0/0 | Tac (1.6) | 2.8 | 18/< 9 | 1/2 |
| 14 | BA | 30/male | - | HVS | Tac (1.2) | 5.3 | 18/29 | 1/1 | Tac (2.0)/MP (1.0)/MMF (1000) | 5.6 | 12/23 | 1/2 |
| 15 | BA | 120/female | - | BDS | Tac (1.5)/PSL (2.5) | 4.4 | 15/14 | 0/0 | Tac (4.0) | 7.0 | 17/< 9 | 0/0 |
| 16 | BA | 163/male M | + | BDS/CMV-I | CsA (150) | CsA 50 | 9/27 | 0/0 | CsA (150)/MMF (1000) | CsA 83 | 91020 | 0/1 |
| 17 | BA | 8/female | + / OKT3 | PVS/CMV-I | Tac (0.8)/MP (0.5) | 2.4 | 30/36 | 0/0 | Tac (2.0) | 5.3 | 15/18 | 1/1 |
| 18 | BA | 12/male | + | - | Tac (0.8) | 3.8 | 14/58 | 0/0 | Tac (0.8) | 0.2 | 8/20 | 1/1 |
| 19 | BA | 13/female | + | CMV-I | Tac (1.6)/MP (2.0) | 9.3 | 22/11 | 0/0 | Tac (1.4)/MP (3.0)/MMF (500) | 2.1 | 15/15 | 0/1 |
| 20 | AD | 19/female | + | CMV-I | Tac (0.8)/MMF (500) | 2.3 | 19/13 | 0/1 | Tac (2.4)/MMF (500) | 3.8 | 14/15 | 1/1 |
| 21 | WD | 112/male | - | - | Tac (4.0) | 1.3 | 16/16 | 0/0 | Tac (5.0) | 1.4 | 19/13 | 0/0 |
| 22 | BA | 170/female | + | BDS | Tac (2.0) | 6.3 | 17/16 | 0/1 | Tac (6.0)/MP (12)/MMF (2000) | 15.5 | 39/22 | 1/0 |
| 23 | BA | 33/F | + | HVS | Tac (1.0)/MP (2.0)/MMF (400) | 3.7 | 10/< 9 | 0/0 | Tac (1.5)/MMF (1000) | 5.4 | 41/19 | 1/0 |
| 24 | BA | 9/female | - | HAT | Tac (0.6) | 0.3 | 14/24 | 0/0 | Tac (0.8) | 0 | 10/10 | 0/0 |
| 25 | BA | 28/female | - | - | Tac (0.4) | 2.8 | 18/< 9 | 0/0 | Tac (1.0) | 0.9 | 15/10 | 1/0 |
| 26 | BA | 9/female | - | IHBDS | Tac (0.4) | 2.1 | 23/< 9 | 0/1 | Tac (2.0)/MP (0.5)/MMF (500) | 5.3 | 41/19 | 1/1 |
| 27 | AD | 19/male | - | - | Tac (0.6) | 3.3 | 12/17 | 0/0 | Tac (2.0)/MMF (500) | 1.5 | 11/59 | 0/0 |
| 28 | BA | 45/female | - | BDS | Tac (1.2) | 3.6 | 13/15 | 0/0 | Tac (1.5) | 4.6 | 11/10 | 0/0 |
| 29 | BA | 9/female | - | - | Tac (0.4) | 0.9 | 11/17 | 0/0 | Tac (0.8) | 1.1 | 13/25 | 0/0 |
| 30 | CEPS | 37/male | - | - | Tac (2.0)/MP (2.5)/MMF (500) | 2.5 | 13/< 9 | 0/0 | Tac (2.0)/MP (1.5)/MMF (500) | 3.9 | 11/< 9 | 0/0 |

PLB: Protocol liver biopsy; LDLT: Living donor liver transplantation; ACR: Acute cellular rejection; IS: Immunosuppressants; Tac: Tacrolimus; ALT: Alanine amino transferase; HA: Hyaluronic acid; A: Activity; F: Fibrosis; OTCD: Ornithine transcarbamylase deficiency; BA: Biliary atresia; AD: Alagille syndrome; WD: Wilson disease; CEPS: Congenital extrahepatic portsystemic shunt; OKT3: Muromonab-CD3; PVS: Portal vein stenosis; BDS: Biliary duct anastomotic stenosis; CMV-I: Cytomegalovirus infection; HVS: Hepatic vein stenosis; HAT: Hepatic artery thrombosis; IHBDS: Intrahepatic biliary duct stenosis; MP: Methylprednisolone; MMF: Mycophenolate mofetil; PSL: Prednisolone; CsA: Cyclosporin A.

suppression. In postoperative year (POY) 5, a PLB was performed; the LFT data were normal, but the Metavir scores were A2 and F2 (Figure 2A). The immunosuppression was subsequently strengthened by increasing the dose of Tac and adding MMF because the PLB histopathology was considered to be abnormal. A follow-up liver biopsy was performed 18 mo after the PLB, at which time the scores were A0 and F0 (Figure 2B).

Case 5: A 58-month-old female girl with biliary atresia

underwent ABO-identical LDLT using a left lateral segment graft. Tac and MP were administered as the standard postoperative immunosuppressive regimen. The patient's postoperative course was uneventful, except for an episode of acute respiratory distress, and she was discharged from the hospital on POD 56 after LDLT. MP was withdrawn at 18 mo after LDLT, and thereafter, only Tac was administered for immunosuppression. In POY 5, PLB was performed; the LFT data were normal, but the Metavir scores were A2 and F2 (Figure 2C). The

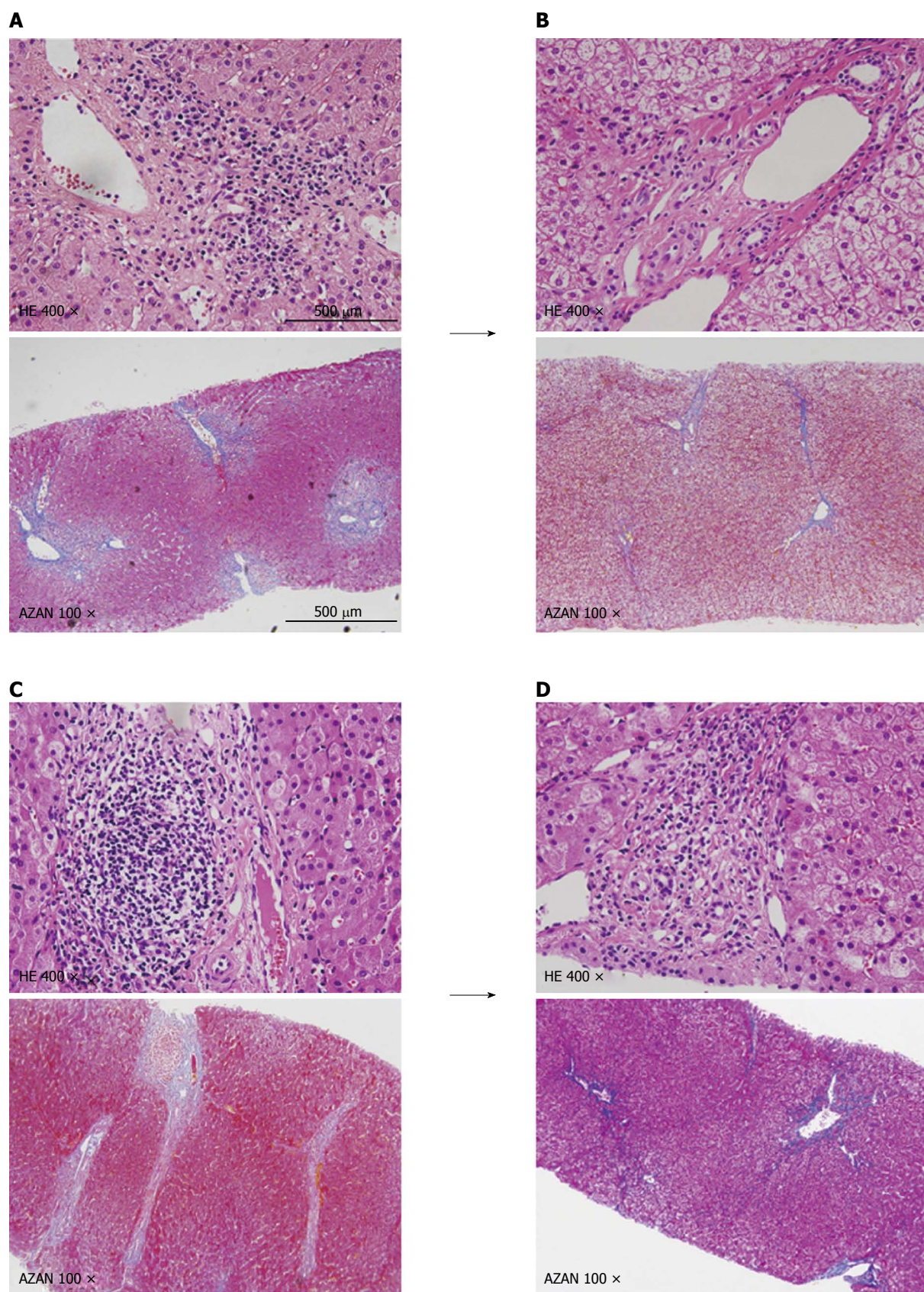


Figure 2 In postoperative year 5, a protocol liver biopsy was performed. A: At which time the Metavir scores were abnormal: A2 (portal inflammation) and F2 (portal and pericellular fibrosis); B: Follow-up liver biopsy was performed at 18 mo after the protocol liver biopsy (PLB), at which time the scores were A0 and F0; C: At which time the Metavir scores were abnormal: A2 (portal inflammation) and F2 (portal fibrosis); D: A follow-up liver biopsy was performed at 20 mo after the PLB, at which time the scores were A1 (portal inflammation) and F0. HE: Hematoxylin and eosin stain; AZAN: Azan stain.

immunosuppression was then strengthened by increasing the dose of Tac and adding MMF because the PLB histopathology was considered to be abnormal. A follow-up liver biopsy was performed 20 mo after the PLB, at which time the scores were A1 and F0 (Figure 2D).

DISCUSSION

LT is an established curative treatment for pediatric patients with end-stage liver disease or acute liver failure^[1-3]. However, histopathological assessments performed during the mid- and long-term period after LT remain insufficient, and it is necessary to further improve the prognosis by maintaining the function of the liver graft using a minimum degree of immunosuppression to obtain an optimal balance between the effectiveness and side effects of individual immunosuppressants.

Histopathological assessments using PLB have recently been reported to be important in adult recipient^[12-15], because the occurrence of graft fibrosis or the recurrence of the original disease cannot be predicted using standard LFTs alone. However, in pediatric recipients, the need for PLB is controversial due to the low incidence of recurrent original diseases. Liver graft fibrosis has recently been reported to be present in 43%-65% and 25%-69% of patients at two and five years after LT, respectively, even if the LFT data are normal^[4,6]. Moreover, there is a relationship between liver graft fibrosis and chronic rejection^[4,5], and the progression to severe fibrosis has been reported to occur in 14%-25% of patients at ten years after LT^[4,6]. Furthermore, the development of liver graft fibrosis after pediatric LT occurs in 69%-97% of cases, including cases of mild fibrosis^[4-8]. The risk factors for fibrosis include an increasingly long interval after LT^[4,7], positivity for antinuclear antibodies^[4], long cold ischemic time^[6], young age at LT^[6], a high donor to recipient graft ratio^[6] and partial LT^[6]. In the present study, independent risk factors in a multivariate analysis of a score of \geq A1 at two years after LDLT included \geq 2 h of cold ischemic time, no acute cellular rejection and an ALT level of \geq 20 IU/L ($P = 0.028$, $P = 0.033$ and $P = 0.012$, respectively); however, no risk factors were identified for a score of \geq F1. Furthermore, no independent risk factors were identified in a multivariate analysis of scores of \geq A1 and \geq F1 at five years. We believe that \geq 2 h of cold ischemic time was found to be an independent risk factor for a score of \geq A1 at two years after LDLT because a prolonged cold ischemic time may induce an immune response by affecting graft liver dysfunction. In addition, we believe that no acute cellular rejection was found to be an independent risk factor for a score of \geq A1 at two years after LDLT because acute cellular rejection may cause an immune response due to the use of less immunosuppression. However, as a result of the ROC curve analysis of ALT at two years after LDLT in the patients with a score of \geq A1, the recommended cutoff value for diagnosing a score of \geq A1 was set at 20 IU/L (sensitivity: 50.0% and specificity: 76.1%). Therefore, the serum

ALT level reflects the degree of portal inflammation in PLB patients at two years after LDLT with an ALT level of \geq 20 IU/L.

With respect to concrete assessment methods for evaluating graft liver fibrosis, portal fibrosis-based liver fibrosis staging systems, such as those reported by Ishak *et al*^[16] and the Metavir Study Group^[11], are widely used, even in studies of pediatric LT recipients^[7,8,17]. Therefore, we applied histopathological assessments using the Metavir score in the present study. Recent reports have indicated that centrilobular perisinusoidal fibrosis occurs in pediatric LT recipients in association with tacrolimus withdrawal or in the presence of donor-specific anti-human leukocyte antigen antibodies^[18,19]. Venturi *et al*^[17] recently developed a novel histopathological scoring system based on the detection of fibrosis in three areas: portal tracts, sinusoids and centrilobular veins. However, the significance of these histopathological findings with respect to morbidity has yet to be clarified and is the most important issue that should be addressed in the future. In the present study, using the Metavir scoring system, the incidence of the scores of \geq F1 at two and five years after LDLT was 24.7% and 34.5%, respectively. However, no risk factors for graft fibrosis were identified, and no serum markers reflected the degree of graft fibrosis. Therefore, detecting graft fibrosis by performing a histopathological assessment using a liver biopsy is important. Furthermore, the PLB represents an important periodic examination in long-term recipients after LDLT because it enables the assessment of the effectiveness of the current immunosuppressive regimen, even when the PLB histopathology is normal. Therefore, at present, PLB is an indispensable examination for the management of patients who have undergone LDLT.

Potential problems associated with PLB include the following: (1) timing; (2) invasiveness; and (3) the obscure definition of abnormal PLB histopathology. The timing of PLB after LT is not definitive. In our department, we performed PLB at two, five, ten and 15 years after LT, considering the examination's effectiveness, invasiveness and potential complications. In the present study, the PLB performed two years after LDLT was found to be an unnecessary examination because the serum ALT level reflected the degree of portal inflammation. At the time, the immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. Gelson *et al*^[20] reported that the histological inflammatory index is correlated with the ALT level. A PLB performed at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect the degree of graft fibrosis.

PLB suffers, however, from a disadvantage. PLB is an invasive procedure that is potentially associated with severe complications, with an incidence of 0.57%^[21]. In the present study, although the rate of PLB-associated complications was only 0.7%, this rate may nevertheless be considered high. Non-invasive examinations, such as imaging, may be used instead of PLB if such examinations

become more effective than PLB in the future. Acoustic radiation force impulse and transient elastography imaging have been reported to exhibit good accuracy in the noninvasive diagnosis of liver fibrosis in the setting of pediatric LT^[22,23].

The most problematic aspect of PLB is the obscure definition of abnormal histopathology. The histopathological findings of PLB after LT include idiopathic post-transplantation hepatitis (4.4%-64.0%)^[4,24-26], central venulitis (16.0%-27.0%)^[13,27], interface hepatitis (14.0%-24.4%)^[28-30] and fibrosis (69.0%-97.0%)^[4-8]. However, the indication for treatment with respect to each histopathological finding is unclear and controversial. In general, liver fibrosis is thought to be irreversible and resistant to treatment. However, in the present cases, the liver fibrosis was reversible, and portal inflammation was ameliorated after strengthening the immunosuppressive regimen. Immunosuppression can be strengthened effectively by increasing the dose of Tac and introducing MMF, given concerns about the side effects of MP^[31-34] and the proven effectiveness of MMF^[35,36]. Our present findings suggest that the early detection of graft liver fibrosis can be achieved using a liver biopsy and that liver fibrosis may be reversible if early treatment is initiated. In our department, we initially defined a histopathological abnormality as a Metavir score of $\geq A2$ or $\geq F2$. However, among 21 patients who underwent PLB at both two and five years after LDLT, the activity and fibrosis scores at two years after LDLT were A0 and F0 in 14 patients, A1 or F1 in six patients and $\geq A2$ or $\geq F2$ in one patient. Seven patients with scores of A0 and F0 at two years after LDLT exhibited worse a score of $\geq A1$ or $\geq F1$. Three patients with a score of A1 or F1 at two years after LDLT exhibited worse a score of $\geq A2$ or $\geq F2$. Therefore, we currently define a histopathological abnormality as a Metavir score of $\geq A1$ or $\geq F1$ and consider such scores to indicate the need for treatment because liver fibrosis is reversible if early treatment is initiated. Both further investigations and the accumulation of more LT cases are required to confirm our present findings.

In a conclusion, A PLB performed at two years after LDLT is an unnecessary examination because the serum ALT level reflects the degree of portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. A PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect the degree of graft fibrosis.

COMMENTS

Background

Histopathological assessments using protocol liver biopsy (PLB) after liver transplantation (LT) have recently been reported to be important. However, few studies have investigated the impact of the timing and effectiveness of PLBs in the field of pediatric LT. This retrospective study assessed the clinical significance of PLBs in pediatric LT.

Research frontiers

The development of liver graft fibrosis after pediatric LT has been reported to

occur in 69%-97% of cases, including cases of mild fibrosis. Because graft dysfunction does not occur unless the fibrosis becomes advanced and because the occurrence of graft liver fibrosis or portal inflammation cannot be predicted using standard liver function test (LFT) alone, histopathological assessments using PLB have recently been reported to be important. However, the significance of mild to severe fibrosis is unknown, and the indications for the treatment of abnormal PLB findings are controversial. In addition, few studies have investigated the impact of the timing and effectiveness of PLB.

Innovations and breakthroughs

The development of liver graft fibrosis after pediatric LT has been reported to occur in 69%-97% of cases, including cases of mild fibrosis. Because graft liver dysfunction does not occur unless the fibrosis becomes advanced and because the occurrence of graft liver fibrosis or portal inflammation cannot be predicted using standard LFT alone, histopathological assessments using PLB have recently been reported to be important. However, the significance of mild to severe fibrosis is unknown, and the indications for the treatment of abnormal PLB findings are controversial. In addition, few studies have investigated the impact of the timing and effectiveness of PLB. This retrospective study assessed the clinical significance of the timing and effectiveness of PLB after pediatric living donor liver transplantation (LDLT). In conclusion, a PLB performed at two years after LDLT is an unnecessary examination because the serum ALT level reflects the degree of portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. A PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect the degree of graft fibrosis.

Applications

The study results suggest the following contents. A PLB performed at two years after LDLT is an unnecessary examination because the serum ALT level reflects the degree of portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. A PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect the degree of graft fibrosis.

Terminology

Protocol liver biopsy: Protocol liver biopsy is a liver biopsy that is periodically performed at two and five years after LT.

Peer review

This is a good descriptive study in which the authors analyzed the histopathological findings using the Metavir scoring system and identified factors associated with scores of $\geq A1$ and $\geq F1$. They, thereafter, clarified the timing and effectiveness of PLB. The results are interesting and suggest the following. A PLB performed at two years after LDLT is an unnecessary examination because the serum ALT level reflects the degree of portal inflammation. In addition, immunosuppressive therapy should be modulated to maintain the ALT concentration at a level less than 20 IU/L. A PLB at five years is an excellent examination for the detection of early reversible graft fibrosis because no serum markers reflect the degree of graft fibrosis.

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Novel method for extracting exosomes of hepatocellular carcinoma cells

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sion electron microscopy. Exosome marker proteins were detected by Western blot analysis. Two potential hepatoma-associated proteins, tissue transglutaminase 2 (TGM2) and annexin A2, were analyzed.

RESULTS: The exosomes separated by the new extraction assay based on the nanomaterial were disc-shaped, intact vesicles with lipid bilayer membranes. They were approximately 30-100 nm in diameter, which is similar to the diameter of exosomes isolated by the traditional method. The protein concentration of exosomes extracted by the new method was approximately 780 $\mu\text{g}/10^8$ cells, and therefore, it was 19 times higher than that of exosomes extracted in the traditional manner. There were differences between the total proteins of Huh-7 cells and the exosomal proteins. Typical exosome proteins, such as the transmembrane protein CD63 and heat shock protein 70, were confirmed. Two potential hepatoma-associated proteins were also identified. TGM2 was first found to exist in the exosomes of human liver cancer cells, but annexin A2 was not secreted into exosomes.

CONCLUSION: The new extraction method based on the nanomaterial is quick and efficient. The cancer-associated protein TGM2 can be secreted through an exosome-mediated non-classical secretion pathway, and it may be a valuable tumor marker.

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Key words: Exosome; Membrane vesicles; Tissue transglutaminase 2; Annexin A2; Nanomaterials

Core tip: The traditional extraction assay of exosomes is usually complicated and time consuming. In this manuscript, we investigate a novel assay based on a nanomaterial to extract exosomes more quickly and efficiently compared with the traditional method. A hepatoma-associated protein, tissue transglutaminase 2, was first found to exist in the exosome of the human

Abstract

AIM: To develop a novel method for the rapid and efficient extraction of exosomes secreted by tumor cells.

METHODS: Unlike the traditional extraction method, the supernatants of cell cultures were concentrated, and the exosomes were isolated promptly and effectively using a novel nanomaterial called ExoQuick. Coomassie brilliant blue staining was used for protein quantification, and the morphology of the exosomes extracted by both methods was visualized by transmis-

liver cancer cell line Huh-7. This protein can be secreted through an exosome-mediated non-classical secretion pathway and is a potential tumor marker.

Zhu L, Qu XH, Sun YL, Qian YM, Zhao XH. Novel method for extracting exosomes of hepatocellular carcinoma cells. *World J Gastroenterol* 2014; 20(21): 6651-6657 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6651.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6651>

INTRODUCTION

The term “exosome” was first used by Pan and Johnstone to describe the small vesicles secreted by reticulocytes during their differentiation into mature red blood cells; in this context, exosomes could carry away plasma membrane proteins that were not required by mature red blood cells, such as transferrin receptor and acetylcholinesterase^[1]. In recent years, it has been demonstrated that exosomes can induce anti-tumor immune responses, and researchers have therefore devoted increasing attention to both basic research on exosomes and applied research regarding the use of exosomes for the development of anti-tumor vaccines. Recent studies have reported that many types of cultured cells can secrete exosomes, and exosomes can also be isolated from many types of body fluids, such as plasma^[2], bile^[3], urine^[4], breast milk^[5], saliva^[6], pleural fluid^[7], ascites^[8], and bronchoalveolar lavage fluid^[9-11]. Traditional exosome extraction methods involve ultracentrifugation or density gradient centrifugation; however, in this study, a new nanomaterial was utilized to isolate and purify exosomes secreted by hepatoma cells. This novel procedure was convenient and saved time and effort relative to traditional procedures. To determine whether this nanomaterial-based approach could quickly and efficiently purify exosomes, exosome morphology was observed by transmission electron microscopy, and biological marker proteins of the isolated exosomes were identified by Western blotting. These validation steps confirmed that the new isolation and purification method served as an efficacious approach for use in subsequent exosome research. Furthermore, in this study, exosomes were isolated and purified from the supernatant of cultured hepatoma cells, and Western blot analysis revealed the presence of tissue transglutaminase 2 (TGM2) in these exosomes. Therefore, these findings provide a theoretical foundation for the secretion of proteins through non-classical exosome-mediated pathways.

MATERIALS AND METHODS

Cell line, antibodies, and reagents

The human liver cancer cell line Huh-7 was obtained from the Human Science Research Resources Bank (Osaka, Japan). Amicon Ultra-15 centrifugal ultrafiltra-

tion units (3 kDa) and polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, United States); ExoQuick Exosome precipitation solution was purchased from SBI (San Francisco, CA, United States). Rabbit anti-human CD63 and heat shock protein 70 (HSP70) polyclonal antibodies, rabbit anti-human annexin A2 monoclonal antibody, and mouse anti-human TGM2 monoclonal antibody were purchased from Santa Cruz (Santa Cruz Biotech., CA, United States). Mouse anti-human β -actin monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, United States). Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG and goat anti-mouse IgG were obtained from ZSGB-BIO (Beijing, China). An ultrasensitive enhanced chemiluminescence solution kit was obtained from Thermo (Waltham, MA, United States).

Cell culture and preparation of concentrated culture medium

Human hepatocellular carcinoma Huh-7 cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C with 5% CO₂. When the cells reached approximately 90% confluence (in total, approximately 1.8×10^8 cells), they were washed three times with 20 mL of phosphate-buffered saline (1 \times PBS) and were cultured for 24 h in serum-free DMEM medium. Approximately 100 mL of culture supernatant was collected and centrifuged at $3000 \times g$ for 15 min at 4 °C ($480 g$ for 5 min followed by $2000 g$ for 10 min) to remove intact cells and cell debris, and then, the remaining supernatant was concentrated to 1 mL using an Amicon Ultra-15 *via* centrifugation in a swing-out rotor at 4 °C and $4000 \times g$. An ultracel centrifugal filter device with a 3 kDa molecular weight cutoff (MWCO) was used.

Ultracentrifugation exosome isolation

The concentrated culture medium (CCM) was then subjected to high-speed centrifugation at $100000 \times g$ (TLA-45 fixed angle, Beckman Coulter) at 4 °C for 2 h. The pellet was resuspended in 1 mL of PBS and washed by recentrifugation at $100000 \times g$ for 3 h. The resulting exosome-enriched pellet was resuspended in 50 μ L 1 \times PBS and either used immediately or stored at -80 °C.

Nanomaterial exosome isolation

The CCM was added to an equal volume of ExoQuick exosome precipitation solution, and the resulting solution was mixed by inverting the tube and allowing it to stand overnight in a refrigerator. This mixture was then centrifuged at $1500 \times g$ for 30 min. The supernatant was discarded, and the precipitate consisted of exosomes. A portion of the precipitate was then re-suspended in 1 \times PBS for morphological observations *via* electron microscopy. Proteins were extracted from the remaining precipitate through resuspension in protein lysis buffer containing protease inhibitors, and the resulting solution was stored at -80 °C for future analysis.

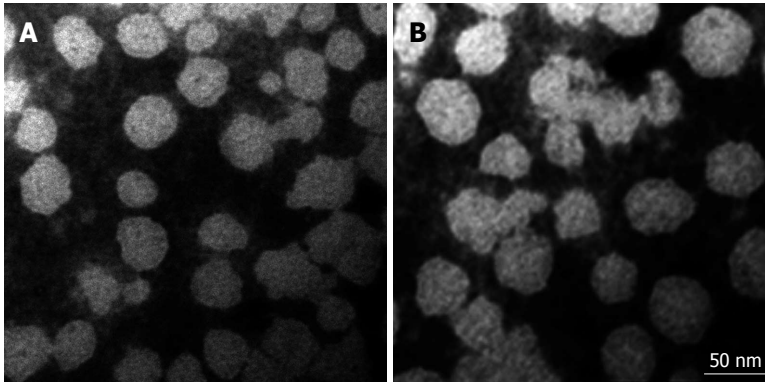


Figure 1 Characterization of the exosomes of Huh-7 cells extracted by two methods. Electron micrographs of exosomes isolated from Huh-7 cells in serum-free concentrated culture medium by the traditional method (A) and the new extraction method based on a nanomaterial (B). Negative-stained images show exosomes with a smooth, saucer-like morphology. The sizes are between 30-100 nm. The scale bar is 50 nm. Direct Mag: 100000 ×, HV = 80.0 kV.

Morphology analysis by transmission electron microscopy

One drop of the solution of exosomes resuspended in $1 \times$ PBS was placed on a copper mesh with a diameter of 2 mm. Fluid was gently absorbed from the edges of the copper mesh with filter paper. A drop of 2% phosphotungstic acid solution was added to the sample, and negative staining was performed for 10 min at room temperature. After the negative staining solution was absorbed by the filter paper, the sample was dried for 2 min under incandescent light. The copper mesh was placed under a transmission electron microscope, and exosome morphology was observed and photographed at 80 kV.

Western blot analysis

Western blotting was used to analyze annexin A2 and TGM2 in the exosomes secreted by Huh-7 hepatoma cells as previously described, with slight modifications^[4,12]. Briefly, approximately 30 μ g of Huh-7 whole cell lysates and exosomal protein were separated, added to loading buffer, and heated at 95 °C for 10 min. The samples were subjected to electrophoresis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer at constant 80 V at 4 °C for 3 h, and then, they were transferred to PVDF membranes. After blocking, the membranes were incubated with primary rabbit anti-CD63 (1:200), rabbit anti-HSP70 (1:200), rabbit anti-annexin A2 (1:500), or mouse anti-TGM2 (1:1000) at 4 °C overnight followed by incubation with the secondary antibody, HRP-labeled goat anti-rabbit IgG or goat anti-mouse IgG (1:3000), at room temperature for 1 h. The membranes were washed three times after each incubation for 5 min and visualized using the LAS4000 imaging system (Fujifilm).

RESULTS

Exosome morphology

The identity of the Huh-7 hepatocellular carcinoma cells was confirmed by short tandem repeats. Observations of the growth of the cells under an inverted phase contrast microscope revealed good adherence. Exosomes isolated from Huh-7 cells by the nanomaterial-based method and ultracentrifugation were viewed with a transmission electron microscope, which indicated the presence of many disc-shaped small vesicles of different sizes with lipid

bilayer membranes. In particular, the diameters of these vesicles were between 30 and 100 nm (Figure 1). Therefore, the exosome morphology resulting from the new extraction assay based on the nanomaterial is similar to that of exosomes isolated by the traditional method.

Comparison of protein concentrations of exosomes isolated by two methods

Exosomal proteins from the supernatants of Huh-7 cells were quantified using the Bradford method. In particular, the actual protein concentrations were calculated using the standard curve equation and the appropriate dilutions. The concentration of exosomal proteins extracted by the new method based on the nanomaterial was approximately 780 μ g/ 10^8 cells, which was more than 19 times higher than that of exosomes extracted in the traditional manner, which typically yields less than 40 μ g/ 10^8 cells.

Exosomal protein identification and secretion mechanisms of human liver cancer-associated proteins

Approximately 30 μ g of Huh-7 whole cell lysates and exosomal proteins were separated by SDS-PAGE. A Coomassie brilliant blue-stained gel obtained after SDS-PAGE demonstrated differences between the total proteins of Huh-7 cells and exosomal proteins secreted by Huh-7 cells (Figure 2). Exosomal proteins were separated by SDS-PAGE, electrotransferred, and probed with exosome markers (HSP70 and CD63). The membrane contained two bands of intense signal at 70 kDa and 53 kDa, which corresponded to the exosomal molecular biomarkers HSP70 and CD63, respectively (Figure 3). It is known that the hepatoma-associated proteins annexin A2 and TGM2 can be extracellularly secreted through non-classical secretory pathways, and the transport of proteins out of cells by exosomes is one of these non-classical secretory pathways. Therefore, we determined whether these proteins were localized to exosomes by Western blot analysis. The results indicated that exosomes secreted by Huh-7 cells contained TGM2 but not annexin A2 (Figure 3), confirming that TGM2 is extracellularly secreted through a non-classical exosome-mediated pathway.

DISCUSSION

Exosomes are small, membranous vesicles with diam-

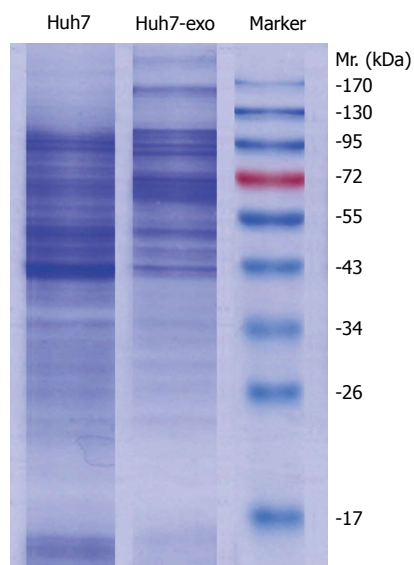


Figure 2 Detection of differences in Huh7 whole cell lysates and exosomal proteins. Approximately 30 μ g of Huh-7 whole cell lysates and exosomal proteins isolated by the nanomaterial procedure were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were visualized by Coomassie brilliant blue staining. The marker is a prestained protein marker; Huh7-exo indicates exosomal proteins isolated from the supernatants of human hepatocellular carcinoma Huh-7 cells; Mr. stands for molecular weight.

eters between 30 and 100 nm that are extracellularly secreted by cells. Their surfaces contain large quantities of protein and lipid components that are closely associated with exosomal origins and functions, and microRNAs (miRNAs), messenger RNAs (mRNAs), and cytoplasmic proteins are contained within exosomes. The unique biological characteristics of exosomes have attracted great interest. For example, the membranous structure of exosomes can protect cytokines or other proteins carried by these vesicles from degradation by serum proteases, and exosomes therefore serve as important carriers that transmit information between cells in the body. Exosomes are derived from endosomes during endocytosis. In particular, during this process, the membranes of endosomes may bud inward into the endosomal lumen to form membrane-enclosed structures. The bases of these structures gradually separate from the endosomal membrane to form small vesicles within endosomes. Multiple small vesicles can be formed at once; these sets of many small vesicles are known as multivesicular bodies (MVBs)^[13]. During exosome formation, cytoplasmic proteins and membrane proteins are selectively incorporated into exosomes. Because these proteins may include a variety of cell surface receptors and/or ligands, exosomes can act in either an autocrine or a paracrine manner and can therefore regulate their own functions or affect distant target cells^[10,14]. However, the specific mechanisms of these interactions and regulatory functions remain unclear. For example, interactions between exosomes and target cells may rely on growth factors or bioactive lipids expressed on the surfaces of exosomes and cells, the transfer of membrane receptors, or direct stimula-

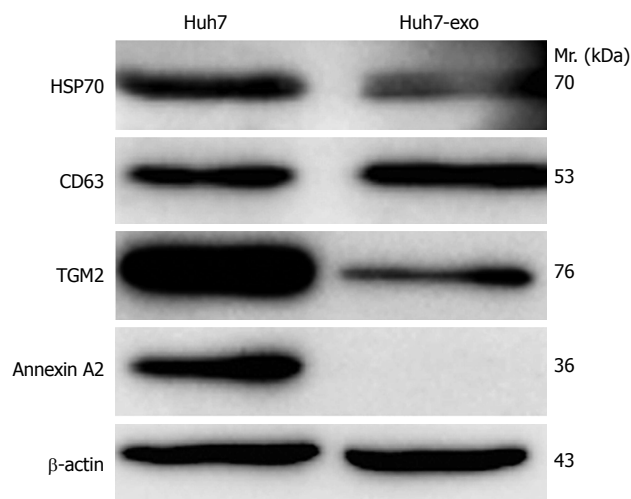


Figure 3 Exosome protein identification and secretion mechanisms of human liver cancer-associated proteins as analyzed by Western blotting.

In total, 30 μ g of proteins from Huh-7 whole cell lysates and exosomal proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, electrotransferred and incubated with antibodies against exosome protein markers (HSP70 and CD63) and hepatocellular carcinoma-associated proteins (TGM2 and annexin A2). β -actin was used as an internal control. Exosome protein markers (HSP70 and CD63) were identified on the surfaces of exosome membranes. Exosomes secreted by Huh-7 hepatocellular carcinoma cells contained the hepatoma-associated protein TGM2 but were negative for annexin A2. Huh7-exo indicates exosomal proteins isolated from the supernatants of human hepatocellular carcinoma Huh-7 cells; Mr. stands for molecular weight.

tion through the delivery of proteins to target cells. The presence of mRNAs and miRNAs in exosomes suggests that the exchange of genetic material may be another aspect of exosome-mediated information transfer between cells^[15]. Exosomes from tumor cells have been proposed to induce the cytotoxic T lymphocyte response, and many scientists initially had high hopes for the antitumor functions of exosomes. 5-Aza-2'-deoxycytidine, an inhibitor of DNA methyltransferase, can increase exosomes produced by hepatoma cells and the immune-associated protein component of exosomes^[16]. However, recent studies have demonstrated that exosomes can downregulate immune responses or induce immune tolerance under certain conditions, leading researchers to re-examine the immunologic properties of exosomes^[17]. The finding that glioblastoma tumor cell-derived exosomes contain mRNAs and microRNAs coupled with the detection of these exosomes in the serum of glioblastoma patients suggests that blood-based exosomes may provide valuable diagnostic information and aid in therapeutic decisions for cancer patients^[18]. Exosomes were quantified by cytometry in the plasma of colorectal cancer patients to evaluate their potential as a tumor indicator. The fraction of exosomes in cancer patients was statistically higher than that in healthy controls^[19]. To better understand the biological role of exosomes, it is important to work with highly purified material.

To date, few studies have examined exosomes. One reason for this lack of research is that the traditional exosome extraction process is relatively complex and cum-

bersome, rendering exosome research difficult. In prior studies in which exosomes have been extracted using conventional methods, such as ultracentrifugation and density gradient centrifugation, the reported quantities of protein obtained from exosomes have generally been no higher than 40 $\mu\text{g}/10^8$ cells^[20]. In contrast, our approach, which involves the use of a new type of nanomaterial to isolate and extract exosomes, yielded protein quantities of up to 780 $\mu\text{g}/10^8$ cells from exosomes secreted by hepatoma cells. In recent years, it has been reported that an immunomagnetic method can be used to purify the exosome proteins and RNA. The quality has been greatly improved^[21], but there still are cumbersome and expensive shortcomings. This novel procedure is convenient and efficient, and it involves only a short period during which the samples are exposed in an open system; as a result, the samples are not readily contaminated by environmental or cell debris and can therefore readily fulfill experimental and clinical needs. We first utilized low-speed centrifugation to remove cells and cell debris from cell culture supernatant samples and then used ultrafiltration tubes with a 3 kDa MWCO to remove proteins smaller than 3 kDa and a large quantity of fluid from each sample. Subsequently, the nanomaterial was mixed with this concentrated sample solution, and the resulting mixture was incubated overnight in a refrigerator and centrifuged; following centrifugation, the exosomes precipitated at the bottom of the centrifuge tube. Observations of exosomal morphology using transmission electron microscopy revealed that the isolated and purified exosomes were circular discs with diameters of 30 to 100 nm, and these findings were consistent with the descriptions of exosomes in the published literature. In addition, the biomarker proteins HSP70 and CD63 were detected in the isolated exosomes by Western blot analysis^[22]. In particular, Western blotting revealed clear bands at the theoretical molecular weights of both proteins, indicating that HSP70 and CD63 are expressed in exosomes and confirming that the bodies extracted using the nanomaterial-based approach were indeed exosomes. These results validate our novel method for rapidly and efficiently isolating and purifying exosomes for exosome-related research.

Annexin A2 and TGM2 are hepatoma-related proteins, and our previous study reported histological and serological findings validating annexin A2 and TGM2 as candidate markers for hepatocellular carcinoma (HCC)^[23]. The results of immunohistochemical examinations of HCC tissues have revealed that annexin A2 is a highly expressed protein in HCC cells. TGM2 is a member of the transglutaminase protein family with Ca^{2+} -dependent transamidation functions. In particular, TGM2 facilitates the crosslinking or polymerization of proteins by crosslinking glutamine residues to the ϵ -amino groups of lysine residues. TGM2 has very diverse functions, including roles in cell differentiation, apoptosis, adhesion, receptor-mediated endocytosis, cell movement, and cell migration. Notably, TGM2 has bidirectional roles in cellular apoptosis, and the specific functions of TGM2 in different

cell types are dependent on various factors, such as the specific apoptotic pathways and types of stimuli that are involved, the subcellular localization of TGM2, and the conversions catalyzed by the various enzymatic activities of TGM2^[24-27]. TGM2 and annexin A2 do not contain signal peptides or transmembrane domains. The extracellular secretion of these proteins is thought to occur through non-classical secretory pathways^[28]. However, these proteins were not detected during the construction of the normal human plasma protein database as part of the Human Plasma Proteome Project (HPPP). Based on the characteristic expression patterns of these proteins and the results indicating that these proteins are potential markers for HCC, it is possible that these proteins could be secreted into the plasma by hepatoma cells. Therefore, Western blot analyses were performed to detect annexin A2 and TGM2 in the isolated and purified exosomes secreted by Huh-7 hepatoma cells. These analyses indicated that exosomes secreted from Huh-7 cells contained TGM2 but not annexin A2. These findings confirmed our hypothesis that TGM2 could be secreted by hepatoma cells and that exosomes constitute one method of TGM2 transport. In contrast, annexin A2 may be extracellularly secreted through other non-classical secretory pathways. Furthermore, an exosome database (ExoCarta) was searched to determine the sources of exosome samples in which annexin A2 and TGM2 had been detected^[22]. This search revealed that these proteins had not previously been detected in exosome samples from human hepatoma cells, and therefore, our experimental results address this gap in previously reported data.

This study experimentally confirmed the feasibility of a novel approach utilizing a nanomaterial to rapidly and efficiently isolate and purify exosomes. In addition, annexin A2 and TGM2, which are candidate markers for HCC, were detected in exosomes secreted by hepatoma cells. The extracellular secretion of TGM2 must occur through non-classical secretory pathways because this protein possesses neither a signal peptide nor a transmembrane domain, and our results revealed that one such non-classical pathway for TGM2 secretion is the transport of this protein in exosomes. Together, these findings provide necessary theoretical foundations for future exosome-related research.

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COMMENTS

Background

Exosomes are small membranous vesicles with diameters of between 30 and 100 nm. They are released from different cell types under both normal and pathological conditions. Exosomes have been reported in many types of cul-

tured cells and body fluids. MicroRNAs (miRNAs), messenger RNAs (mRNAs), and cytoplasmic proteins are contained within exosomes, and exosomes have diverse biological functions including immunomodulatory activity, intercellular communication abilities, and the transport of infectious cargo.

Research frontiers

The molecular composition of exosomes extracted from the cell culture medium from diverse cell types and various body fluids has been analyzed by proteomics. Proteomic studies with large data sets might contribute to the understanding of the biological functions of exosomes, such as the mechanism of regulating tumor development. Scientists try to develop exosomes as tools to target tumor cells. Exosomal proteins, as potential biomarkers, may be studied more frequently in the future.

Innovations and breakthroughs

Exosomes are membrane vesicles that are 40-100 nm in diameter and are of endocytic origin. They are released by most cell types upon the fusion of multi-vesicular bodies with the plasma membrane, presumably as a vehicle for intercellular communication. To better understand the biological role of exosomes, it is important to work with highly purified materials. However, the extraction assay of exosomes is normally complicated and time consuming. Here, the authors describe a novel assay based on a nanomaterial to extract exosomes more quickly and efficiently compared with the traditional method. A hepatoma-associated protein, tissue transglutaminase 2 (TGM2), was found to exist in the exosomes of the human liver cancer cell line Huh-7 for the first time. This protein can be secreted through an exosome-mediated non-classical secretion pathway as a potential tumor marker.

Applications

Improving conventional methods for purifying exosomes might contribute to studying exosome biological function, and an analysis of exosomal proteins may provide insights into the clinical monitoring of cancer patients.

Terminology

Exosomes are small membrane vesicles of endocytic origin secreted by various cell types, and they are thought to play important roles in intercellular communication. Cancer cell-derived exosomes play a role in immune escape, neovascularization, and metastasis. Although exosomes were originally described in 1983, interest in these vesicles has increased dramatically in recent years after the finding that they contain mRNAs and miRNAs.

Peer review

The authors describe a new extraction method for exosomes from hepatocellular carcinoma cells and show that the protein TGM2 can be secreted through an exosome-mediated non-classical secretion pathway. The methodology and the data reported are of great interest.

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Prognostic factors in patients with middle and distal bile duct cancers

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Author contributions: Kwon HJ is the first author and composed the majority of the manuscript; Chun JM prepared the literature review; Hwang YJ designed and coordinated the study and helped to draft the manuscript; Lee WK a statistician and analyzed the data; and Kim SG is the corresponding author and performed the surgery in addition to initiating and guiding the program of identifying the prognostic factors of extrahepatic bile duct cancer; all authors have read and approved the final manuscript.

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Abstract

AIM: To identify the influence of the surgery type and prognostic factors in middle and distal bile duct cancers.

METHODS: Between August 1990 and June 2011, data regarding the clinicopathological factors of 194 patients with surgical and pathological confirmation were collected. A total of 133 patients underwent resections (R0, R1, R2; $n = 102, 24, 7$), whereas 61 patients underwent nonresectional surgery. Either pancreaticoduodenectomy (PD) or bile duct resection (BDR) was selected according to the sites of tumors and comorbidities of the patients after confirming resection

margin by the frozen histology in all cases. Univariate and multivariate analyses of clinicopathologic factors were performed, utilizing the Kaplan-Meier method and Cox hazard regression analysis.

RESULTS: The overall 5-year survival rate for the 133 patients who underwent resection (R0, R1, and R2) was 41.2%, whereas no patients survived longer than 3 years among the 61 patient who underwent non-resectional surgeries. The 5-year survival rate of the patients who underwent a PD ($n = 90$) was higher than the rate of those who underwent BDR ($n = 43$), although the difference was not statistically significant (46.6% vs 30.0% $P = 0.105$). However, PD had a higher rate of R0 resection than BDR (90.0% vs 48.8%, $P < 0.0001$). If R0 resection was achieved, PD and BDR showed similar survival rates (49.4% vs 46.5% $P = 0.762$). The 5-year survival rates of R0 and R1 resections were not significantly different (49.0% vs 21.0% $P = 0.132$), but R2 resections had lower survival (0%, $P = 0.0001$). Although positive lymph node, presence of perineural invasion, presence of lymphovascular invasion (LVI), 7th AJCC-UICC tumor node metastasis (TNM) stage, and involvement of resection margin were significant prognostic factors in univariate analysis, multivariate analysis identified only TNM stage and LVI as independent prognostic factors.

CONCLUSION: PD had a greater likelihood of curative resection and R1 resection might have some positive impact. The TNM stage and LVI were independent prognostic factors.

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Key words: Distal extrahepatic bile duct cancer; Lymphovascular invasion; Tumor node metastasis; Pancreaticoduodenectomy; Bile duct resection; Prognostic factor

Core tip: The prognosis in bile duct cancer is unfavor-

able and varies according to the type of surgery, curability, and pathological factors. We analyzed data collected over a period of 22 years that provide valuable information regarding the prognosis. We show that pancreaticoduodenectomy (PD) has a higher chance of curative resection and suggest that BDR should be applied only to tumors located around the cystic duct or in patients with comorbidity precluding PD. Tumor node metastasis stage and lymphovascular invasion are independent prognostic factors. We believe this study to be of great value for the physician and surgeon treating patients with these rare tumors.

Kwon HJ, Kim SG, Chun JM, Lee WK, Hwang YJ. Prognostic factors in patients with middle and distal bile duct cancers. *World J Gastroenterol* 2014; 20(21): 6658-6665 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6658.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6658>

INTRODUCTION

Cholangiocarcinoma is a rare malignant tumor of the biliary tree that can arise anywhere from the intrahepatic to the extrahepatic bile duct just proximal to the duodenal ampulla, excluding the gallbladder. Extrahepatic bile duct cancer, defined as the presence of malignant tumors arising at the biliary tree distal to second-order branches, accounts for 80% to 90% of all cholangiocarcinomas. Extrahepatic bile duct cancer can be further divided into hilar or middle/distal bile duct cancers. Among these extrahepatic bile duct cancers, middle and distal bile duct cancers comprise approximately 20% to 30%^[1-4].

The site of an extrahepatic bile duct cancer has clinical importance because it affects the selection of the appropriate type of surgical resection and the outcomes after surgery. Compared to hilar bile duct cancer, which requires concomitant bile duct and liver resection, the surgical resection for a middle and distal bile duct cancer requires either segmental bile duct resection or pancreaticoduodenectomy with lymph node dissection. The type of surgery selected depends on the possibility of achieving tumor-free resection margins, and the prognostic influence of the type of resection on long-term prognosis has been controversial^[5,6].

In some patients, the superficial spreading nature of the disease, as well as comorbid diseases, can make it hard to achieve R0 resection, inevitably resulting in R1 resection. Because such cases have been anecdotal, the effect of R1 resection on long-term prognoses must be investigated.

Factors such as tumor node metastasis (TNM) stage, perineural invasion, and lymphovascular invasion have been reported to affect survival, but their influences on prognosis have not always been universal. Tumors with higher T stages tend to run a greater risk of distant metastasis and poorer prognosis, but in some cases, tumors are confined to the bile duct without lymph node metastasis, showing early recurrence in distant areas, such as the

liver, lungs, or multiple bones. The unpredictable prognoses of such cases might be explained by bile duct cancer progressing not only by directly invading into the depths of the bile duct wall but also into the perineural, vascular, and lymphatic spaces^[7]. Nagahashi *et al.*^[8] recently insisted that the presence of lymphovascular invasion resulted in poor prognosis, comparing a pT1 tumor invading the fibromuscular layer to a pT1 tumor confined to the mucosa. The role of such factors as perineural invasion (PNI) and low viscosity index (LVI) must be investigated.

In this study, we analyzed the clinicopathological data of 133 patients who underwent surgical resection for middle and distal bile duct cancers, to identify the influence of the type of surgery selected and of clinicopathological factors on the long-term prognoses of the patients.

MATERIALS AND METHODS

Patient population and diagnosis of middle and distal bile duct carcinoma

Between August 1990 and June 2011, a total of 194 patients with middle and distal bile duct cancers underwent surgery and were pathologically diagnosed in the Department of Surgery, Kyungpook National University Hospital, Daegu, South Korea. Data regarding the clinicopathological factors of the patients were obtained by retrospective review of medical records.

The preoperative diagnosis of middle and distal bile duct cancer was made by imaging studies, including an abdominal computed tomography scan, magnetic resonance imaging, and positron emission tomography. Preoperative endoscopic retrograde cholangiopancreatography was performed to decompress jaundice and to obtain tissue diagnosis whenever possible. The sites of tumors were determined by imaging studies. Middle and distal bile duct cancer was defined by imaging studies and by the operative findings of tumors with the main lesions located at middle and distal third of extrahepatic bile duct. Tumors that were grossly identified to extend toward the hilar bifurcation during surgery were excluded from this study.

Definition of margin status and selection of type of surgery

In all of the cases, the resection margins were sent for frozen biopsy. Resection of the remnant tissue with microscopic involvement of carcinoma in situ or with invasive carcinoma was defined as R1 resection, and resection of remnant tissue with gross involvement was defined as R2 resection.

Pancreaticoduodenectomy was performed for tumors located in the distal third of the extrahepatic bile duct. When proximal resection margins were microscopically negative, we proceeded to perform PD. If positive, the proximal bile duct was repeatedly resected until a negative margin was achieved. If a negative margin could not be achieved from the uppermost bile duct, we tried to perform an R1 or R2 PD in selected cases.

Either PD or BDR was performed for tumors in the middle third of extrahepatic bile duct. When the proximal resection margin was microscopically negative, we proceeded to resect the bile duct, including the tumor. If the lowest distal resection margin was negative after BDR, no additional resection was performed; however, if the lowest resection margin was positive, PD was added unless the patient had a serious comorbidity precluding an additional PD. When the proximal resection margin was microscopically positive, the proximal bile duct was repeatedly resected until a negative margin was achieved. If a negative margin could not be achieved from the uppermost bile duct, we performed R1 or R2 BDR. Lymph node dissection was routinely performed around the hepatoduodenal ligament, common hepatic artery, and retropancreas when resection was possible.

Nonresectional surgeries, including exploration only, simple cholecystectomy, and bypass surgery, were indicated for patients with very advanced bile duct carcinoma. These cases included tumors directly invading the common hepatic artery, superior mesenteric artery, inferior vena cava, long segment, or more than half the circumference of the portal vein, as well as tumors with peritoneal or liver metastases.

Patient follow-up after surgery

Follow-up examinations were performed based on abdominal ultrasonography, computed tomography, and measurement of the serum carcinoembryonic antigen and carbohydrate antigen 19-9 levels every 3 to 6 mo. None of the patients received chemotherapy before or after surgery. Information on long-term outcomes after surgery was collected by personal interview or on the telephone. If a patient died, we recorded the survival time after surgery and the cause of death. For surviving patients, the postoperative length of survival and status of recurrence were recorded.

Statistical analysis

A prognostic analysis was performed using the data from 133 patients who underwent resectional surgery including R0, R1 and R2 resection. For the survival analysis after surgical resection, the patients with non-resectional surgery or mortality cases were excluded from this study. The survival data were processed using the Kaplan-Meier method and were compared using the log-rank test. A *P* value less than 0.05 was considered statistically significant. Multivariate analysis was performed using the clinicopathologic factors that were statistically significant in univariate analysis or other marginal predictors, which were obtained using Cox proportional hazards regression.

RESULTS

Clinicopathological features and types of surgery of patients who underwent surgery

In total, the study enrolled 120 men and 74 women, and

the mean age was 66.4 ± 8.6 years old. Among these 194 patients, 133 patients received resection, and the resection rate was 68.6%. Ninety patients (67.7%, 90/133) underwent PD, and 43 (32.3%) patients underwent BDR. The remaining 61 patients underwent nonresectional surgeries, such as bypass, cholecystectomy, or exploration only.

The numbers of patients with R0, R1, and R2 resections were 102, 24, and seven, respectively. The PD group had a higher rate of R0 resection than the BDR group [90% (81/90) *vs* 48.8% (21/43) *P* < 0.0001]. In detail, the PD group included eight patients with R1 and one patient with R2 PD, whereas the patients who underwent BDR included 16 patients with R1 and six patients with R2 BDR. The reason for R1 PD and R1 BDR was a microscopic positive margin at the uppermost resection margin in eight and 12 patients, respectively. The remaining four patients with R1 BDR had positive margins at their lowermost resection margins, but the comorbidity of the patients precluded an additional PD. In one patient with portal vein invasion, a combined portal vein wedge resection was performed.

According to the 7th AJCC-UICC classification, the frequencies of T1S, T1, T2, and T3 were 1.6%, 28.1%, 19.5% and 50.8%, respectively. Lymph node dissection was performed routinely whenever R0 or R1 resection was possible. The average number of lymph nodes which were harvested was 10.9 ± 4.5 . Lymph node metastasis was present in 25%, and perineural invasion was present in 41.4%. The rates of perineural invasion for T1, T2 and T3 tumors were 18.4%, 60% and 46.2%, respectively, showing significant associations (*P* = 0.017).

Lymphovascular invasion was present in 25 of 128 (19.5%). The frequencies of lymphovascular invasion in T1, T2, and T3 were 5.3%, 20.0%, and 26.2%, respectively; invasion was associated with T staging at a statistically significant level (*P* = 0.013). Lymphovascular invasion was not significantly associated with nodal metastasis (*P* = 0.071).

Well, moderately, and poorly differentiated cases were found in 33.6%, 55.5% and 10.9% of patients, respectively.

Long-term survival of all patients with resections and univariate analysis of their clinicopathologic factors

The overall 1-, 3-, and 5-year survival rates for the 133 patients who underwent resection (R0, R1, and R2) were 86.8%, 54.4% and 41.2%, respectively, whereas no patients survived longer than 3 years among the 61 who underwent nonresectional surgeries (Figure 1).

To elucidate the factors influencing long-term survival after resection, 10 clinicopathologic factors for 133 patients who underwent R0, R1 and R2 resection were entered into univariate analysis (Table 1). The 5-year survival rates, according to the type of resection, were 46.6% for PD and 30.0% for BDR, although the differences between the two groups did not reach statistical significance (*P* = 0.105). The 5-year survival rates ac-

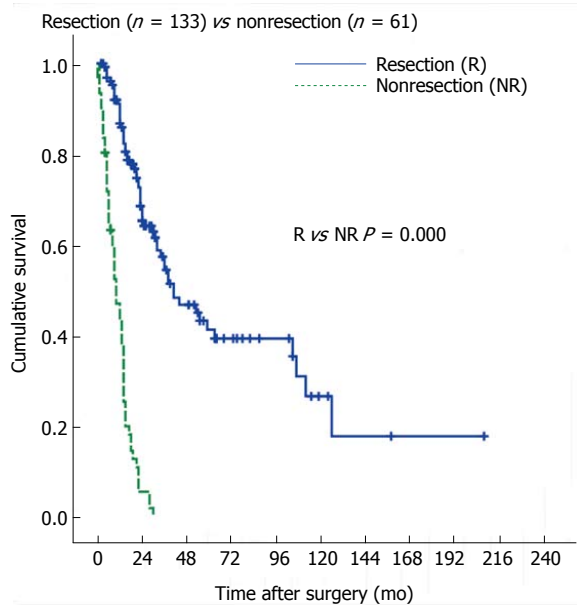


Figure 1 Overall survival of 194 patients who underwent resection ($n = 133$) or nonresectional surgeries ($n = 61$) for middle and distal bile duct cancers.

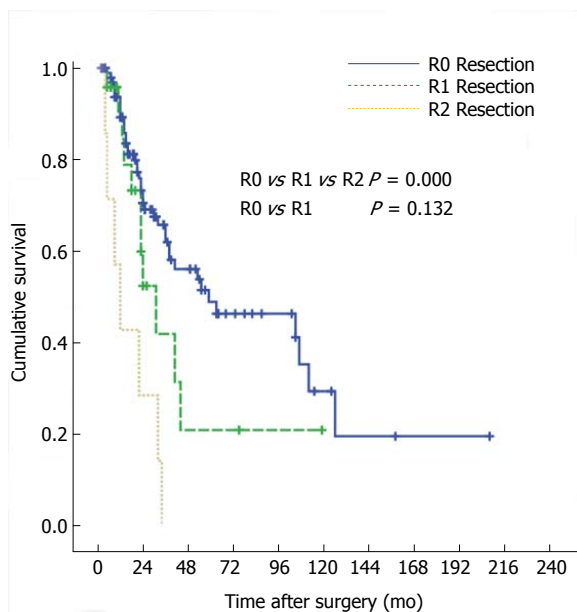


Figure 2 Overall survival of the 133 patients who underwent resection according to the status of their resection margins.

cording to margin status were 49.0% for R0 resections ($n = 102$), 21.0% for R1 resections ($n = 24$), and 0% for R2 resections ($n = 7$) ($P < 0.0001$) (Figure 2). When an R0 resection was achieved, the 5-year survival rates after PD and BDR were similar (49.4% *vs* 46.5%, $P = 0.762$) (Figure 3A).

According to the 7th edition of the AJCC T-staging system, the 5-year survival rates of Tis/T1, T2, and T3 were 52.3%, 56.8% and 34.1%, respectively ($P = 0.144$).

The 5-year survival rate of patients without lymph node metastasis was higher than that of patients with

Table 1 Univariate analysis of survival, according to the clinicopathological factors of patients who underwent surgical resection (R0, R1 and R2) for mid-distal extrahepatic bile duct cancer n (%)

| Variables | | Total (<i>n</i> = 133) | | | |
|-------------------|-----------|-------------------------|---------------|--------|----------------|
| | | No. | 5 yr survival | Median | <i>P</i> value |
| Age | | Mean 65.8 | | | |
| | ≤ 70 | 86 (64.7) | 39.1 | 41.0 | 0.917 |
| | > 70 | 47 (35.3) | 44.8 | 38.0 | |
| Sex | M | 81 (60.9) | 33.6 | 36.0 | 0.359 |
| | F | 52 (39.1) | 59.4 | 105.0 | |
| CA19-9 (U/mL) | ≤ 35 | 27 (30) | 57.9 | 85.0 | 0.734 |
| | > 35 | 63 (70) | 44.9 | 37.0 | |
| Type of resection | PD | 90 (67.7) | 47.2 | 59.0 | 0.105 |
| | BDR | 43 (32.3) | 30.0 | 32.0 | |
| T classification | Tis, T1 | 38 (29.7) | 52.3 | 63.0 | 0.144 |
| | T2 | 25 (19.5) | 56.8 | - | |
| | T3 | 65 (50.8) | 34.1 | 36.0 | |
| N classification | N0 | 96 (75.0) | 51.3 | 63.0 | 0.033 |
| | N1 | 32 (25.0) | 17.0 | 30.0 | |
| M classification | M0 | 130 (97.7) | 42.2 | 41.0 | 0.242 |
| | M1 | 3 (2.3) | 0 | 16.0 | |
| TNM stage | 0, I | 51 (39.5) | 64.5 | 105.0 | 0.006 |
| | II | 75 (58.1) | 30.1 | 36.0 | |
| | III | 0 (0) | - | - | |
| | IV | 3 (2.3) | 0 | 16.0 | |
| PNI | - | 75 (58.6) | 55.8 | 105.0 | 0.022 |
| | + | 53 (41.4) | 17.5 | 38.0 | |
| LVI | - | 103 (80.5) | 52.3 | 63.0 | 0.000 |
| | + | 25 (19.5) | 6.7 | 23.0 | |
| Resection margin | R0 | 102 (76.7) | 49.0 | 59.0 | 0.000 |
| | R1 | 24 (18.0) | 21.0 | 31.0 | |
| | R2 | 7 (5.3) | 0 | 12.0 | |
| Differentiation | Papillary | 4 (3.2) | 66.7 | 105.0 | 0.409 |
| | W/D | 40 (32.5) | 48.8 | 59.0 | |
| | M/D | 66 (53.7) | 36.7 | 36.0 | |
| | P/D | 13 (10.6) | 56.1 | 63.0 | |

PD: Pancreatoduodenectomy; BDR: Segmental bile duct resection; W/D: Well differentiated; M/D: Moderately differentiated; P/D: Poorly differentiated; LVI: Lymphovascular invasion; PNI: Perineural invasion.

lymph node metastasis (51.3% *vs* 17.0%, $P = 0.033$).

The five-year survival rates of the patients at TNM stage 0 and 1 were higher than those of patients with TNM stage 2 and 4 respectively (64.5% *vs* 30.1%, and 0%; $P = 0.006$).

The 5-year survival rate for patients with perineural invasions was worse than that for patients without perineural invasion in univariate analysis (17.5% *vs* 55.8%; $P = 0.022$).

Lymphovascular invasion was present in 25 patients (19.5%). The presence of lymphovascular invasion unfavorably affected long-term survival. The 5-year survival rate of the 103 patients without lymphovascular invasion was 52.3%, compared to 6.7% for the 25 patients with lymphovascular invasion.

The pathological grading of differentiation was not associated with prognosis in this study ($P = 0.409$).

Recurrence after surgery occurred in 58.8% (60/102) of the patients with R0 resection during the period of follow up. The most common site of first recurrence was abdominal lymph nodes only (38.3%, periportal, around Superior mesenteric artery, paraaortic) followed

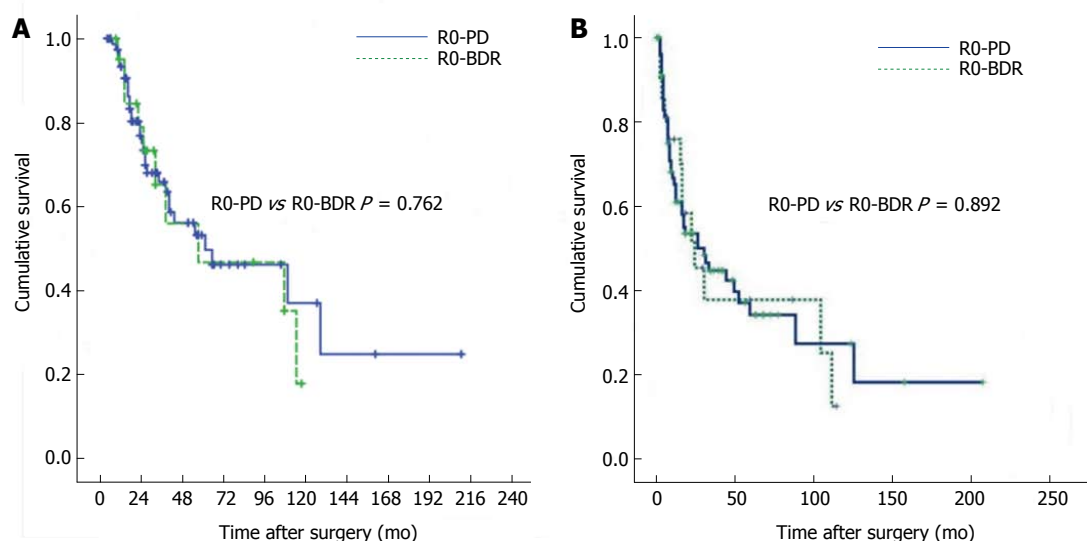


Figure 3 Overall (A) and disease free (B) survival of the patients according to R0-pancreatoduodenectomy and R0-bile duct resection.

Table 2 Multivariate analysis of survival according to the clinicopathological factors of patients who underwent surgical resection (R0, R1 and R2) for middle and distal extrahepatic bile duct cancers

| Variable | | Hazard ratio | 95%CI | P value |
|------------|------|--------------|---------------|---------|
| AJCC stage | 0, I | 1 | | 0.023 |
| | II | 4.572 | 1.473-14.183 | |
| | III | - | - | |
| | IV | 11.185 | 1.061-117.922 | |
| LVI | - | 1 | | 0.002 |
| | + | 2.942 | 1.510-5.733 | |

LVI: Lymphovascular invasion.

by liver only (35%), anastomosis site (8.3%), and lung (1.7%). The abdominal lymph node metastasis was present in combination with local recurrence and liver metastasis at the time of first recurrence in 5% and 1.7% respectively. Lymph node metastases were found in 55.0% of the patients with first recurrence in the pattern of lymph node metastasis only (38.3%) or combination with other organ metastases (16.7%). The long term disease free survival of R0 PD was similar to R0 BDR (Figure 3B).

In summary, univariate analysis revealed that lymph node metastasis ($P = 0.033$), TNM stage ($P = 0.006$), perineural invasion ($P = 0.022$), lymphovascular invasion ($P < 0.0001$), and resection margins ($P < 0.0001$) were significant factors for long-term survival.

Multivariate analysis of clinicopathologic factors

A multivariate analysis was performed using the seven clinicopathologic factors that had been proved to be significant, or at least marginally predictive, in univariate analysis: type of resection; T classification; N classification; TNM stage; perineural invasion; lymphovascular invasion; and resection margin. In multivariate analysis, the TNM stage and lymphovascular invasion were identified

as independent prognostic factors associated with poor survival (Table 2).

Influence of lymphovascular invasion on survival, according to T and N stage

Lymphovascular invasion was present in 25 of 128 cases (19.5%), unfavorably affecting the 5-year survival rate (52.3% *vs* 6.7%, $P < 0.0001$). pT1 tumors without lymphovascular invasion showed significantly better survival, compared to pT1 tumors with lymphovascular invasion (5-year survival rate, 57.40% *vs* 0%; $P < 0.0001$).

The presence of lymphovascular invasion in patients with nodal metastasis did not affect survival, whereas the presence of lymphovascular invasion in patients without nodal metastasis strongly affected the 5-year survival rate (0% *vs* 60.4%; $P < 0.0001$).

DISCUSSION

Middle and distal bile duct cancers are rare malignancies with poor prognoses, and only complete surgical resection of bile duct cancer offers a chance for long-term survival. The prognosis of middle and distal bile duct cancers remains poor, even with radical resection, due to the high incidence of recurrence. Furthermore, it is difficult to determine the optimal extent of resection and to identify the prognostic factors. Although several prognostic factors, such as surgical radicality, nodal status, depth of invasion, differentiation, perineural invasion, and lymphovascular invasion, have been reported for middle and distal extrahepatic bile duct cancers^[9-13], the prognostic values of these factors have not been consistent. Among the aforementioned factors, nodal metastasis, differentiation, and R0 resection have been widely recognized to be associated with long-term survival^[3,14-17].

In this study, the overall survival was longer in patients with resection, compared to patients without resection. However, the prognosis of patients with R2

resection was much poorer compared that of patients with R0 or R1 resection. As shown in the results, patients with R0 and R1 resections showed 5-year survival rates of 49.0% and 21.0%, respectively, whereas none of the patients with nonresection or R2 resection survived longer than 3 years (medians of 10 mo and 12 mo, respectively).

Many studies have reported that R0 resection is necessary for long-term survival^[5,17-19]. However, it is sometimes very difficult to achieve a tumor-free margin of the bile duct due to superficial microscopic spreading and multifocal tumors. In such cases, R1 resection is inevitable, and some authors have reported that patients with R1 resections have survived longer than expected^[3,5,6,20].

In our study, R1 resection was performed in 24 patients, including eight patients with R1 PD and 16 patients with R1 BDR. Positive uppermost bile duct margins accounted for the 20 patients with R1 resections (8 PD and 12 BDR), and positive lowermost bile duct margins accounted for the four patients with R1 BDR resections, for whom the addition of further PD was precluded by the presence of co-morbidities. The 5-year survival rates after R0-resection were higher than those after R1-resection, but the difference did not reach statistical significance (49.0% *vs* 21.0%; $P = 0.132$) (Lymphovascular. 2). Thus, it can be assumed that R1 resection, if inevitable, should be recognized as prolonging survival, according to our data, which reflected that no patients with R2 resections or nonresections survived longer than 36 mo. The definition of R1 resection in this study was a microscopically positive margin of the resected bile duct. The major drawbacks of this study were that the pathological description was not made by a single pathologist and that the invasiveness of the positive margin was not subclassified into carcinoma in situ or invasive carcinoma. These means of analysis will be further investigated in the near future.

According to the primary site and the extent of the tumor, different surgical procedures can be applied. PD is most commonly performed for tumors because bile duct resection is sometimes not sufficient to achieve adequate surgical margins, compared to PD^[5,6]. In our study as well, the microscopic involvement of margins was more frequent after bile duct resection, compared to PD [37.2% (16/43) *vs* 8.9% (8/90); $P < 0.0001$]. The 5-year survival rates of patients with PD and BDR were 46.6% and 30.0% ($P = 0.105$), respectively; this difference, although not statistically significant, can be attributed to a higher rate of positive resection margins in patients with bile duct resection. The lack of statistical significance in the difference between the two groups might have been due to the limited number of patients. However, if R0 resection had been achieved, the 5-year survival rates after bile duct resection would have been similar after PD (46.5% *vs* 49.4%; $P = 0.762$) (Figure 3A). Thus, it is possible that PD offers a greater likelihood of complete resection and, consequently, better survival. Therefore, PD is recommended unless patient co-morbidities pre-

clude its implementation. BDR is an option only if the co-morbidities of patients preclude PD or if an R0 resection seems to be achieved for tumors limited to the area surrounding the cystic duct.

The frequency of lymph node metastasis has been reported to range from 23.8% to 68%, and lymph node involvement has been determined to be an important predictor of survival in patients with middle and distal bile duct cancers^[3,6,16,19,21,22]. In our study, the frequency of nodal metastasis was 25%, and patients with nodal metastasis had worse survival than patients without nodal metastasis in univariate analysis (5-year survival rate 17.0% *vs* 51.3%; $P = 0.033$).

Perineural invasion is one of the pathways through which local infiltrations spread and metastasize. Although perineural invasion has recently been accepted as a prognostic factor in a number of different malignancies, its clinical significance for mid-distal extrahepatic cholangiocarcinoma remains unclear. According to Bhuiya *et al*^[7], perineural invasion had a profound impact on the survival of patients with extrahepatic bile duct cancer. The 5-year survival rate for patients with perineural invasion was 32%, compared to 67% for patients without invasion. A distinct and significant correlation was potentially reported between the depth of tumor invasion and perineural invasion. In our study as well, the frequency of perineural invasion was 41.4%, and the rates of perineural invasion for T1, T2 and T3 tumors were 18.4%, 60% and 46.2%, respectively. Thus, a significant association with depth of invasion was shown ($P = 0.017$). The survival of patients with perineural invasion was worse than that of patients without perineural invasion in univariate analysis (5-year survival rate 17.5% *vs* 55.8%; $P = 0.022$).

Lymphovascular channel invasion is one of many different manners by which tumors spread. The clinical significance of LVI was first described as far back as 1967, when studies reported higher recurrence and poorer survival in cervical cancer patients with lymphovascular invasion^[23]. Nevertheless, few reports have been published regarding the significance of lymphovascular invasion, and its clinical significance has not been established in middle and distal bile duct cancers^[8,24]. Our study showed that the presence of lymphovascular invasion was an independent prognostic factor and unfavorably influenced long-term survival. Lymphovascular invasion was present in 19.5% of patients, and the overall 5-year survival rates of these patients with lymphovascular invasion were poor, compared to the rates of patients without lymphovascular invasion (6.7% *vs* 52.3%, $P < 0.0001$).

Additionally, lymphovascular invasion was significantly associated with the depth of invasion (T classification) ($P = 0.013$). The frequency of lymphovascular invasion, according to the depth of the invasion (T1, T2 and T3), was 5.3%, 20% and 26.2%, respectively. Although pathologic T1 (pT1) tumors generally have favorable prognoses after resection, the presence of lympho-

vascular invasion, even in pT1 tumors, affected survival unfavorably, compared to pT1 tumors without lymphovascular invasion (5-year survival rate, 0% *vs* 57.4%; $P < 0.0001$). Our results were similar to those from a study published by Nagahashi *et al.*^[8], who reported poorer prognoses in patients with invasion into the fibromuscular layer, compared to those with mucosal layer invasion. These authors insisted that the cause of these adverse effects was related to lymphovascular invasion. Contrary to our expectations, the present study did not find that lymphovascular invasion was strongly associated with nodal metastasis ($P = 0.071$). This lack of association has also been reported in other studies examining nodal status and lymphovascular invasion in breast cancer^[25,26]. A direct correlation between the presence of lymphovascular invasion and nodal metastasis might not always be apparent. The presence of lymphovascular invasion in patients with nodal metastasis did not affect survival, but in patients without nodal metastasis, the presence of lymphovascular invasion affected 5-year survival (0% *vs* 60.4%; $P < 0.0001$). This result suggests that lymphovascular invasion has a profound impact on the survival of patients with lymph node-negative middle and distal extrahepatic bile duct cancers.

Pancreaticoduodenectomy had higher rate of R0 resection than bile duct resection, although the long-term survival between the two groups was not significantly different. BDR can be applied when only R0 resection is possible or if the co-morbidity of the patient precludes PD. R1 resection, if inevitable, can be performed because it offers better survival than R2 or no resection. The TNM stage and LVI were identified as independent factors influencing the survival of patients with middle and distal bile duct cancers.

COMMENTS

Background

Cholangiocarcinoma is a rare malignant tumor of the biliary tree that can arise anywhere from the intrahepatic to the extrahepatic bile duct just proximal to the duodenal ampulla, excluding the gallbladder. The prognosis in bile duct cancer is unfavorable and varies according to the type of surgery, curability, and pathological factors.

Innovations and breakthroughs

Authors analyzed the clinicopathological data of 133 patients who underwent surgical resection for middle and distal bile duct cancers, to identify the influence of the type of surgery selected and of clinicopathological factors on the long-term prognoses of the patients.

Peer review

This is a well-designed retrospective study with a quite big sample size. The conclusions are reasonable and credible.

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Toll-like receptor 4 polymorphisms to determine acute pancreatitis susceptibility and severity: A meta-analysis

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Abstract

AIM: To investigate the correlation of toll-like receptor 4 (*TLR4*) gene *Asp299Gly* and *Thr399Ile* polymorphisms and acute pancreatitis (AP) risk and severity.

METHODS: To get a more precise estimation of the relationship, a comprehensive search was performed to examine all the eligible studies of *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms and AP risk. The odds ratios with 95% confidence intervals were used to assess the strength of the association. Publication bias was analyzed by Begg's funnel plots.

RESULTS: In total, six studies with 1255 cases and 998 controls were included in this meta-analysis. Totally, no significant associations were found between

TLR4 Asp299Gly or *Thr399Ile* polymorphisms and AP risk using five models with high homogeneity ($P > 0.05$). Furthermore, stratification analysis by ethnicity or assay also found no significant association in these two polymorphisms ($P > 0.05$), and *TLR4 Asp299Gly* was not associated with AP severity ($P > 0.05$). In addition, no publication bias was found in these studies ($P > 0.05$).

CONCLUSION: Our current meta-analysis suggests that *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms may not be risk factors to AP susceptibility.

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Key words: Toll-like receptor 4; Acute pancreatitis; Risk; Single nucleotide polymorphisms

Core tip: Toll-like receptor 4 (*TLR4*) is one of the central proinflammatory factors in the pathology of acute pancreatitis (AP). Nevertheless, the relationship between *TLR4* polymorphisms and AP susceptibility has been controversial. Here, we performed a systematic meta-analysis of *TLR4* polymorphisms and AP risk, and our data showed that *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms may not be associated with AP susceptibility.

Zhou XJ, Cui Y, Cai LY, Xiang JY, Zhang Y. Toll-like receptor 4 polymorphisms to determine acute pancreatitis susceptibility and severity: A meta-analysis. *World J Gastroenterol* 2014; 20(21): 6666-6670 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6666.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6666>

INTRODUCTION

Acute pancreatitis (AP) is a potentially lethal disease with a mortality rate ranging from 10% to 25% depending on the infectious status of the disease^[1]. Therefore, AP is

one of the major problems encountered by many clinical specialists. Clinically, AP is divided into two groups by the disease severity: mild AP (MAP) and severe AP (SAP). MAP is a self-limited disease while SAP has a fast malignant progression, even resulting in multiple organ failure and death^[2]. However, the molecular mechanisms that explain why some people suffer from SAP and others have MAP, remains largely unknown up to now.

Recently more and more solid evidence has demonstrated that the involvement of the immune system and, largely, release of multiple proinflammatory factors have played a fundamental role in the pathogenesis of AP. Toll-like receptor 4 (TLR4) is one of these key factors in the inflammatory process of AP disease. It has been reported that there are two common single nucleotide polymorphisms (SNPs) exist in the coding region of TLR4: *Asp299Gly* and *Thr399Ile*. Many studies have investigated the relationship between these two TLR4 polymorphisms and AP risk. Nevertheless, the conclusions are still controversial. Therefore, we conducted a systematic meta-analysis of current data to clarify the association of TLR4 *Asp299Gly* and *Thr399Ile* polymorphisms and AP susceptibility.

MATERIALS AND METHODS

Databases search

A literature search was conducted to looking for eligible studies that explored the association between TLR4 polymorphisms and AP risk using Pubmed, Embase, Web of Science, CBM (China Biological Medicine Database) on September 27, 2013 with combinations of the following key words including (“toll like receptor” or “TLR”) and (“polymorphism” or “genotype” or “variant” or “mutation”) and “pancreatitis”. There was no language restriction in the literature search. All reference lists from relevant studies and reviews were hand searched for additional eligible studies. The studies with the latest sample size were included when there were republished studies.

Eligible studies and data extraction

Eligible studies had to meet all of the following criteria: (1) evaluating the association of TLR4 *Asp299Gly* or *Thr399Ile* polymorphisms and AP risk; (2) a case-control study; (3) it is of Hardy Weinberg equilibrium (HWE) in the control group; and (4) sufficient genotyping information to evaluating an odds ratio (OR) and 95% confidence interval (CI).

The following information of each study was extracted independently by two reviewers: the name of first author, year of publication, country, ethnicity, genotypes distribution in both AP and controls, *P* values for HWE evaluation, source of controls, sample size (case/control), and genotyping methods.

Statistical analysis

The pooled OR with its 95%CI was calculated to evaluate the strength of association between TLR4 polymor-

phisms and AP susceptibility in five different genetic models, and the Z test was used to determine the significance of the pooled OR. Cochran's χ^2 -based *Q* statistic test was performed to assess possible heterogeneity between the individual studies^[3]. The fixed-effects model was applied to calculate the pooled OR with its 95%CI when there was no obvious between-study heterogeneity, otherwise, the random-effects model was used^[4,5]. In the case of zero cells, an appropriate continuity correction (addition of 0.5) was implemented^[6]. Publication bias analysis was performed by the funnel plot and Egger's test^[4]. All *P* values are two-sided, and *P* < 0.05 was considered statistically significant. Statistical analyses were done with Stata software (version 12.0).

RESULTS

Characteristics of studies

We collected 24 studies after database searches. After evaluation of title and abstract for the association of TLR4 polymorphisms and AP susceptibility, nine relevant studies were identified and retrieved for further investigation. Finally, six studies were identified according to the selection criteria. A total of 6 studies^[7-12] with 1255 cases and 998 controls were included in this meta-analysis. In these studies, six studies^[7-12] with 1255 cases and 998 controls were about the association of TLR4 *Asp299Gly* polymorphism and AP risk; three studies^[7,8,11] with 815 cases and 744 controls were about the association of TLR4 *Thr399Ile* polymorphism and AP risk. Among these studies, five studies were published in English^[7-11], and one study was in Chinese^[12]. There were two studies of subjects of Caucasian descent^[7,11], and four studies of subjects of Asian descent^[8-10,12]. A classic polymerase chain reaction restriction fragment length polymorphism assay (PCR-RFLP) was used in four studies^[8-10,12], a Taqman assay was conducted in two studies^[7,11]. Table 1 listed the main characteristics of these six studies for two SNPs of TLR4 and AP. All five studies were consistent with HWE in the controls except for one study^[11] (Table 1). Moreover, TLR4 *Asp299Gly* polymorphism and the severity of AP susceptibility from 4 studies^[7,9,11,12] are also summarized in Table 2.

Meta-analysis of TLR4 *Asp299Gly* polymorphisms and AP susceptibility

When those six studies were included in the meta-analysis, there was no obvious heterogeneity between the individual studies using five genetic models (*P* > 0.05). In overall analysis, TLR4 *Asp299Gly* polymorphism was not associated with AP risk when all studies were pooled into the meta-analysis using five genetic models (for A *vs* G: OR = 1.022, 95%CI: 0.748-1.397, *P* = 0.891; for AA *vs* GG: OR = 1.537, 95%CI: 0.466-5.075, *P* = 0.480; for AG *vs* GG: OR = 1.828, 95%CI: 0.454-7.368, *P* = 0.396; for AA + AG *vs* GG: OR = 1.576, 95%CI: 0.477-5.205, *P* = 0.456; for AA *vs* AG + GG: OR = 0.764, 95%CI: 0.458-1.277, *P* = 0.305, Table 3, Figure 1). Moreover, the

Table 1 Studies included in the meta-analysis

| SNP | Ref. | Year | Country | Ethnicity | AP | | | Control | | | P value for HWE | Source of controls | AP | Control | Assay |
|------------------|--------------------------------------|------|------------------------|-----------|------------|----|----|------------|----|----|-----------------|--------------------|-----|---------|----------|
| | | | | | AA | AB | BB | AA | AB | BB | | | | | |
| <i>Asp299Gly</i> | Hofner <i>et al</i> ^[11] | 2006 | Hungary | Caucasian | 84 | 7 | 1 | 64 | 7 | 2 | 0.01 | PB | 92 | 73 | Taqman |
| | Gao <i>et al</i> ^[10] | 2007 | China | Asian | 101 | 22 | 0 | 71 | 9 | 0 | 0.59 | PB | 123 | 80 | PCR-RFLP |
| | Zhang <i>et al</i> ^[9] | 2008 | China | Asian | 238 | 0 | 0 | 121 | 0 | 0 | 1.00 | PB | 238 | 121 | PCR-RFLP |
| | Takagi <i>et al</i> ^[8] | 2009 | Japan | Asian | 202 | 0 | 0 | 286 | 0 | 0 | 1.00 | PB | 202 | 286 | PCR-RFLP |
| | Chen <i>et al</i> ^[12] | 2009 | China | Asian | 64 | 15 | 0 | 47 | 6 | 0 | 0.76 | PB | 79 | 53 | PCR-RFLP |
| | Guenther <i>et al</i> ^[7] | 2010 | Germany, United States | Caucasian | A 991 G 51 | | | A 725 G 45 | | | | PB | 521 | 385 | Taqman |
| <i>Thr399Ile</i> | Hofner <i>et al</i> ^[11] | 2006 | Hungary | Caucasian | 85 | 6 | 1 | 64 | 7 | 2 | 0.01 | PB | 92 | 73 | Taqman |
| | Takagi <i>et al</i> ^[8] | 2009 | Japan | Asian | 202 | 0 | 0 | 286 | 0 | 0 | 1.00 | PB | 202 | 286 | PCR-RFLP |
| | Guenther <i>et al</i> ^[7] | 2010 | Germany, United States | Caucasian | C 977 T 49 | | | C 728 T 36 | | | | PB | 521 | 385 | Taqman |

SNP: Single nucleotide polymorphisms; AP: Acute pancreatitis; PB: Population-based study; HWE: Hardy-Weinberg equilibrium in controls; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

Table 2 Studies of *TLR4 Asp299Gly* polymorphism and severity of acute pancreatitis

| Ref. | Year | Country | Ethnicity | Assay | MAP | | | SAP | | |
|--------------------------------------|------|------------------------|-----------|----------|------------|----|----|------------|----|----|
| | | | | | AA | AG | GG | AA | AG | GG |
| Hofner <i>et al</i> ^[11] | 2006 | Hungary | Caucasian | Taqman | 41 | 1 | 0 | 43 | 6 | 1 |
| Zhang <i>et al</i> ^[9] | 2008 | China | Asian | PCR-RFLP | 104 | 2 | 0 | 128 | 4 | 0 |
| Chen <i>et al</i> ^[12] | 2009 | China | Asian | PCR-RFLP | 32 | 8 | 0 | 32 | 7 | 0 |
| Guenther <i>et al</i> ^[7] | 2010 | Germany, United States | Caucasian | Taqman | A 587 G 33 | | | A 404 G 18 | | |

PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

five studies possessed highly homogeneity in all four genetic models (Pheterogeneity > 0.05, Table 3, Figure 1). Additionally, the further subgroup analysis by ethnicity or test assay showed that *TLR4 Asp299Gly* polymorphism was not a risk for in Asian or Caucasian populations or using PCR-RFLP assay or Taqman assay ($P > 0.05$, Table 3) with highly homogeneity.

Considering the two different types of AP (MAP and SAP), we then assessed the association of *TLR4 Asp299Gly* polymorphism and two different types of AP risk using an allele genetic model. Our meta-analysis showed that the *TLR4 Asp299Gly* polymorphism has no association with MAP risk or SAP risk or the severity of AP using a fixed-effects model ($P > 0.05$, Table 4).

Meta-analysis of *TLR4 Thr399Ile* polymorphisms and AP susceptibility

For *TLR4 Thr399Ile* polymorphism, overall no association was found between *TLR4 Thr399Ile* polymorphism and AP risk using four genetic models (for C *vs* T: OR = 1.090, 95%CI: 0.736-1.614, $P = 0.667$; for CC *vs* TT: OR = 1.523, 95%CI: 0.258-9.012, $P = 0.643$; for CT *vs* TT: OR = 1.455, 95%CI: 0.166-12.756, $P = 0.735$; for CC + CT *vs* TT: OR = 1.494, 95%CI: 0.253-8.844, $P = 0.658$; for CC *vs* CT + TT: OR = 1.530, 95%CI: 0.580-4.041, $P = 0.390$, Table 5) with highly homogeneity.

Publication bias analysis

A funnel plot of these six included studies was symmetrical and didn't suggest a possibility of publication bias (Figure 2). The statistical results from Egger's test still did

not show publication bias for *TLR4 Asp299Gly* polymorphism (for A *vs* G $P_{\text{egger}} = 0.659$; for AA *vs* GG $P_{\text{egger}} = 0.204$; for AG *vs* GG $P_{\text{egger}} = 0.051$; for AA + AG *vs* GG $P_{\text{egger}} = 0.250$; for AA *vs* AG + GG $P_{\text{egger}} = 0.594$) in all five genetic models (Figure 2).

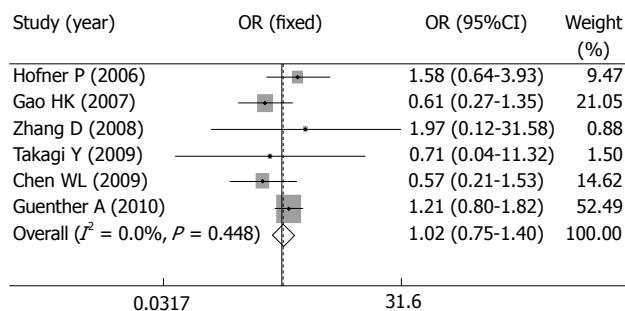
DISCUSSION

The early phase of severe AP progression is commonly accompanied by activation of monocytes, polymorphonuclear granulocytes and macrophages, and the activated monocytes are the index of AP severity. Many factors and multiple pathways participated in the regulation of innate immune response of AP. Toll-like receptors (TLRs) can recognize pathogen-associated molecular patterns and protect bodies by initiating inflammatory reactions to destroy the invaders, thus playing pivotal roles in immune regulation. TLR4 has been extensively explored in inflammatory reactions and immune responses among the TLR family. TLR4 is commonly secreted by immune cells and can bind to its receptor Gram-negative bacterial lipopolysaccharide (LPS) as well as to a series of diverse ligands, such as heat-shock proteins, in both exogenous and endogenous situations^[13,14]. It has been reported that about 29 SNPs have been found in the TLR4 gene till now^[15]. Among these SNPs, *Asp299Gly* and *Thr399Ile* are the most common mutations in TLR4 gene. *Asp299Gly* is an A to G conversion which results in the replacement of Asp by Gly, while *Thr399Ile* is a C to T conversion which results in the replacement of Thr by Ile. Mutant Gly299 and Ile399 change the fourth exon structure of TLR4

Table 3 Meta-analysis of *TLR4 Asp299Gly* polymorphism and acute pancreatitis risk

| | Test of association | | | Model | Test of heterogeneity | |
|-------------|---------------------|--------------|---------|-------|-----------------------|--------------------|
| | OR | 95%CI | P value | | P value | I ² (%) |
| Total | | | | | | |
| A vs G | 1.022 | 0.748-1.397 | 0.891 | F | 0.446 | 0.000 |
| AA vs GG | 1.537 | 0.466-5.075 | 0.480 | F | 0.971 | 0.000 |
| AG vs GG | 1.828 | 0.454-7.368 | 0.396 | F | 0.992 | 0.000 |
| AA vs AG/GG | 1.576 | 0.477-5.205 | 0.456 | F | 0.973 | 0.000 |
| AA/AG vs GG | 0.764 | 0.458-1.277 | 0.305 | F | 0.570 | 0.000 |
| Asian | | | | | | |
| A vs G | 0.628 | 0.349-1.133 | 0.122 | F | 0.874 | 0.000 |
| AA vs GG | 1.273 | 0.316-5.126 | 0.734 | F | 0.965 | 0.000 |
| AG vs GG | 1.760 | 0.338-9.153 | 0.502 | F | 0.966 | 0.000 |
| AA vs AG/GG | 1.326 | 0.330-5.339 | 0.691 | F | 0.961 | 0.000 |
| AA/AG vs GG | 0.606 | 0.330-1.115 | 0.107 | F | 0.861 | 0.000 |
| Caucasian | | | | | | |
| A vs G | 1.264 | 0.869-1.839 | 0.221 | F | 0.592 | 0.000 |
| AA vs GG | 2.625 | 0.233-29.591 | 0.435 | F | - | - |
| AG vs GG | 2.000 | 0.146-27.447 | 0.604 | F | - | - |
| AA vs AG/GG | 2.563 | 0.228-28.840 | 0.446 | F | - | - |
| AA/AG vs GG | 1.477 | 0.540-4.039 | 0.448 | F | - | - |
| PCR-RFLP | | | | | | |
| A vs G | 0.628 | 0.349-1.133 | 0.122 | F | 0.874 | 0.000 |
| AA vs GG | 1.273 | 0.316-5.126 | 0.734 | F | 0.965 | 0.000 |
| AG vs GG | 1.760 | 0.338-9.153 | 0.502 | F | 0.966 | 0.000 |
| AA vs AG/GG | 1.326 | 0.330-5.339 | 0.691 | F | 0.961 | 0.000 |
| AA/AG vs GG | 0.606 | 0.330-1.115 | 0.107 | F | 0.861 | 0.000 |
| Taqman | | | | | | |
| A vs G | 1.264 | 0.869-1.839 | 0.221 | F | 0.592 | 0.000 |
| AA vs GG | 2.625 | 0.233-29.591 | 0.435 | F | - | - |
| AG vs GG | 2.000 | 0.146-27.447 | 0.604 | F | - | - |
| AA vs AG/GG | 2.563 | 0.228-28.840 | 0.446 | F | - | - |
| AA/AG vs GG | 1.477 | 0.540-4.039 | 0.448 | F | - | - |

PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; F: Fixed-effects mode.

**Figure 1** Forest plot of *TLR4 Asp299Gly* polymorphism and acute pancreatitis risk for A vs G genotype.

protein, thus affecting the binding sites of ligands and bringing about interruption of TLR4 to LPS pathway^[16].

In the past decade, accumulated studies demonstrated that bacterial infection in the necrotic tissues of pancreas is the major reason for death from SAP, and the chief pathogenic bacteria of infection in necrotic tissues are Gram-negative bacteria^[2,17]. Many studies showed that Asp299Gly mutation of the TLR4 gene has changed the susceptibility of hosts to Gram-negative bacteria and turnover of individual bacterial infection^[18-20]. The recent

Table 4 Meta-analysis of *TLR4 Asp299Gly* polymorphism and severity of acute pancreatitis (A vs G)

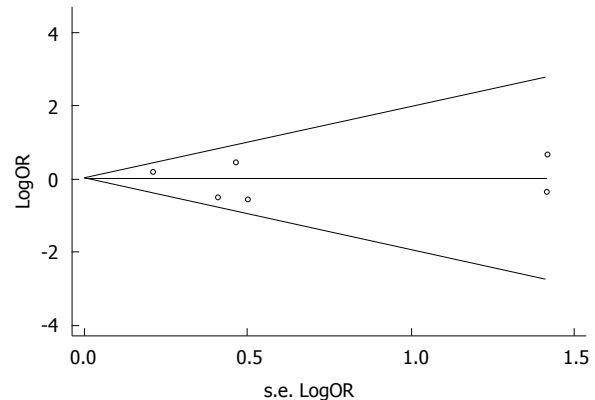
| Comparison | Test of association | | | Model | Test of heterogeneity | | Test of publication bias |
|----------------|---------------------|-------------|---------|-------|-----------------------|--------------------|--------------------------|
| | OR | 95%CI | P value | | P value | I ² (%) | |
| MAP vs SAP | 1.040 | 0.658-1.643 | 0.866 | F | 0.219 | 32.300 | 0.124 |
| MAP vs Control | 1.112 | 0.747-1.655 | 0.601 | F | 0.159 | 42.000 | 0.955 |
| SAP vs Control | 1.054 | 0.691-1.609 | 0.807 | F | 0.334 | 11.800 | 0.020 |

MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis; F: Fixed-effects model.

Table 5 Meta-analysis of *TLR4 Thr399Ile* polymorphism and acute pancreatitis risk

| Genetic models | Test of association | | | Model | Test of heterogeneity | |
|----------------|---------------------|--------------|---------|-------|-----------------------|--------------------|
| | OR | 95%CI | P value | | P value | I ² (%) |
| C vs T | 1.090 | 0.736-1.614 | 0.667 | F | 0.503 | 0.000 |
| CC vs TT | 1.523 | 0.258-9.012 | 0.643 | F | 0.481 | 0.000 |
| CT vs TT | 1.455 | 0.166-12.756 | 0.735 | F | 0.823 | 0.000 |
| CC vs CT/TT | 1.494 | 0.253-8.844 | 0.658 | F | 0.493 | 0.000 |
| CC/CT vs TT | 1.530 | 0.580-4.041 | 0.390 | F | 0.560 | 0.000 |

F: Fixed-effects model.

**Figure 2** Funnel plot of *TLR4 Asp299Gly* polymorphism and acute pancreatitis risk for A vs G genotype.

findings have been inconclusive for the association of *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms and AP susceptibility^[6-12,15,21], therefore, we performed this meta-analysis to clarify this association.

In this meta-analysis, we finally collected six studies with 1255 cases and 998 controls, and our meta-analysis indicated that no significant associations were found between *TLR4 Asp299Gly* or *Thr399Ile* polymorphisms and AP risk using five models with high homogeneity. Furthermore, subgroup analysis showed no significant association in these two polymorphisms by ethnicity or assay, and *TLR4 Asp299Gly* was not associated with AP severity. Our current meta-analysis indicates that both

Asp299Gly and *Thr399Ile* polymorphisms of TLR4 gene may not be risk factors to AP susceptibility, implying that the polymorphisms of TLR4 have little effect in the pathogenesis of AP although TLR4 is one of the key genes in AP progression.

In summary, our meta-analysis implies that TLR4 gene polymorphisms were not significantly associated with AP susceptibility. However, the connection between TLR4 gene polymorphisms to AP susceptibility remains to be addressed in future investigations with a larger number of subjects.

COMMENTS

Background

Acute pancreatitis (AP) is a potentially lethal disease and many proinflammatory factors play important roles in the pathogenesis of AP.

Research frontiers

Toll-like receptor 4 (TLR4) *Asp299Gly* and *Thr399Ile* polymorphisms have been found to interrupt the binding of TLR4 to lipopolysaccharide pathway, however, the results remains unclear.

Innovations and breakthroughs

In this paper, the authors for the first time conducted a systematic meta-analysis to evaluate the association between TLR4 polymorphisms and AP risk, and the results suggest that *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms play little role in the pathogenesis of AP.

Applications

This study helped people to further understand the relationship between *TLR4 Asp299Gly* and *Thr399Ile* polymorphisms and AP susceptibility.

Peer review

This paper deals with a hot topic of association between gene single nucleotide polymorphisms and AP risk.

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Rapid improvement in post-infectious gastroparesis symptoms with mirtazapine

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Abstract

We report the case of a 34-year-old woman with severe post-infectious gastroparesis who was transferred from an outside medical facility for a second opinion regarding management. This patient had no prior history of gastrointestinal symptoms. However, in the aftermath of a viral illness, she developed two months of intractable nausea, vomiting, and oral intake intolerance that resulted in numerous hospitalizations for dehydration and electrolyte disturbances. A solid-phase gastric emptying scan had confirmed delayed emptying, confirming gastroparesis. Unfortunately, conventional pro-kinetic agents and numerous anti-emetic drugs provided little or no relief of the patient's symptoms. At our institution, the patient experienced a cessation of vomiting, reported a significant reduction in nausea, and toler-

ated oral intake shortly after taking mirtazapine. Based on mirtazapine's primary action as a serotonin (5-HT) 1a receptor agonist, we infer that this receptor system mediated the clinical improvement through a combination of peripheral and central neural mechanisms. This report highlights the potential utility of 5-HT_{1a} agonists in the management of nausea and vomiting. We conclude that mirtazapine may be effective in treating symptoms associated with non-diabetic gastroparesis that are refractory to conventional therapies.

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Key words: Nausea; Vomiting; Gastroparesis; Symptoms; Mirtazapine; Anti-emetics

Core tip: The management of symptoms associated with severe gastroparesis remains challenging because current therapeutic options are fairly limited. This case report documents the rapid improvement of nausea and vomiting in a patient with severe post-infectious gastroparesis with mirtazapine. Because mirtazapine acts primarily as a serotonin 1a receptor agonist, this receptor system may be an important adjunctive target for nausea and vomiting refractory to standard therapies. Thus, mirtazapine should be considered as a treatment option for gastroparesis.

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INTRODUCTION

Gastroparesis is a disorder defined by delayed gastric emptying in the absence of mechanical gastric outlet

obstruction^[1]. Patients with gastroparesis typically experience symptoms including anorexia, nausea and vomiting, abdominal pain, and other dyspeptic symptoms (e.g., early satiety, post-prandial bloating)^[1-3]. Patients can have a wide range of symptom severity, and those with more severe cases may require multiple hospitalizations^[4]. Patients tend to have a clinical course that lasts several months to years^[5,6]. The prevalence of gastroparesis has been extrapolated to include up to approximately 2% of the general population, but even this estimate has been thought to represent the “tip of the iceberg”^[7,8].

While the etiology of gastroparesis was once thought to be primarily caused by uncontrolled diabetes mellitus (*i.e.* visceral neuropathy) or prior gastric surgery resulting in vagal nerve injury, it is now appreciated that the majority of cases of gastroparesis are idiopathic^[3,6]. However, a significant subset of patients with idiopathic gastroparesis can associate the onset of symptoms following a viral-like illness, and this subset of patients tends to present with more acute and severe symptoms. Fortunately, most cases of post-viral gastroparesis ultimately resolve, but patients typically have fairly durable symptoms during the course of the illness^[6].

The management of symptoms associated with severe gastroparesis is challenging because current therapeutic options are fairly limited. The current mainstay of treatment focuses on symptom control using a variety of pharmacologic approaches including prokinetic agents such as metoclopramide, domperidone, and erythromycin, and anti-emetic agents such as phenothiazines and 5-HT₃ antagonists (e.g. ondansetron)^[1-3,7]. We report a case of a patient whose symptoms attributable to gastroparesis were refractory to such conventional treatments. This patient experienced significant improvement of her symptoms following initiation of treatment with mirtazapine.

CASE REPORT

A 34-year-old woman was referred to the inpatient gastroenterology and consult-liaison psychiatry services for evaluation of intractable nausea, vomiting, and intolerance of oral intake. She had no significant past medical history other than occasional migraine headaches responsive to sumatriptan. Two months prior to presentation at our institution, she developed a presumptive upper gastrointestinal viral illness manifesting as nausea with vomiting, as the patient's two younger children had similar symptoms. However, despite the rapid resolution of symptoms in her children, the patient subsequently developed constant nausea and intractable vomiting requiring intermittent emergency room visits and eventual hospitalization. Initial workup at an outside hospital was consistent with mild dehydration, but multiple diagnostic studies were fairly unremarkable except for the finding of a decreased gallbladder ejection fraction. Based on this result, coupled with the continued symptoms of nausea and vomiting, she proceeded with a laparoscopic cholecystectomy. Subsequent to the surgery, she devel-

oped *C. difficile* colitis, which was effectively treated. Unfortunately, the patient's nausea and vomiting persisted for several weeks after the other issues had resolved. A 2 h, solid phase gastric emptying study using a standardized test meal was performed at an outside facility. This study demonstrated gastric emptying of < 23% at 2 h, and the patient was diagnosed with gastroparesis. This result was obtained without the patient having taken narcotics or anti-cholinergic agents. Ultimately, the patient had lost 16 lbs over the two months of illness prior to presenting to our institution for a second opinion regarding management.

Review of the patient's prior therapy showed that she had trialed numerous oral and intravenous forms of anti-emetics including ondansetron, prochlorperazine, promethazine, scopolamine (*via* transdermal patch), dronabinol, and aprepitant. The prokinetic agents erythromycin and metoclopramide had both been tried without significant clinical benefit, and she experienced central side effects with metoclopramide. At our institution, she continued to have significant nausea and was unable tolerate oral intake without vomiting. A head computed tomography was negative for any intracranial pathology to explain symptoms. On psychiatric evaluation, she did not meet DSM-IV criteria for any psychiatric illness such as depression, anxiety, somatoform disorder, or factitious disorder that could contribute to symptoms. Because of the poor oral intake and subsequent weight loss, she had a post-pyloric nasojejunal tube placed for enteral feeding, along with continuous IV fluids. She was also trialed on proton pump inhibitors and benzodiazepines, in addition to scheduled dosing of several other antiemetics. Several days after initiation of enteral feeding, she continued to have ongoing severe symptoms attributed to gastroparesis. Using an 11-point verbal rating scale, she reported that her nausea was 8/10 in severity with only brief and mild relief from combination therapy using clonazepam, promethazine, ondansetron, and a transdermal scopolamine patch. She continued to have increased nausea with vomiting after attempts at solid food ingestion and had generally poor tolerance of even minimal volumes of ingested liquids.

Given the lack of efficacy of conventional approaches, she was started on mirtazapine 15 mg PO qhs, in addition to the other agents as above. Within a couple of days after starting mirtazapine, she had a complete cessation of her vomiting and reported an improvement in nausea to 5/10 in severity. However, because nausea still remained and the patient still did not tolerate significant oral intake, a standardized, 4 h solid-phase gastric emptying study was repeated to clarify the diagnosis of gastroparesis. This study was performed while she was using the transdermal scopolamine patch. Interestingly, the study demonstrated some improvement in gastric emptying as compared to the previous study obtained at the outside hospital, with a normalization of early phases of gastric emptying - at 2 h, there was 55% emptying (normal > 40%). However, the same study still demonstrated per-

sistent delay in the later stages of gastric emptying, with a 3 h emptying of 65% (normal > 70%) and 4 h emptying of 70% (normal > 90%). Given that there was some clinical improvement in symptoms with the lower dose of mirtazapine, the medication was increased to a dose of 30 mg PO qhs. There were no discernible side effects reported with the increased dose. Within a couple of days, the patient subsequently reported further improvement in nausea to 4/10 in severity, a level which was deemed tolerable to her. She also experienced improved appetite and was able to drink larger volumes of liquids and to tolerate small volumes of soft foods. Intravenous hydration was stopped and she remained in stable condition. She was subsequently discharged home without readmission for the first time in 3 mo on a regimen only including mirtazapine 30 mg PO qhs, clonazepam 1 mg PO BID, and ondansetron PRN. While nocturnal nasojejunal feeding was continued over the ensuing 6 wk, this therapy was ultimately discontinued because the patient had continued to improve with no episodes of vomiting, generally improved nausea, further improvements in oral intake (including some tolerance of solid foods), and weight gain near to her prior baseline.

DISCUSSION

To our knowledge, this is the first report of mirtazapine successfully used to treat symptoms of post-infectious gastroparesis. A previous report documented the successful use of mirtazapine in a patient with treatment-refractory diabetic gastroparesis^[9]. Mirtazapine has well documented efficacy in managing symptoms of nausea and vomiting in other clinical contexts, such as in cancer chemotherapy^[10] and in perioperative settings^[11,12]. Therefore, we felt mirtazapine could be an effective medication for the treatment of similar symptoms related to gastroparesis.

Mirtazapine is a unique antidepressant that is currently approved for use in the treatment of major depression. It specifically blocks histamine H1^[13] and serotonin 5-HT_{2A}, 5-HT_{2C}, and 5-HT₃ receptors, and stimulates 5-HT_{1A} receptors^[14] both peripherally and in the central nervous system. Stimulation of 5-HT_{1A} receptors is believed to be responsible for its antidepressant and anxiolytic effects, whereas blockade of H1, 5-HT_{2A} and 5-HT_{2C} receptors may also relate to some of its anxiolytic and sedating effects^[13,14]. Mirtazapine's impact on central neural systems involved in mood regulation could have contributed to symptom improvement in this case. While our patient did not have any overt depression or anxiety disorder, some degree of dysregulated mood in the context of prolonged and repeated hospitalizations could have influenced the perceived severity of symptoms.

Mirtazapine blocks 5-HT₃ receptors with similar efficacy to ondansetron^[10], and this mechanism may have contributed to the anti-emetic effects observed in the present case. However, 5HT-3 receptor blockade alone was unlikely the primary mechanism driving this patient's clinical

improvement, given that high doses of intravenous ondansetron had not been particularly effective. Furthermore, while we observed mildly improved gastric emptying following the administration of a lower dose of mirtazapine, symptoms of nausea and general intolerance to oral intake still persisted at that time. This observation fits with the generally poor correlation of gastroparesis symptom severity with the degree of delay in gastric emptying^[15]. Therefore, it is unlikely that the beneficial effects of the medication were driven by improved gastric emptying *per se*.

Interestingly, receptive fundic relaxation, a process largely controlled by the activity of nitrergic neurons, is influenced by the stimulation of 5-HT_{1A} receptors^[16]. Selective 5-HT_{1A} receptor agonism using the agent buspirone has been shown to dose-dependently and acutely increase gastric accommodation in healthy subjects^[16]. This mechanism also operates in those with functional dyspepsia, and buspirone can improve symptoms in this patient population^[17]. Improved gastric accommodation would be predicted to allow for larger ingested volumes of liquids or solid food. Gastric wall tone influences intragastric pressures and may drive the perception of nausea *via* increased vagal afferent activity. While mirtazapine has not been studied in this specific context, its shared pharmacology with buspirone implicates improved gastric accommodation *via* stimulation of 5-HT_{1A} receptors as a potential mechanism that could account for some of the clinical improvement seen in our patient. Finally, given the pleiotropic pharmacological effects of mirtazapine, it is possible that its therapeutic effect could also be mediated by an impact on multiple central neural systems important for the generation of the percept of nausea, autonomic sensorimotor integration important for stomach motility and sensation, and the central regulation of appetite. Thus, it is clear that mirtazapine could be beneficial through multiple potential mechanisms. However, regardless of mechanism, the dramatic improvement in this patient's symptoms after the initiation of mirtazapine suggests that this medication may be an effective treatment for severe gastroparesis-related symptoms.

While the current case is confounded by the concurrent use of other medications (benzodiazepine, 5-HT₃ antagonist, and anti-cholinergic agents), these other medications had been used for a week or more with only modest clinical benefits. The significant improvement in both the nature and severity of the symptoms after only two doses of mirtazapine would suggest that mirtazapine was primarily responsible for the clinical effect. Secondly, while post-viral gastroparesis symptoms can improve as a part of the natural history of the illness, symptoms tend to last at least several months. This patient's symptoms were durable for nearly 3 mo and refractory to standard therapies at the time mirtazapine was started. It appears quite unlikely that her illness spontaneously improved in the days during which mirtazapine was started. Also, the fact that symptoms were only partially improved on mirtazapine, would argue against a spontaneous remission of

the underlying gastroparesis.

Treatment for post-infectious gastroparesis can be challenging because symptoms are often severe and conventional therapies may not be effective. We report a case of patient with post-infectious gastroparesis who had an excellent clinical improvement after starting mirtazapine. The specific mechanism driving this response remains to be clarified, but mirtazapine could exert a therapeutic effect on nausea and vomiting *via* 5-HT₁ receptor agonism. Mirtazapine could represent an effective, alternative treatment for patients with gastroparesis who are refractory to conventional prokinetic and anti-emetic medications.

COMMENTS

Case characteristics

A 34-year-old woman with no major past medical history presented with two months of intractable nausea and vomiting.

Differential diagnosis

Gastroparesis, functional dyspepsia, chronic idiopathic nausea

Laboratory diagnosis

On initial presentation, the patient had a K 3.2 mmol/dL with a slight anion gap acidosis of 16 mEq/L and 2+ ketonuria; otherwise, CBC and liver function tests were within normal limits.

Imaging diagnosis

A 4-h solid phase gastric emptying scan was obtained at the institution, using the ingestion of 1.06 mCi of Tc-99m sulfur colloid mixed with a solid meal. This demonstrated a delay in emptying at the 3- and 4-h time points.

Treatment

Along with stable doses of clonazepam, ondansetron, and scopolamine, the patient was then treated with mirtazapine 15 mg PO qhs, with the dose increased to 30 mg PO qhs after several days.

Related reports

There is one other case report documenting the use of mirtazapine for symptoms of diabetic gastroparesis.

Term explanation

Post-infectious gastroparesis refers to a subset of patients who can clearly identify an acute infectious gastroenteritis, typically viral in nature, in the days to weeks prior to developing gastroparesis.

Experiences and lessons

To our knowledge, this is the first report of mirtazapine successfully used to treat symptoms of post-infectious gastroparesis.

Peer review

This report describes the use of mirtazapine to induce a rapid improvement in nausea and vomiting in a patient with post-viral gastroparesis.

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Deep endometriosis with pericolic lymph node involvement: A case report and literature review

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Key words: Deep infiltrating endometriosis; Gastrointestinal tract; Recto-sigmoid endometriosis; Recto-vaginal node; Lymph node endometriosis; Lymph node removal

Core tip: We present the case of a 37-year-old female patient with an endometrial growth on the sigmoid colon wall causing pain, diarrhea and the presence of blood in the feces associated with the involvement of the utero-vesical fold, the recto-vaginal septum and a pericolic lymph node, which are quite uncommon findings. We also reviewed the literature to examine the behavior of deep infiltrating endometriosis, analyzing the risk of recurrence related to the possible treatments.

Cacciato Insilla A, Granai M, Gallippi G, Giusti P, Giusti S, Guadagni S, Morelli L, Campani D. Deep endometriosis with pericolic lymph node involvement: A case report and literature review. *World J Gastroenterol* 2014; 20(21): 6675-6679 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6675.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6675>

Abstract

Deep infiltrating endometriosis is an often-painful disorder affecting women during their reproductive years that usually involves the structures of the pelvis and frequently the gastrointestinal tract. We present the case of a 37-year-old female patient with an endometrial growth on the sigmoid colon wall causing pain, diarrhea and the presence of blood in the feces. The histology of the removed specimen also revealed the involvement of the utero-vesical fold, the recto-vaginal septum and a pericolic lymph node, which are all quite uncommon findings. To identify the endometrial cells, we performed immunohistochemical staining for CD10 and the estrogen and progesterone receptors.

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INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory dysfunction first described by Rokitsansky in 1860 and characterized by the presence of glands and stroma that histologically resemble functional endometrial tissue but are located outside of the uterus^[1,2].

Endometriosis occurs in an estimated 1% to 20% of asymptomatic women, 10% to 25% of sterile patients and 60% to 70% of women with chronic pelvic pain. Different manifestations of this disorder have been described, such as endometriosis genitalis externa, adenomyosis externa, endometriosis extra-genitalis and deep infiltrating endometriosis (DIE)^[3].

Within the abdomen, endometriosis can be divided into intra- and extra-peritoneal disease. In decreasing or-



Figure 1 Double-contrast barium enema examination. Endometriosis implant in sigmoid colon: extrinsic compression with protruding polypoid appearance and mucosal pleating.

der of frequency, the prevalent intra-peritoneal locations are the ovaries (30%), the utero-sacral and large ligaments (18%-24%), the fallopian tubes (20%), the pelvic peritoneum, Douglas' pouch, the appendix and the small and large intestines. In contrast, it is uncommon to find endometriosis in an extra-peritoneal structure, such as the cervix (0.5%), vagina and recto-vaginal septum, the round ligament and inguinal hernia sac (0.3%-0.6%), the navel (1%), abdominal scars resulting from gynecological surgery (1.5%) and the abdominal rectus muscle (0.5%). Finally, endometriosis rarely affects extra-abdominal organs, such as the lungs, urinary system, skin and central nervous system^[2].

Gastrointestinal involvement of endometriosis has been found in 3% to 37% of women, most commonly in the sigmoid colon, rectum, and terminal ileum^[2,4]. Although bowel endometriosis may cause severe gastrointestinal symptoms, these disturbances are not often adequately investigated at the time of gynecologic evaluation^[1]. As a result, bowel endometriosis may be an unexpected finding at the time of surgery.

Lymph node involvement in endometriosis is usually considered to be uncommon. However, some studies^[5] emphasize that lymph node involvement in endometriosis might just be an underestimated event related to the minimal removal of tissues by surgeons.

We report a case of DIE with recto-sigmoid and paracolic lymph node involvement and compare our remarks with the current literature.

CASE REPORT

A nulliparous 37-year-old woman was referred to the general surgery department of our hospital in April 2012 for widespread abdominal pain associated with diarrhea and bloody stools. The patient had started to complain about the pain seven months earlier, in September 2011. Episodes of pain relapsed regularly every month, approximately three days after menstruation. Initially, the pain did not match the diarrhea and presence of blood in the feces, which both appeared only two months later

(November 2011). In 2004, the patient had undergone exploratory laparoscopy with the removal of some endometrial cysts from both ovaries, the extra-pelvic abdominal peritoneum and the pouch of Douglas, resulting in a diagnosis of stage four endometriosis. A forced menopause *via* treatment with a GnRH agonist (Decapeptyl) was induced for one year. Between 2005 and 2011, the patient had four IVF attempts to become pregnant, all unsuccessful. During these attempts, she satisfactorily contained the symptomatology with an estroprogestinic pill. At the worsening of symptoms in November 2011, she consulted her gynecologist and underwent an abdominal ultrasound examination, revealing the presence of a 3 cm diameter node on the surface of the sigmoid colon suspected for endometriosis growth. A subsequent double-contrast barium enema highlighted a stenosis of the proximal tract of the sigmoid colon with an extrinsic compression on the medial wall, approximately 35 cm in length (Figure 1). An abdominal-pelvic magnetic resonance imaging (MRI), performed on January 31st, 2012, confirmed the presence of a node infiltrating the sigmoid colon wall approximately 17 cm from the pectinate line. MRI examination also revealed the presence of an adhesion between the sigmoid colon and an adjacent intestinal loop; a secondary subcentimetric nodule at the level of the recto-vaginal septum was also identified, with the same radiological aspects as the largest nodule. Finally, on April 6th, a colonoscopy performed to refine the diagnosis revealed the presence of an inflamed and hyperemic mucosa roughly 20 cm from the anus. Due to the stenosis, the examination was unable to reach the ileocecal valve. The biopsy only revealed the presence of inflammatory cells and edema. The physical examination prior to the operation showed mild diffuse abdominal tenderness and negative Blumberg's sign. Auscultation detected normal bowel sounds and peristaltic rushes. Laboratory analysis revealed values of white blood cell 4.58 K/uL, hemoglobin 13.2 g/dL, carbohydrate antigen (CA)125 29.7 U/mL, CA15.3 14.3 U/mL, CA19.9 7.3 U/mL, and carcinoembryonic antigen 0.4 mg/mL; metabolic panel and liver function tests were within normal limits. The patient underwent laparoscopic surgery on May 2nd, 2012 during which a minimal left hemicolectomy was performed with the resection of only 7 cm of the sigmoid colon. Both radiologically described nodes were detected and removed during the surgical procedure. Another 1.5 cm node was revealed at the level of the utero-vesical fold during the operation (Figure 2). There was no evidence of adhesion between the sigmoid colon and the near intestinal loops as documented by MRI. The post-operative course was uneventful, and the patient left the hospital 5 d later. The evaluation of the excised specimen showed endometriosis involving the removed sigmoid colon tract and causing its convoluted course. Histological analysis revealed infiltration of the bowel wall (Figure 3), but the mucosa was not ulcerated. Endometriosis was evident in the serosa, the subserosa, the entire thickness of the muscularis propria and focally within the submu-

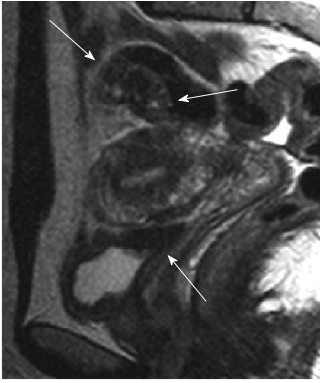


Figure 2 Magnetic resonance imaging shows two hypointense nodular implants of endometriosis infiltrating the proximal sigmoid wall and the utero-vesical fold, with the latter retrospectively recognized after the surgery (arrows). The presence of high signal intensity foci within the nodules is due to hemorrhagic foci.

cosa. The submucosa also showed diffuse congestion, fibrosis and focal clusters of hemosiderin-laden macrophages. All of the removed nodes were diagnosed as endometrial growths. Endometrial involvement, with a cystic glandular pattern, was also detected in a pericolic lymph node measuring 3 mm, as confirmed by immunohistochemical staining for CD10 and the estrogen and progesterone receptors (Figure 4). Approximately one year after the surgical operation (July 2013), the patient referred to her physician for an episode of constipation and pain associated with defecation. A transvaginal ultrasound examination performed in September 2013 revealed the presence of a cystic structure around the rectum and in the utero-vesical fold, strongly suspected for a relapse of the disease.

DISCUSSION

Endometriosis is a common condition that affects women during the reproductive years. It occurs when normal tissue from the uterine lining, the endometrium, attaches to other organs and starts to grow. This displaced endometrial tissue causes irritation in the pelvis, which may lead to pain and infertility. Experts do not understand why some women develop endometriosis. Although we know the factors potentially involved in the etiology and pathogenesis of endometriosis, the exact mechanism by which this disease develops, with its associated signs and symptoms, remains obscure. Nevertheless, it is recognized that three separate entities exist (peritoneal, ovarian, and recto-vaginal endometriosis) based on the different locations, possible origins, appearances and hormone responsiveness of all these lesions^[6]. Several theories have been developed to account for the pathogenesis of different implants, which can be divided into implants originating from the uterine endometrium and those arising from tissues other than the uterus^[7,8]. The most widely accepted theory is the retrograde menstruation theory proposed by Sampson in 1920^[8]. According to this theory, endometrial tissue refluxes through the fal-

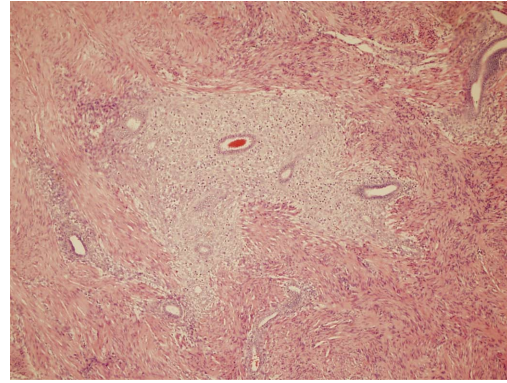


Figure 3 Histology of the sigmoid wall showing endometrial tissue in the muscular layer.

lopian tubes during menstruation, in turn implanting and growing on the serosal surface of the abdominal and pelvic organs. The usual anatomic distribution of endometriotic lesions also favors the retrograde menstruation theory^[8]. Some authors^[6,8,9] sustain that this theory is not sufficient to explain the origin of the so-called “deep endometriosis,” which includes recto-vaginal and infiltrative lesions involving vital structures such as the bowel, ureters, and bladder. Koninckx and Martin^[9] were the first to define deep endometriosis, having distinguished posterior cul-de-sac and recto-vaginal lesions in three different subgroups: type I, conically shaped, developed from infiltration; type II, deeply located, covered by extensive adhesions, most likely formed by retraction; and type III, the most severe, having one or more spherical nodules located in the recto-vaginal septum with the largest size under the peritoneum, possibly to be considered as adenomyosis externa. As a matter of fact, these latter endometriosis growths should be considered a different entity than peritoneal endometriosis, likely with another pathogenesis. Since 1997, it has been suggested that these growths could correspond to an adenomyotic nodule originating from müllerian rests through a metaplastic process^[7]; however, this hypothesis remains very disputed and is not universally shared^[10]. Endometriosis usually occurs in the pelvic organs and peritoneum but rarely in the rectum, colon, small intestine, kidney, ureter, appendix, external female genital organs, lymphatic nodules or the surrounding area of the anus. Endometriosis infiltrates the bowel with a frequency of 5% to 37%^[11] in the following order: rectum, sigmoid colon, appendix, ileum and cecum. Clinical symptoms of endometriosis include dysmenorrhea, chronic pelvic pain, dyspareunia and infertility, but the clinical presentation is often non-specific. DIE, especially if it involves the bowel, is often also associated with constipation or diarrhea, abdominal bloating, bowel movements and occasionally bloody stools. Symptoms are often cyclical but may become permanent when the lesion progresses. Furthermore, the symptoms are not always the same in each woman, and in some women with endometriosis, they are totally absent.

The status of the lymph nodes in endometriosis re-

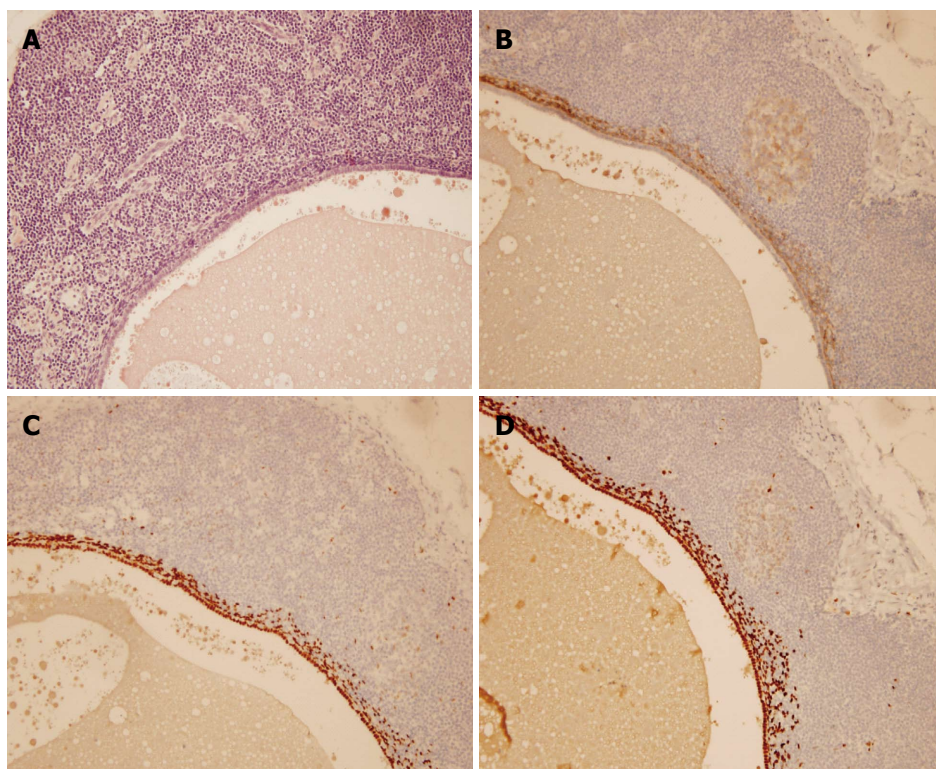


Figure 4 Lymph node endometriosis with a cystic glandular pattern. A: Hematoxylin/eosin stain $\times 200$; B: CD10 immunostaining $\times 200$; C: Estrogen receptor immunostaining $\times 200$; D: Progesterone receptor immunostaining $\times 200$. A BX-51 Olympus microscope connected to a computer by a color CCD camera was used to obtain and edit histology images. The analySISB software (Olympus) was used to acquire images at different magnifications.

mains obscure or, more likely, underestimated. The greatest limit to their identification is that dissection is not usually performed for benign diseases. Noël *et al*^[5] carefully studied 26 cases of recto-sigmoid endometriosis, finding lymph node involvement in 42.3% of the cases and demonstrating that lymph node involvement in the recto-sigmoid endometriosis should not be considered an uncommon occurrence. A shared hypothesis is that lymph node endometriosis represents a lymphatic drainage from endometriotic tissue. Similar to true malignant tumors, DIE, in particular the recto-sigmoid endometriosis, has an aggressive potential and ability to invade the adjacent tissue extensively with probable lymphovascular invasion. Endometriosis is considered a benign disease, but it occasionally becomes severe and progressive with a high rate of recurrence: endometriotic and cancer cells have similar characteristics, such as responsiveness to growth factors, resistance to antiproliferative factors, decreased apoptosis, the promotion of neoangiogenesis and metastatic potential^[12].

Finally, there are numerous reported cases of malignancy arising from endometriotic deposits and substantial histologic evidence according to which endometriosis is associated with endometrioid carcinoma and clear cell carcinoma of the ovary^[13]. At the present moment, there are many controversies regarding the therapeutic approach to DIE, in particular if bowel involvement is associated. In our case, the sigmoid resection was necessary due to the deep infiltration of the wall by the endometri-

osis lesion and the heavy symptomatology of the patient. Despite the fact that bowel resection has become a popular treatment modality, even with the improved operative laparoscopy techniques, many authors emphasize that an aggressive surgery for DIE involving a bowel resection is rarely justified^[14]. In their studies on DIE, Acien *et al*^[14] strongly suggest that the efficacy of an aggressive surgery may be lower than that of medical treatment. For instance, in the case of widespread disease with bowel involvement, nonaggressive sharing (NAS), with or without a hysterectomy and salpingo-oophorectomy (HBSO), followed by hormone replacement therapy may be considered a valid alternative to intestinal surgery. Indeed, it has been proven that surgery associated with post-operative hormone therapy can provide better results than an exclusively surgical or pharmacological treatment^[15]. In general, these studies show that patients who undergo an aggressive operation have a worse outcome than those treated with NAS and/or HBSO. Nevertheless, these studies also underline the fact that NAS is deeply associated with a higher risk of recurrences and reoperations over the years: approximately 55% of patients experience a recurrence, with approximately 38% needing a more extensive operation, versus patients treated with HBSO who experienced a follow-up free of recurrences^[14]. In our case, the detection of the involved lymph node has been an infrequent event, proving, however, the existence of some possible endometriosis foci beyond the obvious ones. If the aim of surgery is to remove all areas of

endometriosis, then it could appear irrational to remove only all visible foci when there is a high risk of associated lymph node involvement and/or of recurrence^[5,16].

After these considerations, a more conservative type of surgery aimed at removing all visible endometriosis foci followed by pharmacological therapy could be the best choice to have a positive outcome, but it does not appear as good at reducing the risk of recurrence as a wide surgical operation. Only randomized studies of medical treatments with or without conservative surgery versus HBSO, alone or with bowel resection, will allow us to determine which option provides the best balance between patient satisfaction and the risk of disease recurrence.

COMMENTS

Case characteristics

A 37-year-old female with a history of deep infiltrating endometriosis presented with abdominal pain, diarrhea and blood in her feces.

Clinical diagnosis

Diffuse abdominal tenderness and negative Blumberg's sign, auscultation with normal bowel sounds and peristaltic rushes.

Differential diagnosis

Endometriosis, inflammatory bowel disease, cancer.

Imaging diagnosis

Double-contrast barium enema highlighted a stenosis of the proximal tract of the sigmoid colon, and abdominal-pelvic magnetic resonance imaging confirmed the presence of a node infiltrating the sigmoid colon wall previously detected by ultrasound examination and also revealed a secondary subcentimetric nodule at the level of the recto-vaginal septum.

Pathological diagnosis

All specimens surgically removed were diagnosed as endometriosis foci, with one of them infiltrating the sigmoid wall.

Term explanation

CD10, estrogen and progesterone receptors are immunohistochemical markers typically expressed by the endometrial stromal cells.

Experiences and lessons

Conservative surgical treatment of only visible endometriosis foci, even followed by pharmacological therapy, might expose the patient to a higher risk of disease recurrence.

Peer review

Based on the belief that finding a lymph node involved in endometriosis is uncommon, we reflected on the relevance of leaving undetected endometriosis foci behind after a surgical operation.

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Ehealth monitoring in irritable bowel syndrome patients treated with low fermentable oligo-, di-, mono-saccharides and polyols diet

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Abstract

In the present study we report on changes in irritable bowel syndrome-severity scoring system (IBS-SSS) and irritable bowel syndrome-quality of life (IBS-QoL) in 19 IBS patients, aged 18 to 74 years (F/M: 14/5), during 12 wk registering their symptoms on the web-application (www.ibs.constant-care.dk). During a control period of the first 6-wk patients were asked to register their IBS-SSS and IBS-QoL on the web-application weekly without receiving any intervention. Thereafter,

low fermentable oligo-, di-, mono-saccharides and polyols (FODMAP) diet (LFD) was introduced for the next 6 wk while continuing the registration. Though a small sample size a significant improvement in disease activity (IBS-SSS) was observed during both the control period, median: 278 (range: 122-377), $P = 0.02$, and subsequently during the LFD period, median: 151 (range: 29-334), $P < 0.01$. The IBS-QoL solely changed significantly during the LFD period, median: 67 (37-120), $P < 0.01$. The significant reduction in disease activity during the control period shows a positive effect of the web-application on IBS symptoms when presented as a "traffic light". However adding the diet reduced IBS-SSS to < 150 , inactive to mild symptoms. In the future results from larger scale trials are awaited.

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Key words: Irritable bowel syndrome; Low fermentable oligo-, di-, mono-saccharides and polyols diet; Self-management; Disease-specific quality of life; Disease activity

Core tip: The treatment of irritable bowel syndrome (IBS) symptoms provides a challenge for clinicians in everyday practice. In our case report we present the changes in disease activity and quality of life of 19 IBS patients using web-application as a tool for self-management over 6-wk and then applying the low Fermentable Oligo-, Di-, Mono- saccharides and Polyols diet while continuing the registration of their symptoms over a second 6-wk period.

Pedersen N, Vegh Z, Burisch J, Jensen L, Ankersen DV, Felding M, Andersen NN, Munkholm P. Ehealth monitoring in irritable bowel syndrome patients treated with low fermentable oligo-, di-, mono-saccharides and polyols diet. *World J Gastroenterol*

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INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder of partly known etiology. The condition is characterized by relapsing symptoms of abdominal pain and discomfort associated with bloating, distension and altered bowel habit (ranging from diarrhoea to constipation) but without any pathological abnormality of the gut wall^[1]. The low FODMAP (Fermentable Oligo-, Di-, Mono- saccharides and Polyols) diet (LFD) provides a new therapeutic approach for patients with IBS^[2-4]. FODMAPs are short-chain, poorly absorbed and readily fermented carbohydrates^[5]. Their ingestion induces luminal distension and gastrointestinal symptoms by gas production (H₂, CH₄) in and water delivery to the colon^[6]. The efficacy of the LFD in decreasing gastrointestinal symptoms in IBS was previously confirmed^[7-9]. Web-based self-management programs have proven to be feasible approaches in chronic diseases, among others inflammatory bowel diseases (IBD), by influencing disease course and increasing self-adherence, compliance and quality of life (QoL)^[10-13]. The web-app (web-application) and homepage www.ibs.constant-care.dk, ConstantMed Inc., was developed in order to investigate whether the same encouraging effect could be achieved for patients with IBS^[16]. The application contains education about IBS symptoms and treatment options, IBS disease activity score and QoL scoring presented in a traffic light and e-learning. Further it carries a web-ward round system applicable for *e.g.* nurses or doctors, ranking patients in colours in accordance with IBS severity. We report on changes in irritable bowel syndrome-severity scoring system (IBS-SSS) and IBS-QoL of 19 IBS patients in a pilot study.

CASE REPORT

We hereby report on 19 IBS patients aged 18 to 74 years fulfilling the Rome III criteria of IBS. Eight patients were diagnosed with IBS-D (diarrhoea predominant subtype), 7 with IBS-A (alternating subtype) and 4 with IBS-C (constipation predominant subtype). The patients were selected consecutively in 2011-2012 from Herlev Hospital and Hamlet Hospital in Copenhagen^[17]. Patient characteristics at baseline are shown in Table 1. IBS was differentiated from IBD by negative outcome of sigmoidoscopy/colonoscopy, normal histological specimens from the bowel, negative inflammatory markers (white blood cells, platelets, CRP, faecal calprotectin) and by no presence of blood in the stool. Negative bacterial culture results, negative transglutaminase antibodies and negative lactose intolerance gene test were required for the participation as well as no history of known food allergy. Negative bacterial culture results were required

Table 1 Demographic and disease characteristics of patients with irritable bowel syndrome at baseline *n* (%)

| Males | 5 (26) |
|-------------------------------------|------------|
| Median age at inclusion, yr (range) | 35 (18-74) |
| IBS subtype | |
| IBS-D, diarrhoeal | 8 (42) |
| IBS-C, constipating | 4 (21) |
| IBS-A, alternating | 7 (37) |
| Abdominal surgery before study | 4 (21) |
| Appendectomy | 2 (11) |
| Hysterectomy | 1 (5) |
| Laparoscopy | 1 (5) |
| IBS medication at baseline | 7 (37) |
| Antidepressivum ± laxativum | 4 (50) |
| Laxativum ± spasmolyticum | 3 (38) |
| Smoking status | |
| Never | 11 (58) |
| Former | 7 (37) |
| Currently | 1 (5) |
| Median BMI (range) | 22 (17-27) |

IBS: Irritable bowel syndrome; BMI: Body mass index.

for the differentiation from infectious gastroenteritis. Patients having any alarm symptoms as fever (> 38.5 °C), anaemia, unintended weight loss > 5 kg, familiar disposition to colorectal cancer or any other significant diseases were excluded. During the first 6-wk, non-interventional control period, patients were asked to fill in the IBS-SSS (Irritable Bowel Syndrome-Severity Scoring System) and IBS-QoL (Irritable Bowel Syndrome-Quality of Life) questionnaires weekly in the web-app. At the end of the control period they were instructed by a FODMAP-certified dietician regarding the LFD during a 45-min consultation. The end of the control period was considered as the start of the LFD period, because the patients started the LFD between week 6 and 7. They were requested to follow strictly the LFD during the second 6-wk period and continue the registration of their IBS-SSS and IBS-QoL in the web-app. The patients and the study investigator could monitor the disease course by the changes in IBS-SSS and IBS-QoL in a traffic light system (a patient case is illustrated in Figure 1): red indicated severe IBS-SSS, yellow moderate IBS-SSS and green mild IBS-SSS and remission (= no symptoms). All statistical analyses were carried out using SPSS software Version 20.0 for Windows (SPSS Inc., Chicago, IL). Standard descriptive statistics were performed, including calculation of median and range for continuous variables. Differences between the IBS-SSS and IBS-QoL values between the start and the end of the control period, between the start and the end of the LFD period and between the start of the control period and the end of the LFD period were analysed by Wilcoxon two-related-samples test. A *P* value of < 0.05 was considered statistically significant. The power of the study was not performed as it was planned to be a case/pilot report. The study was approved by the Ethical Committee, Denmark (protocol number H-2-2011-095-IBS). All patients included in this study signed an informed consent. For measuring the IBS severity the

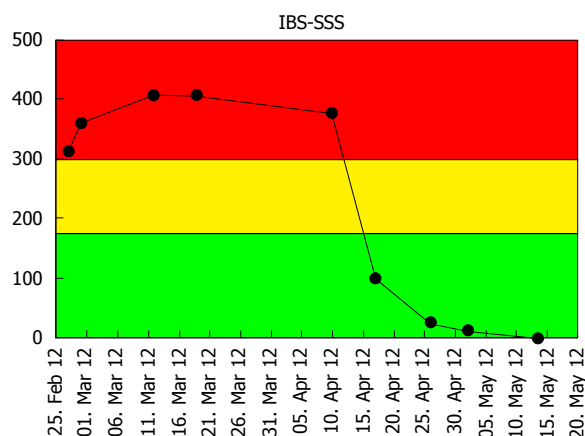


Figure 1 Patient self-managed web-based registry of irritable bowel syndrome symptom severity score during twelve weeks. Y-axis: Irritable bowel syndrome symptom severity score (IBS-SSS) score which ranges from 0-500 points [Green: Remission and/or mild (0-175); yellow: Moderate disease severity (175-300); and red: Severe disease severity (> 300)]; X-axis: Control period (normal diet): week 1-6 and LFD period: week 7-12. FODMAP: Fermentable, oligosaccharides, disaccharides, monosaccharide's and polyols; LFD: Low FODMAP diet.

IBS-SSS questionnaire was used^[18]. Each of the five questions generates a maximum score of 100 using prompted visual analogue scales (VAS), leading to a total possible score of 500. Below 175 indicates mild or remission, 175 to 300 moderate and above 300 severe IBS-SSS. The IBS-QoL is a 34-item validated, 1-5 options, self-administered questionnaire for assessing the perceived quality of life for persons with IBS (range: 34-170, lower scores indicate better IBS-QoL)^[19]. Each questionnaire was administered at least once a week during the study period.

During the non-interventional control period there was a significant reduction in IBS-SSS from baseline to week six in all patients, start median: 320 (range: 260-406), end median: 278 (range: 122-377); $P = 0.02$. During the LFD period a significant reduction was observed from week 6 to week 12 in all patients, start median: 278 (range: 122-377), end median: 151 (range: 29-334); $P < 0.01$ (Figure 2). Out of 19 patients, 6 had obtained remission, 5 had mild activity, 5 had moderate, 3 had severe activity. Investigating the changes in IBS-SSS in each IBS subtype, we found significant difference only in IBS-D during the LFD period, control period: IBS-D: $P = 0.06$, and after LFD period: IBS-D: $P = 0.01$.

During the control period we found no significant difference in IBS-QoL from baseline to week six in all patients, start median: 82 (range: 56-131), end median: 81 (range: 47-127); $P = 0.33$. During the LFD period IBS-QoL improved significantly from week 6 to week 12 in all patients, start median: 81 (range: 47-127), end median: 67 (37-120), $P < 0.01$ (Figure 3). Similarly to IBS-SSS, significant difference in IBS-QoL was observed only in the IBS-D during the LFD period: $P = 0.02$.

DISCUSSION

We demonstrate in 19 patients a difference in IBS-SSS

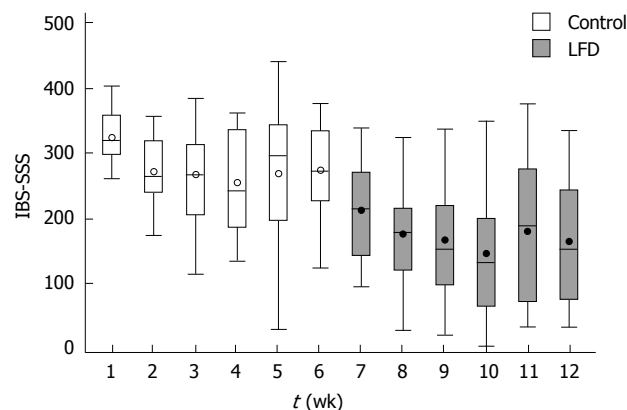


Figure 2 Box plot irritable bowel syndrome symptom severity score (median, range per week) during 12-wk in 19 irritable bowel syndrome patients. Y axis: IBS-SSS scores from 0-500 points; X axis: Week 1-12. Control period (normal diet): Week 1-6; LFD period: Week 7-12. FODMAP: Fermentable, oligosaccharides, disaccharides, monosaccharide's and polyols; LFD: Low FODMAP diet.

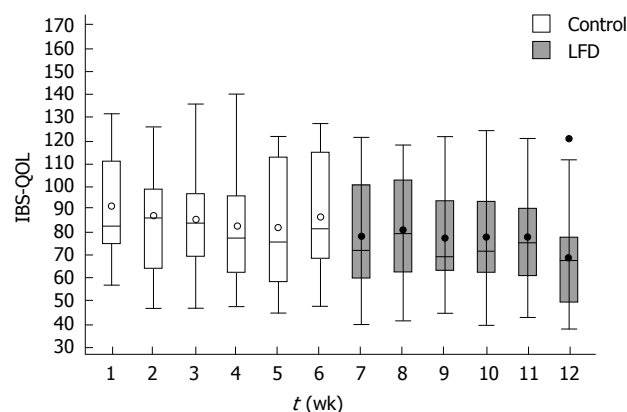


Figure 3 Box plot of irritable bowel syndrome quality of life (median, range per week) during 12-wk in 19 irritable bowel syndrome patients. Y axis: Irritable bowel syndrome quality of life (IBS-QoL) scores from 170 (worst) to 34 (best) points; X axis: Week 1-12. Control (normal diet) period: Week 1-6; LFD period: Week 7-12. FODMAP: Fermentable, oligosaccharides, disaccharides, monosaccharide's and polyols; LFD: Low FODMAP diet.

observed during the control and the LFD period as well. However, stratifying between the three IBS subtypes, only a significant reduction in the diarrhoea predominant subtype was observed, but because of the small sample size final interpretation can only be carried out in a larger scale randomized trial. At the outset of the LFD period the IBS-SSS value improved from moderate to mild IBS severity. IBS-SSS scoring at the end of LFD showed that out of the 19 patients, 11 had obtained remission to mild IBS severity.

An interesting finding was the significant reduction of IBS-SSS during the control period, from severe to moderate IBS symptoms. The web-based program seems to be feasible and efficient for IBS patients. One of the explanations could be that with the help of the web-program patients recognize the disease pattern of their IBS, thus could manage their disease course also by themselves possibly by identifying the cause of their in-

crease of their symptoms and subsequently eliminate the specific environmental components (diet, stress, polyols in candy *etc.*). Furthermore, the web-app tool might have a placebo effect in this patient group: the fact, that a physician follows the changes in patients' IBS symptoms, this might also have a positive effect on their disease course. Similarly to previous studies, the efficacy of the LFD in decreasing IBS-SSS was confirmed also in our present case report^[2-4]. The natural disease course of IBS should also be taken into account when evaluating our results, as it has a chronic course with intermittent flares. The strength of our study is that this is the first case report on the use of the web-application as a new initiative in the management of IBS patients applying LFD. The weakness of the manuscript is that the small simple size impedes interpretation of the significant results observed in the IBS diarrhea predominant subtype. Furthermore, the adherence to the LFD was not measured, however the patients had close contact to the dieticians and they could easily reach them *via* e-mail and telephone if they had any questions regarding the LFD.

In conclusion, the web-app and the LFD might be feasible concepts and help the IBS patients to control and lower their IBS symptom severity scoring. Larger randomized controlled trials are awaited.

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COMMENTS

Case characteristics

Hereby the authors report on 19 patients fulfilling the Rome III criteria of irritable bowel syndrome (IBS) registering their symptoms on the web-application before and during the use of the low fermentable oligo-, di-, mono-saccharides and polyols (FODMAP) diet, which was especially developed for IBS patients.

Clinical diagnosis

IBS patients fulfilling the Rome III criteria were selected consecutively in 2011-2012 from Herlev Hospital and Hamlet Hospital in Copenhagen in the study. Cases were aligned to the three subtypes of IBS: IBS-D (diarrhoea predominant subtype), IBS-A (alternating subtype) or IBS-C (constipation predominant subtype).

Differential diagnosis

IBS was differentiated from inflammatory bowel diseases by negative inflammatory markers (FC = faecal calprotectin) and by no presence of blood in the stool.

Laboratory diagnosis

All of our patients had negative laboratory test results for transglutaminase antibodies, lactose intolerance gene test, white blood cells (WB > 3.0 × 10⁹/L, -9.0 × 10⁹/L), hemoglobin (hemoglobin > 7.5 mmol/L), platelets (platelets 100-100-350 × 10⁹/L) and for C-reactive protein (CRP) (CRP < 3 mmol/L).

Imaging diagnosis

Negative outcome of sigmoidoscopy or colonoscopy was required before the inclusion in the study.

Pathological diagnosis

Normal histological specimens from the bowel were required before the inclusion in the study.

Treatment

The included patients applied normal western diet (ND) during first 6-wk and the late finish date during the second 6-wk period, which was developed especially for IBS patients.

Term explanation

The low FODMAP diet (Fermentable oligo-, di-, mono-saccharides and polyols)

(LFD) is based on the reduced intake of the short chain carbohydrates and polyols and it proved to be an effective treatment option in IBS patients in previous studies. The IBS-SSS (Irritable Bowel Syndrome-Severity Scoring System) was used for measuring the disease severity in IBS patients. The IBS-QoL (Irritable Bowel Syndrome-Quality of Life) was applied for measuring the quality of life of IBS patients.

Experiences and lessons

Both interventions, the web-application and the LFD had a positive effect on the disease severity of these patients.

Peer review

The strength of their study is that this is the first case report on the use of the web-application as a new initiative in the management of IBS patients applying LFD. The weakness of the manuscript is that the small simple size impedes interpretation of the significant results observed in the IBS diarrhoea predominant subtype.

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Solitary schwannoma of the gallbladder: A case report and literature review

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Abstract

Schwannomas occurring in the gallbladder are extremely rare. Preoperative diagnosis of gallbladder schwannomas appears to be very difficult because they are normally asymptomatic and are often found incidentally. Until now, only five cases have been reported in the literature. To our knowledge, the contrast-enhanced ultrasound (CEUS) features of gallbladder schwannomas have not been reported before in other studies. We treated a 55-year-old male patient with gallbladder schwannoma in China. He had no symptoms, and the lesion was incidentally found by conventional ultrasound (US) when performing a health examination. The patient had normal liver function; moreover, serum carcinoembryonic antigen and alpha-fetoprotein were within the normal ranges. The lesion

showed no blood flow signals on color Doppler US, and the wall beneath the lesion was intact on CEUS. The lesion was believed to be a benign entity; in addition, gallbladder adenomyomatosis was suspected. A laparoscopic cholecystectomy was performed to remove the mass. Pathological examination revealed that the tumor was mainly composed of spindle-shaped cells; neither atypical cells nor signs of malignancy were found. Immunohistochemical staining showed a strong positive S-100 protein reaction. Vimentin and CD56 staining were also positive, whereas CD34 and CD117 were negative. Finally, the lesion was diagnosed as schwannoma. Herein, we report the case; the associated literature is also reviewed.

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Key words: Schwannoma; Gallbladder; Ultrasound; Contrast-enhanced ultrasound

Core tip: Gallbladder schwannoma, a benign tumor derived from the Schwann's cells in the gallbladder wall, is extremely rare. This paper describes the case of a 55-year-old man with a mass in the gallbladder but no other symptoms. The patient was treated by cholecystectomy. After 1 year of follow-up with clinical evaluation and ultrasound (US), it did not show any evidence of local recurrence. We learned through this rare case of gallbladder schwannoma that the imaging findings have not been reported in detail before. The US, especially contrast-enhanced ultrasound, features seemed to be helpful in excluding malignancy, and it was essential for treatment planning and the alleviation of patient anxiety in this case.

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INTRODUCTION

Schwannomas are benign neurogenic tumors that originate from the Schwann's cells of the peripheral nerves in young to middle-aged patients^[1]. Schwannoma can develop in any part of the body, but the most common sites include the head, neck, and flexor surfaces of the extremities^[2]. Schwannomas arising in the digestive tract are quite rare, and they occur most commonly in the stomach, followed by the colon and rectum^[3,4]. Primary benign schwannoma of the gallbladder is extremely rare. In this paper, we present a case of gallbladder schwannoma, with an emphasis on the imaging and pathological findings. Biliary system schwannomas in the literature are also reviewed.

CASE REPORT

A 55-year-old male patient was admitted to the hospital because a 2.1-cm mass was incidentally detected in his gallbladder by ultrasound (US) during a health examination. The patient had neither complaints nor symptoms. According to the physical examination, his abdomen was soft and flat; there was no evidence of jaundice or abdominal tenderness. He had no previous history of any other major illness, and his vital signs were stable. The blood laboratory study results were as follows: leukocyte count, $6.86 \times 10^9/L$; hemoglobin, 159 g/L; total protein, 80 g/L; total albumin, 50 g/L; total bilirubin, 13.4 $\mu\text{mol/L}$; direct bilirubin, 10.8 $\mu\text{mol/L}$; serum alanine aminotransferase 16.1 U/L; serum alkaline phosphatase, 53.4 U/L; and serum gamma-glutamyltranspeptidase, 31.4 U/L. The laboratory studies revealed almost normal liver function. Carcinoembryonic antigen and alpha-fetoprotein were within the normal ranges.

US was performed using a LogiQ E9 scanner (GE Healthcare, Milwaukee, WI, United States) with a convex transducer (frequency range, 2-6 MHz). Conventional US showed a 2.1-cm iso-echoic mural mass in the gallbladder. The mass was solid, homogeneously echogenic, and well defined with no infiltration into the liver (Figure 1A). Color Doppler US showed no flow signals within the mass (Figure 1B). Contrast-enhanced ultrasound (CEUS) was then performed using the low acoustic power contrast-specific imaging mode. The contrast agent used was SonoVue (BR1; Bracco SpA, Milan, Italy), a sulfur hexafluoride-filled microbubble contrast agent. The contrast agent was injected into the antecubital vein as a bolus (within 1-2 s) at a dose of 1.5 mL, followed by a flush of 5 mL of normal saline. The mass appeared slightly hyper-enhanced in the arterial phase and slightly hypo-enhanced in the venous phase. The gallbladder wall under the mass was intact (Figure 1C, 1D and 1E). A diagnosis of gallbladder adenomyomatosis was suspected before surgery.

Contrast-enhanced computed tomography (CECT) was subsequently performed to examine the gallbladder within 1 wk after the US examination, using a 64-slice computed tomography (CT) scanner. An un-enhanced CT scan of the gallbladder region showed a well-defined

round mass in the gallbladder. The mass showed homogeneous hypo-attenuation without internal calcification or liquefaction (Figure 2A). On CECT, the mass showed slight enhancement in the arterial phase (Figure 2B) and delayed enhancement until the late venous phase (Figure 2C). A CT diagnosis of a gallbladder polyp was suspected before surgery.

A laparoscopic cholecystectomy was performed to remove the mass. The gross specimen showed a 2.5-cm-sized, well-circumscribed, localized mass, which was surrounded by a fibrous capsule. Microscopic examination revealed that the tumor mainly consisted of spindle-shaped cells; neither atypical cells nor signs of malignancy were found (Figure 3A). Immunohistochemical staining showed a strong positive S-100 protein reaction (Figure 3B). Vimentin and CD56 staining were also positive (Figure 3C), whereas CD34 and CD117 were negative (Figure 3D). The final diagnosis of gallbladder schwannoma was made. Until now, twelve months after operation, the patient remained alive and in good status, without signs of recurrence of the lesion.

DISCUSSION

Schwannoma is a benign tumor derived from the Schwann's cells that encapsulates the nerve sheath, so it is known as a neurilemoma. Malignant schwannomas are extremely rare and are always associated with von Recklinghausen's disease^[5,6]. Schwannoma usually occurs in the extremities, but it can also be found in head, neck, trunk, retroperitoneum or mediastinum^[5]. It can also develop in the gallbladder due to the abundant anastomotic network of sympathetic and parasympathetic nerve fibers in the wall of the gallbladder and bile duct^[7]. However, schwannomas arising in gallbladder are extremely rare.

As a result of lacking of adequate knowledge of this tumor and low incidence in clinical practice, correct pre-operative diagnosis is hard to achieve. When tumor was small, patient normally appeared asymptomatic; on the contrary, jaundice and vague pain may happen when tumor was too large to compress surrounding organs. Until now, only five similar cases have been reported (Table 1). In the reported cases of gallbladder schwannomas, two cases revealed obstructive jaundice and vague pain; the other three cases were asymptomatic.

Imaging modalities, such as CT, magnetic resonance imaging (MRI), and US, are useful for detecting and locating tumors; however, definitive diagnosis is rarely achievable. Some cases might even be not visible on imaging examinations when the lesions coexist with cholecystolithiasis or are overly small^[5]. Generally speaking, an unenhanced CT scan depicts a schwannoma as a well-defined hypoattenuating area, and CECT shows peripheral enhancement with an irregular pattern. Delayed peripheral enhancement until the late venous phase on the CT scan reflects a fibrous capsule and an internal fibrillary element^[1,8]. In our case, however, no obvious enhancement during the arterial phase was observed on

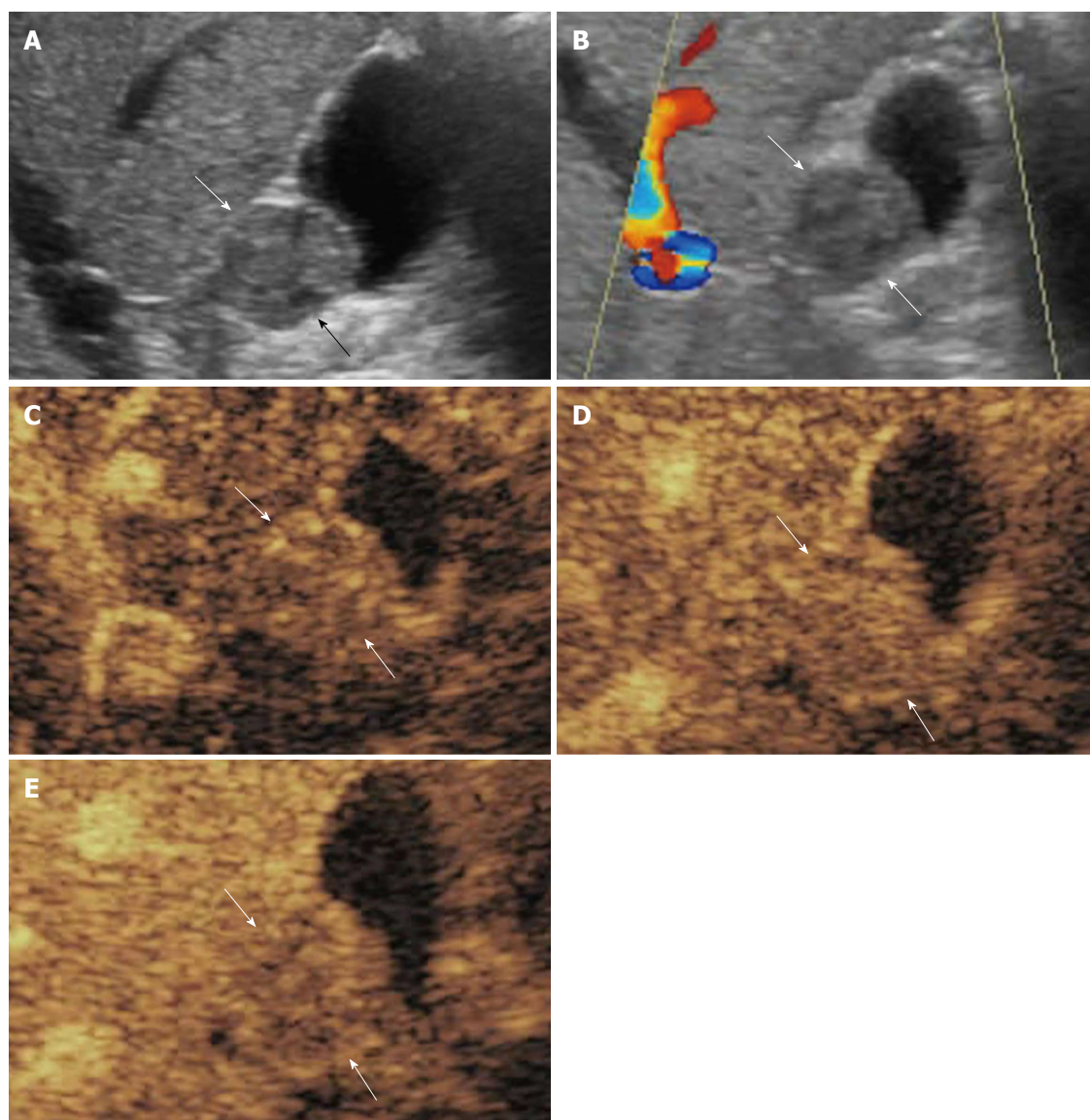


Figure 1 Ultrasound and contrast-enhanced ultrasound imaging. A: Conventional ultrasound shows a lesion (arrows) sized 2.1 cm in diameter in the gallbladder; B: Color Doppler ultrasound shows no blood supply in the mass (arrows); C: The lesion (arrows) shows hyper-enhancement 21 s after contrast agent injection; D: The lesion (arrows) shows iso-enhancement 34 s after contrast agent injection; E: The lesion (arrows) shows slight hypo-enhancement 54 s after contrast agent injection.

CECT. On MRI, schwannomas present as masses of low signal intensity on T1-weighted images and of high signal intensity on T2-weighted images^[2,9].

The US features of this lesion are unknown and non-specific. Ohta *et al*^[5] reported a lesion appearing on US as local gallbladder wall thickening at the fundus of the gallbladder with cholecystolithiasis. The lesion was diagnosed as chronic cholecystitis before surgery. In the present case, the lesion was visualized as a well-defined round isoechoic mass originating from the gallbladder wall on gray-scale US, and no intra-lesional blood flow signals were visible on color Doppler US. Although the lesion was larger than 2 cm in diameter, the wall beneath the mass was intact. No infiltration into the adjacent liver was present. CEUS with SonoVue was used for the preoperative diagnosis, which has not been reported before. The contrast arrival time to the lesion was 19 s after administration of the contrast agent. The lesion showed hyper-

enhancement in the arterial phase of CEUS and began to be hypo-enhanced 54 s after contrast administration. The gallbladder wall under the mass was intact in all of the phases. According to the previous literature regarding gallbladder CEUS^[10-12], malignant gallbladder lesions usually fade more quickly than benign gallbladder lesions, and most malignant gallbladder lesions begin to be hypo-enhanced 50 s before contrast administration. In addition, the gallbladder wall beneath the malignant lesion is usually destroyed, whereas it remains intact with benign gallbladder lesions.

Immunohistochemical analysis is necessary to distinguish schwannomas from neurofibromas, gastrointestinal stromal tumors and leiomyomas^[13]. Schwannomas are strongly positive for vimentin and S100 protein while negative for muscle cell markers; CD117 and CD34 are also useful clues for schwannoma, whereas positive staining has been helpful for the immunohistochemical diag-

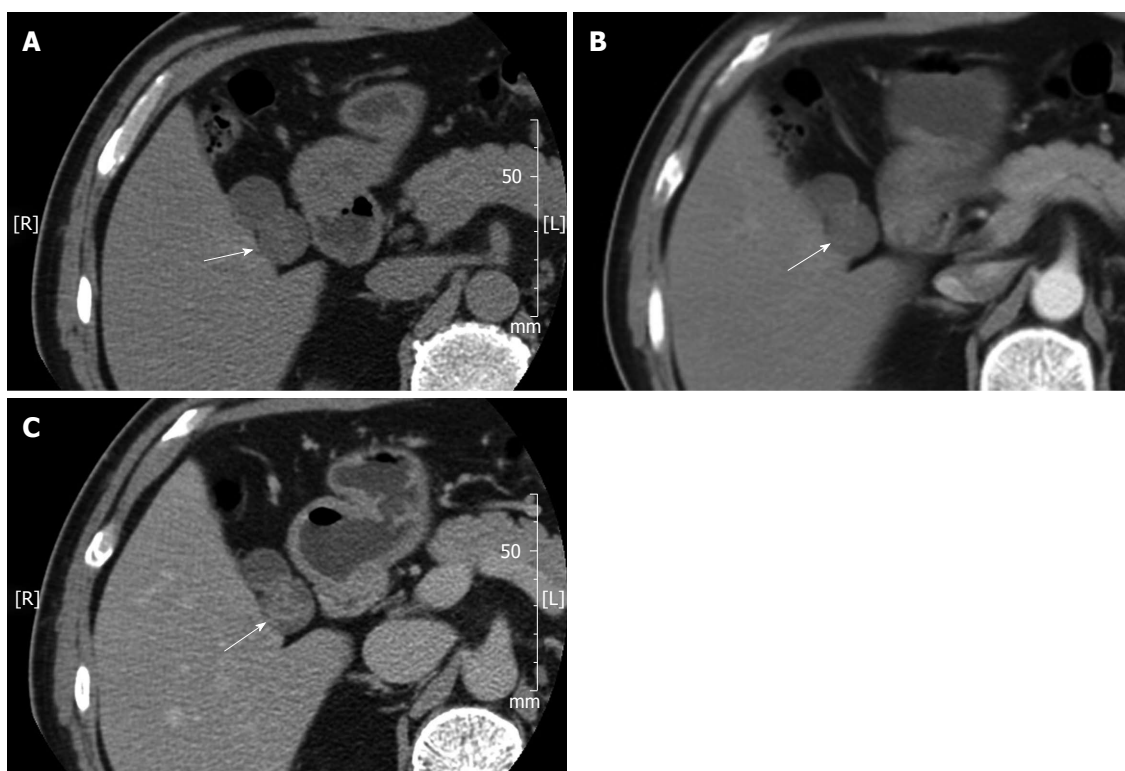


Figure 2 Gallbladder computed tomography imaging. A: Unenhanced computed tomography shows an iso-attenuating mass (arrow) in the gallbladder; B: No obvious enhancement (arrow) was found in the arterial dominant phase; C: The mass (arrow) shows slight hyper-attenuating in the late venous phase.

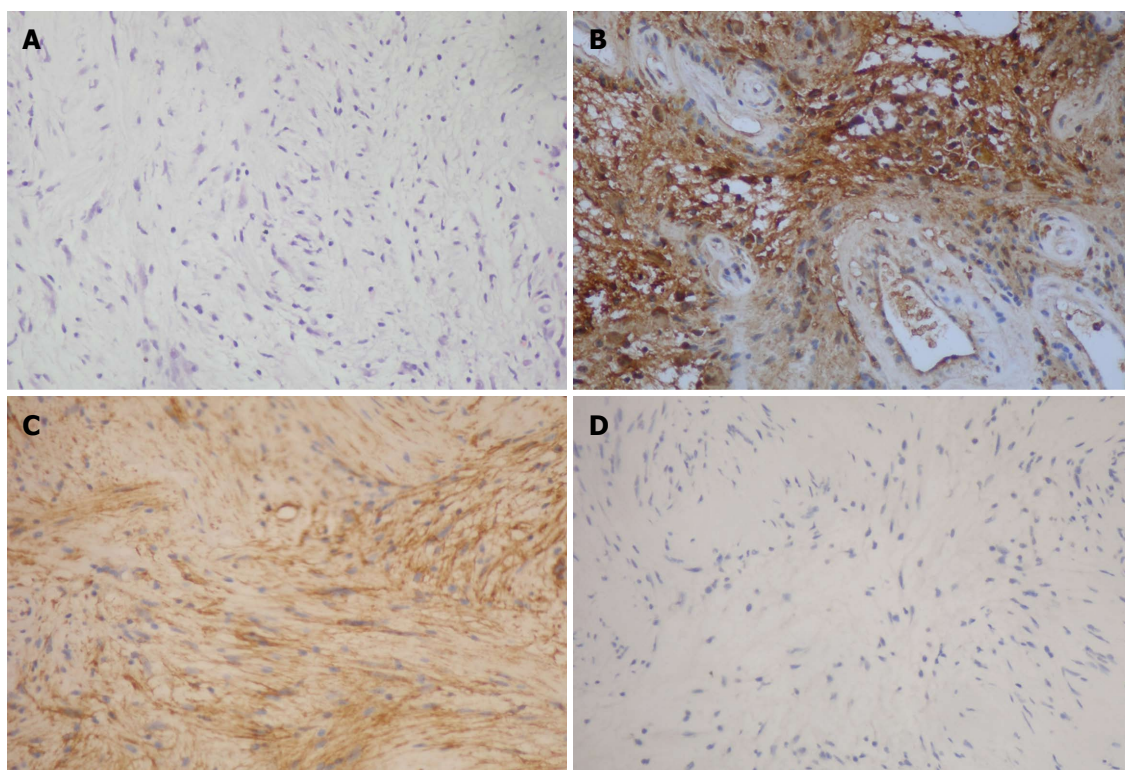


Figure 3 Pathological examinations. A: Microscopic examination shows that the tumor mainly composes of spindle-shaped cells and no atypical cells are found (HE staining; magnification: 10×30); B, C, D: Immunohistochemical staining shows the tumor is positive for S-100 protein (10×30) (B) and CD56(10×30) (C), whereas negative for CD117 (10×30) (D).

Table 1 Reported cases of schwannomas of the gallbladder

| Ref. | Year | Sex | Age | Symptom | Imaging method | Location | Size (mm) | Preoperative diagnosis | With GB stones | Treatment | Follow-up | Status |
|---------------------------------------|------|-----|-----|--------------|-------------------------|----------|-----------|------------------------|----------------|-------------------------------|-----------|----------|
| Yamagiwa <i>et al</i> ^[18] | 1991 | M | 58 | Jaundice | NA | Neck | 4 | Bile duct cancer | NA | Cholecystectomy | NA | NA |
| Matsuoka <i>et al</i> ^[19] | 1996 | M | 74 | Asymptomatic | US MRI CT and endoscopy | Fundus | 10 | Adenomyomatosis | N | Cholecystectomy + hepatectomy | NA | NA |
| Ren <i>et al</i> ^[20] | 2001 | F | 26 | Vague pain | US and CECT | Neck | 110 | Tumor of GB | N | Cholecystectomy | NA | NA |
| Colović <i>et al</i> ^[21] | 2003 | F | 61 | NA | NA | Whole | 90 | Tumor of GB | N | Cholecystectomy | 10 Y | Survived |
| Ohta <i>et al</i> ^[5] | 2008 | M | 58 | Asymptomatic | CT and US | Fundus | 3 | Cholecystolithiasis | Y | Cholecystectomy | 16 M | Survived |
| Current case | 2012 | M | 55 | Asymptomatic | CEUS and CECT | Neck | 22 | Adenomyomatosis | N | Cholecystectomy | 12 M | Survived |

NA: Not available; CEUS: Contrast-enhanced ultrasound; CECT: Contrast-enhanced computed tomography; US: Ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging.

nosis of gastrointestinal stromal tumors^[5,14].

Histologically, schwannomas originating in the digestive tract are S-100 protein-positive spindle cell tumors that consist mainly of cellular (Antoni A) areas, and they generally do not show a nuclear palisading pattern, which is usually found in conventional schwannomas of the soft tissue and central nervous system^[2,15,16]. Whether lack neurofibromatosis-2 genetic alterations might be the key point to distinct schwannomas of the digestive tract from conventional schwannomas^[2,17].

Schwannomas of the gallbladder can be successfully treated surgically, like schwannomas in other locations^[1]. The treatment of choice is cholecystectomy due to the diagnostic uncertainty before surgery, even with extensive application of various imaging modalities. To our knowledge, this was the first time to evaluate the features and usefulness of CEUS in diagnosing solitary schwannoma of the gallbladder. CEUS can easily excluding the possibility of malignant GB disease.

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COMMENTS

Case characteristics

A rare case of gallbladder schwannoma was incidentally detected in a 55-year-old man by ultrasound (US) during a health examination.

Clinical diagnosis

The patient had no symptoms, such as obstructive jaundice or pain.

Differential diagnosis

Gallbladder polyp; gallbladder adenomyomatosis; gallbladder cancer.

Laboratory diagnosis

Leukocyte count, $6.86 \times 10^9/L$; hemoglobin, 159 g/L; total protein, 80 g/L; total albumin, 50 g/L; total bilirubin, 13.4 $\mu\text{mol/L}$; direct bilirubin, 10.8 $\mu\text{mol/L}$; serum alanine aminotransferase 16.1 U/L; serum alkaline phosphatase, 53.4 U/L; serum gamma-glutamyltranspeptidase, 31.4 U/L; carcinoembryonic antigen, alpha-fetoprotein and liver function tests were within the normal limits.

Imaging diagnosis

US showed a 2.1-cm, iso-echoic, well-defined, mural mass in the gallbladder;

contrast-enhanced ultrasound (CEUS) showed the mass slightly was hyper-enhanced in the arterial phase and slightly hypo-enhanced in the venous phase; moreover, the gallbladder wall under the mass was intact.

Pathological diagnosis

Microscopic examination revealed that the tumor mainly consisted of spindle-shaped cells; it was S-100/Vimentin/CD56-positive and CD34/CD117-negative.

Treatment

The patient was treated with cholecystectomy.

Related reports

Schwannomas arising in the gallbladder are extremely rare, and preoperative diagnosis appears to be very difficult because these tumors are commonly asymptomatic and are often discovered incidentally.

Term explanation

To our knowledge, the CEUS features of gallbladder schwannomas have not been reported before in the other literature.

Experiences and lessons

This report not only presents the details of the US and CEUS features in this case, but it also indicates the usefulness of CEUS in excluding malignancy before surgery.

Peer review

This article presents a case of gallbladder schwannoma with an emphasis on the imaging and pathological findings; also, biliary system schwannomas in the literature are reviewed.

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Reversible posterior leukoencephalopathy syndrome induced by bevacizumab plus chemotherapy in colorectal cancer

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Key words: Bevacizumab; Reversible posterior leukoencephalopathy syndrome; Colorectal cancer; Hypertension; Coma

Core tip: This is the first report of reversible posterior leukoencephalopathy syndrome (RPLS) induced by bevacizumab in China. RPLS is a rare complication of bevacizumab, but may present with life-threatening symptoms such as coma. Sudden blood pressure increase is the most common risk factor, and early recognition and prompt control of blood pressure may make this complication reversible.

Abstract

Reversible posterior leukoencephalopathy syndrome (RPLS) is a rare brain-capillary leak syndrome, characterized by clinical symptoms of headache, visual loss, seizures and altered mental functioning. This syndrome is usually reversible and is associated with hypertension, nephropathy, and use of immunosuppressive medication and cytotoxic agents. We describe two rare cases of RPLS occurring in colorectal cancer, both of which presented with coma, that we believe can be directly attributed to bevacizumab, a monoclonal antibody that inhibits the angiogenesis of tumours by specifically blocking vascular endothelial growth factor. We analysed the clinical features, risk factors and outcomes of RPLS in these two patients, and although no typical finding was identified on imaging examination, we found that inadequate blood pressure control was one of the risk factors leading to RPLS and that supportive treatment including intensive blood pressure control improved outcomes. Due to the increasing use of bevacizumab in colorectal cancer, clinicians should be aware of this potential complication.

Wang W, Zhao LR, Lin XQ, Feng F. Reversible posterior leukoencephalopathy syndrome induced by bevacizumab plus chemotherapy in colorectal cancer. *World J Gastroenterol* 2014; 20(21): 6691-6697 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i21/6691.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6691>

INTRODUCTION

Reversible posterior leukoencephalopathy syndrome (RPLS), first described by Hinchey *et al*^[1] in 1996, is also known as posterior reversible encephalopathy syndrome. RPLS is an underappreciated syndrome characterized by clinical symptoms of headache, altered mental functioning, visual loss, and seizures, and radiological findings by magnetic resonance imaging (MRI) of sub-cortical oedema predominantly in the posterior cerebral white matter. In most cases both the symptoms and radiological features of RPLS are reversible. The precise pathophysiology of RPLS remains uncertain. Deficiency in cerebrovascular auto-regulation is a favoured hypothe-

A 56-year-old female patient was diagnosed as having Stage IV rectal cancer and developed retroperitoneal and left supraclavicular lymph node metastasis in March 2011, as confirmed by PET-CT scan and colonoscopy. She was treated with three cycles of “FOLFIRI + bevacizumab” regimen (bevacizumab 300 mg IV infusion on D1, iri-

notecan 270 mg IV infusion on D1, leucovorin 0.3 IV infusion on D1, 5-FU 0.6 IV bolus on D1, 5-FU 3.6 continuous IV 46 h, repeated every 2 wk). The patient had no history of hypertension. Her blood pressure and urine protein were normal during treatment. The third cycle of chemotherapy was given on April 9th, 2011. The patient developed coma and convulsion of the limbs at 6:00 AM on April 11th, and her blood pressure was 126/81 mmHg at that point. She had a normal neurological examination. Infusion of 5-FU was withdrawn immediately. The patient was treated intravenously to reduce intracranial hypertension and was given medication for sedation. Continuous blood pressure monitoring recorded that blood pressure was stable at around 105-165/63-92 mmHg. Eight hours after coma an enhanced computed tomography (CT) scan of the brain showed mild brain atrophy (Figure 1). After 12 h of coma, she recovered with some verbal response but was still aphasic. Her limbs demonstrated some involuntary movements. The administration was withdrawn. On the morning of April 14th, she showed complete recovery. She could answer questions correctly and walk around. Enhanced MRI scan and venography (MRV) on April 16th showed (Figure 2) mottled lesions in the left parietal lobe, which was a possible microhaemorrhage, but no other abnormal findings. The patient was discharged on April 18th. Subsequent follow-ups documented partial response to treatment. Given the toxicity of the previous treatment, her subsequent regimen was switched to palliative chemotherapy alone without monoclonal antibody.

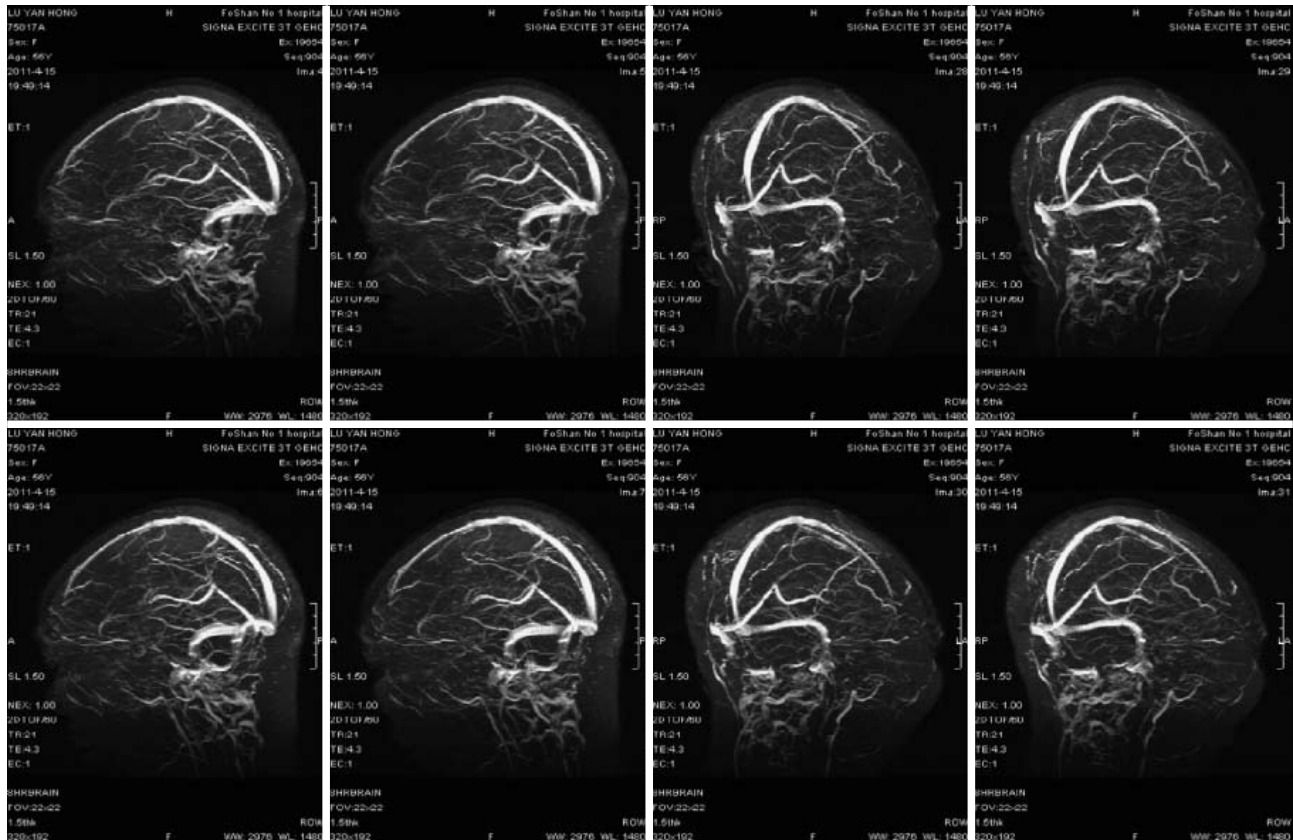


Figure 2 Enhanced brain magnetic resonance imaging and magnetic resonance venography scan of case 1 (2 d after recovery). Mottled lesions of the left parietal lobe, indicating intracerebral microbleeds.

Case 2

A 58-year-old female was admitted with abdominal pain for more than 1 mo. She was previously diagnosed as having Stage IV carcinoma of the descending colon in January 2011 with extensive metastasis, including mesenteric, mesenteric root, retroperitoneal, bilateral hilar, mediastinal and bilateral supra- and infra-clavicular lymph nodes based on enteroscopy, lymph node puncture and biopsy, and PET-CT scan. She received eight cycles of “mFOLFOX6 + bevacizumab” combination regimen (bevacizumab 300 mg IV infusion on D1, oxaliplatin 150 mg IV infusion on D1, leucovorin 0.68 IV bolus on D1, 5-FU 0.68 IV bolus on D1, 5-FU 4.0 CIV 46 h, repeated every 2 weeks). The last dose of chemotherapy was administered on May 21st, 2011. The patient had a history of hypertension and bronchitis for 10 years. No records were available about her medication. Blood pressure monitoring and urine protein were normal on admission. On the morning of June 4th, the patient presented severe headache and dizziness. Her blood pressure was 225/135 mmHg. Other vital signs were normal. She was treated intravenously with uradil hydrochloride as well as oral amlodipine, hydrochlorothiazide and spironolactone, but the symptom was not relieved. Continuous blood pressure monitoring showed blood pressure around 127-191/80-165 mmHg. The patient then developed coma, restlessness, muscle weakness of the left limbs, and pathological signs in the left lower

limb on June 7th. Right cerebral infarction was suspected. CT scan and CTA (CT angiography) of the brain (Figure 3) showed mild cerebral atherosclerosis; narrowing in the A1 segment of the right anterior cerebral artery and the intracranial segment of the right vertebral artery, but appropriate distal blood supply; mild brain atrophy and a small lacunar infarction in the bilateral region of the basal ganglia. Symptomatic treatment was maintained. On the morning of June 8th, her blood pressure was 152/82 mmHg. She recovered consciousness and the muscle strength of her limbs and could eat food. On June 9th, the patient experienced mild dizziness but blood pressure was normal. The intravenous antihypertensive drug was discontinued. The results of a CT scan and CTA of the brain were similar to those obtained previously (Figure 4). The patient then received oral medications to maintain normal blood pressure. She was discharged from hospital on June 15th. Follow-up examination showed that her tumour partially responded to the chemotherapy. The patient is currently undergoing regular follow-up.

DISCUSSION

The two cases described above were diagnosed as RPLS according to current clinical guidelines. To the best of our knowledge these are the first series of cases in China of RPLS-associated coma induced by bevacizumab com-

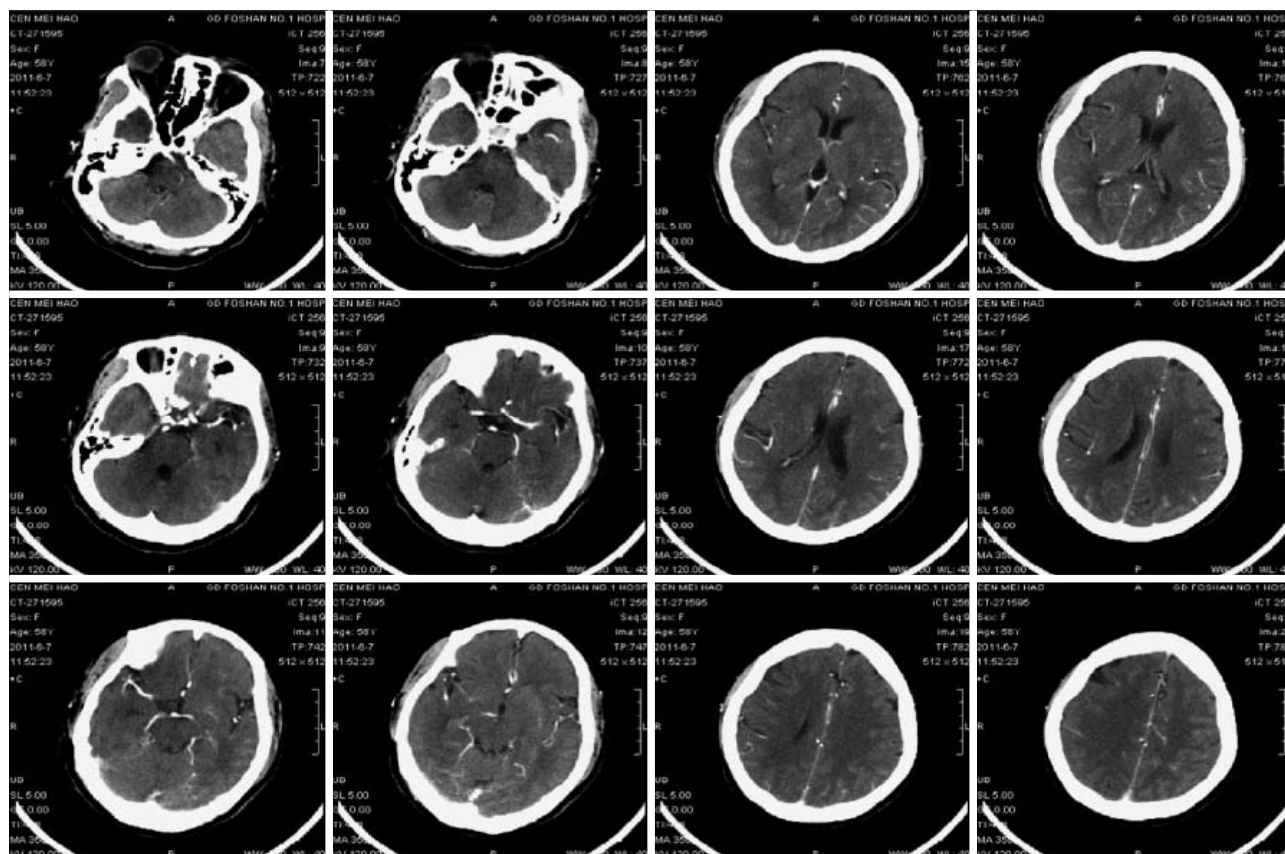


Figure 3 Enhanced computed tomography and magnetic resonance venography scan of the brain of case 2. Mild cerebral arteriosclerosis, stenosis of the A1 segment of the right anterior cerebral artery and right intracranial artery, and effective blood supply of distal vascular and mild lacunar infarction of bilateral basal ganglia.

bined with chemotherapy. RPLS is a rare brain-capillary leak syndrome, and is associated with hypertension, nephropathy, and use of immunosuppressive medication and cytotoxic agents (*e.g.*, cyclosporin A, tacrolimus). The clinical manifestations include rapidly progressing intracranial hypertension, seizure, visual disorder, disturbance of consciousness, and mental disorder. Coma is rarely seen in RPLS. The imaging findings of RPLS are characteristic of reversible extensive oedema in the white matter of bilateral posterior cerebral hemispheres, frontal lobe, region of basal ganglia, brain stem, cerebellum, and cerebral cortex. The diagnosis of RPLS primarily depends on MRI, however typical imaging findings may not always be present.

Although the pathogenesis of RPLS is not yet fully understood two common theories have been described. The first one argues that a sudden increase in blood pressure could lead to the dysfunction of cerebral vascular autoregulation, including deficiency of vasodilative prostaglandin release and cerebral vascular endothelial dysfunction. Even a mild rise in blood pressure, if acute, especially in the presence of an underlying endothelial dysfunction, may result in breakdown of the blood-brain barrier, pathological vasodilation, and capillary leakage, leading to extravasation of fluids into the brain parenchyma. As a result, vasospasm and brain hypoperfusion, activation of the coagulation system and fluid effusion eventually take place. For this reason RPLS is also

known as “hypertensive encephalopathy”. The posterior cerebral circulation is more susceptible to such injury, probably due to the presence of fewer adrenergic nerves in the posterior cerebral circulation system, which makes the blood vessels more sensitive to sudden changes in blood pressure^[4].

The second theory states that toxic substances such as immunosuppressive agents could directly lead to transient impairment of the blood-brain barrier by injuring the vascular endothelium. Reconditioning vasoconstriction or microthrombosis will result in occlusion of the cerebral artery, cerebral ischaemia/hypoxia and vasogenic oedema^[5].

Bevacizumab is a monoclonal antibody that inhibits the angiogenesis of tumours by specifically blocking vascular endothelial growth factor (VEGF). Bevacizumab is commonly used in combination with various chemotherapy regimens to provide additional survival benefits to patients with metastatic colorectal cancer^[6]. Currently, the NCCN guideline recommends bevacizumab in combination with chemotherapy as the standard treatment regimen for metastatic colorectal cancer. The two patients in this report received bevacizumab as well as chemotherapy agents, including oxaliplatin, irinotecan, 5-FU, *etc.* It has been reported that 5-FU can cause a rare kind of encephalopathy, known as multifocal inflammatory leukoencephalopathy, which usually occurs from 6 wk to 5 mo after 5-FU treatment. However, the clinical and imaging

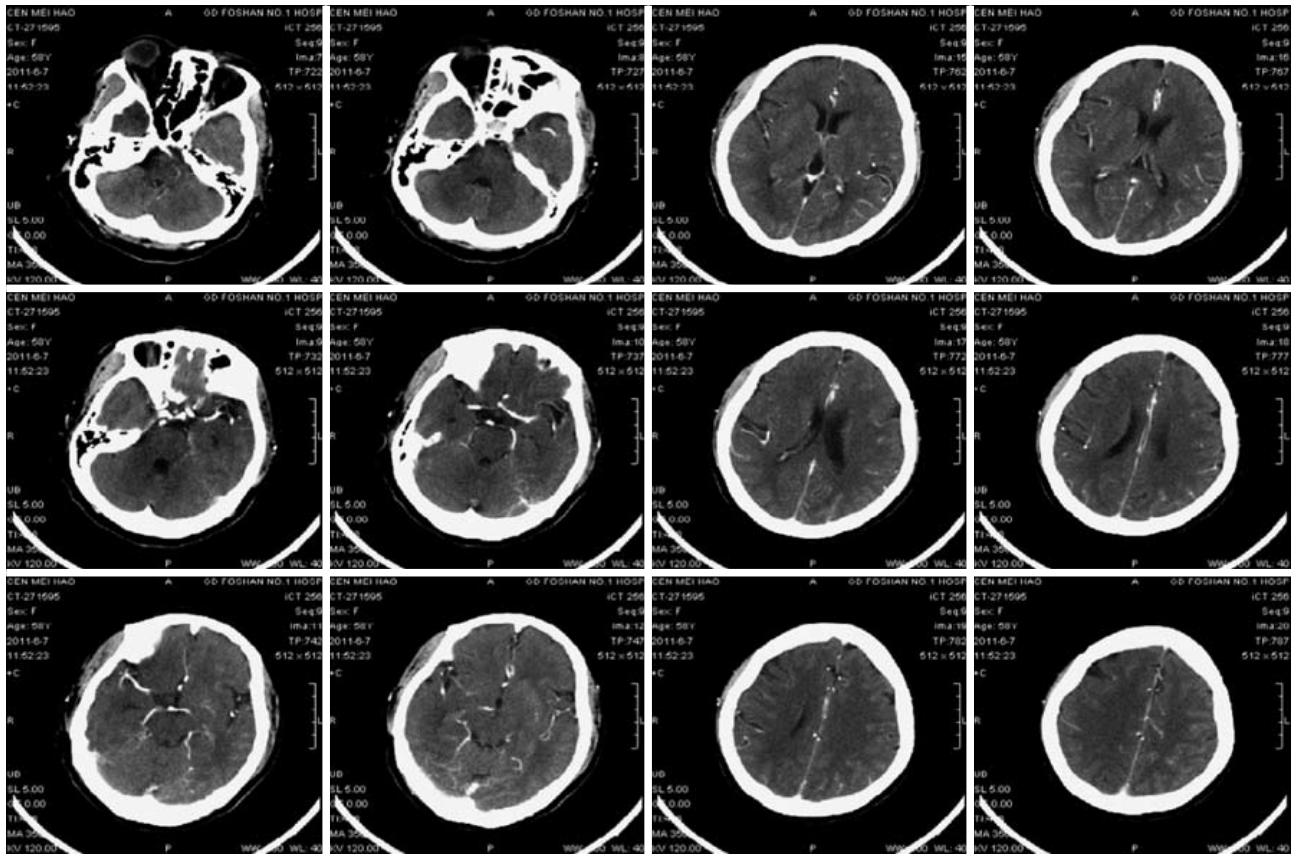


Figure 4 Repeat enhanced computed tomography and magnetic resonance venography scan of the brain of case 2 on the following day showed the same image changes as previously described.

features differ from those of RPLS. In our cases leukoencephalopathy can be excluded^[7]. To date, neither RPLS nor other relevant encephalopathies have been reported to be induced by irinotecan or oxaliplatin. In March 2006, Professor Glusker *et al*^[8] of Stanford University reported the first case of bevacizumab-induced typical RPLS in the New England Journal of Medicine. Furthermore, the United States Food and Drug Administration updated the safety information of bevacizumab on September 25, 2006, indicating that healthcare professionals should pay attention to RPLS, a rare adverse reaction during bevacizumab treatment. Hypertension is the most frequently reported adverse reaction during bevacizumab treatment. Bevacizumab may cause RPLS *via* the following possible mechanisms: sudden blood pressure rise during bevacizumab treatment causes dysfunction of cerebral vascular autoregulation. Moreover, bevacizumab can disrupt the blood-brain barrier by extensively impairing the endothelium. When blood pressure in the systemic circulation increases, the above changes can cause vasogenic oedema and eventually RPLS^[9,10].

RPLS may occur at any time during bevacizumab treatment. However, in most cases it develops within the half-life (about 20 d) of bevacizumab^[8,11,12]. Typical RPLS-related adverse symptoms have been observed in colorectal and renal cancers treated with bevacizumab combination chemotherapy^[12,13]. RPLS usually occurs during the first seven cycles of bevacizumab treatment.

The interval between the administration of bevacizumab and onset of RPLS ranges from 16 h to 11 d. The first patient in our report developed RPLS on day 2 of the third cycle of bevacizumab therapy. The second patient developed RPLS on day 17 after eight cycles of treatment. These are consistent with previous reports.

Poor blood pressure control is the most important risk factor for RPLS. Most cases of RPLS are associated with increased blood pressure. The second patient in this report developed RPLS when her blood pressure was not controlled appropriately. The first patient also experienced increased blood pressure before she developed RPLS. Generally, if grade 2 or higher hypertension (according to NCI-CTC, grade 2 hypertension is defined as diastolic blood pressure increase > 20 mmHg, or > 150/100 mmHg if previously normal blood pressure) is documented, it is recommended that the offending agent should be withdrawn as soon as possible and blood pressure should be controlled^[14].

Fortunately RPLS is reversible. Immediate diagnosis, proper blood pressure control and withdrawal of the implicated drugs will enable recovery of the clinical and imaging findings. Although some patients may develop progressive neurological symptoms, these will generally improve or resolve within several days. Instant and effective blood pressure control is the primary objective of managing RPLS. If malignant hypertension is present, the diastolic blood pressure must be reduced at a steady

speed to below < 100 mmHg within several hours. Blood pressure control is recommended for even mild hypertension. Intravenous antihypertensive agents, *e.g.*, sodium nitroprusside and nicardipine, are recommended for rapid onset. Such intravenous therapy can also maintain adequate cerebral perfusion pressure.

It is not clear whether it is safe for patients who have experienced RPLS to continue bevacizumab, although discontinuation of bevacizumab is recommended. Since it became available on the market 6 years ago, five cases of bevacizumab-induced RLPS have been reported worldwide^[15], while no similar case has ever been reported in China since it entered the Chinese market in 2010. Indeed, only two out of 30 cases developed RLPS induced by bevacizumab in combination with chemotherapy.

The lack of typical imaging or thrombotic changes in the central nervous system makes early recognition of RPLS crucial. Whenever coma is present during bevacizumab treatment, RPLS should be considered, especially when complicated with hypertension. Moreover, bevacizumab combination with chemotherapy should be carefully used in patients with a history of hypertension, and blood pressure should be monitored closely during bevacizumab therapy. Precaution and timely management are vital to prevent coma. RLPS is a reversible complication if handled appropriately.

In conclusion, these are the first cases of coma of RPLS induced by bevacizumab combination chemotherapy reported in China. Although usually reversible, RPLS is a serious and potentially life-threatening syndrome and its association with hypertension in the setting of bevacizumab combination chemotherapy should be recognized. In addition, a history of hypertension should be addressed prior to the combination regimen. If RPLS develops, a less toxic regimen should be considered to prevent possible effects on future cognitive function.

COMMENTS

Case characteristics

Two colorectal cancer patients treated with bevacizumab plus chemotherapy presented with the rare complication of reversible posterior leukoencephalopathy syndrome (RPLS).

Clinical diagnosis

Reversible clinical symptoms of coma after treatment with bevacizumab.

Differential diagnosis

Multifocal inflammatory leukoencephalopathy, hypertensive encephalopathy, encephalitis, demyelinating diseases.

Imaging diagnosis

No typical finding was identified on imaging examination.

Pathological diagnosis

Rectal cancer and colon cancer.

Treatment

The two patients received "FOLFIRI + bevacizumab" and "mFOLFOX6 + bevacizumab" anticancer treatment separately.

Related reports

Bevacizumab combination with chemotherapy should be carefully used in patients with a history of hypertension, and blood pressure should be monitored closely during bevacizumab therapy.

Term explanation

RPLS is a syndrome characterized by clinical symptoms of headache, altered mental functioning, visual loss and seizures, and is associated with hypertension, nephropathy, and use of immunosuppressive medication and cytotoxic agents.

Experiences and lessons

RPLS is a rare complication of bevacizumab, and may present with life-threatening symptoms such as coma; however, early recognition and prompt control of blood pressure may make this complication reversible.

Peer review

This article demonstrates a rare complication of bevacizumab in colorectal cancer, and given the increasing use of bevacizumab, clinicians should be aware of this potential complication.

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Application of blunt dissection in ESD of a gastric submucosal tumor

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Author contributions: Wen ZQ and Wu GY were the main contributors to this work, and are co-first authors; Yu SP, Lin XD and Li SH guided and helped to diagnosis the patient; Wu GY, Huang XG and Zhang F obtained the data; Wu GY analyzed the data; Wen ZQ and Zeng XY performed the surgery; Huang HY and Li AM assisted in the surgery; Wen ZQ and Wu GY wrote the report; Yu SP guided the work; all authors approved the final version.

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Key words: Blunt dissection; Endoscopic submucosal dissection; Gastric fundus submucosal tumor

Core tip: We performed endoscopic submucosal dissection (ESD) of a gastric fundus tumor. It was difficult to strip the tumor completely due to space limitation, therefore, we used blunt dissection and removed the tumor quickly and safely. This is a new method based on traditional ESD, and ensured quick and safe removal of the tumor in this patient.

Wen ZQ, Wu GY, Yu SP, Lin XD, Li SH, Huang XG, Zhang F, Zeng XY, Huang HY, Li AM. Application of blunt dissection in ESD of a gastric submucosal tumor. *World J Gastroenterol* 2014; 20(21): 6698-6700 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v20/i21/6698.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i21.6698>

Abstract

We performed endoscopic submucosal dissection of a gastric fundus tumor. It was difficult to strip the tumor completely due to space limitation, and we used blunt dissection to remove the tumor quickly and safely. Firstly, the basal area of the 2.5 cm submucosal tumor located in the gastric fundus was cut open, and the mucosa was dissected. The tumor was difficult to peel, therefore, a snare was used and the tumor was pulled and tightened slightly. Short electronic coagulation was used during the procedure. The tumor was then bluntly dissected. This method ensured rapid and complete removal of the tumor.

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INTRODUCTION

Endoscopic submucosal dissection of a submucosal tumor located in the gastric fundus adjacent to the gastric cardiac region or which has extended into the cardioesophageal junction, is difficult to perform due to space limitation. Recently, we performed endoscopic submucosal dissection (ESD) of a 2.5 cm gastric fundus submucosal tumor adjacent to the gastric cardiac region. The tumor was removed safely and quickly using blunt dissection.

CASE REPORT

Patient

A 30-year-old female was admitted due to recurrent nausea of 3 years, which had worsened in the previous 2 wk. A neoplasm was found in the stomach. Computed to-

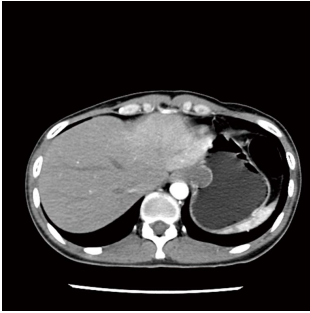


Figure 1 Computed tomography revealed a tumor located in the gastric fundus adjacent to gastric cardiac region.

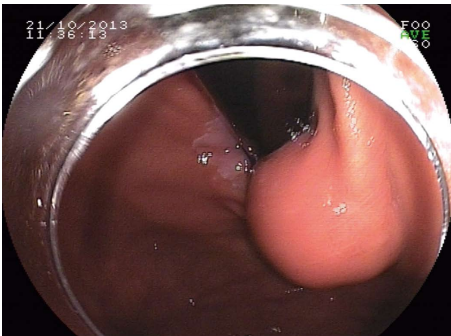


Figure 2 Gastroscopy showed the gastric fundus tumor.

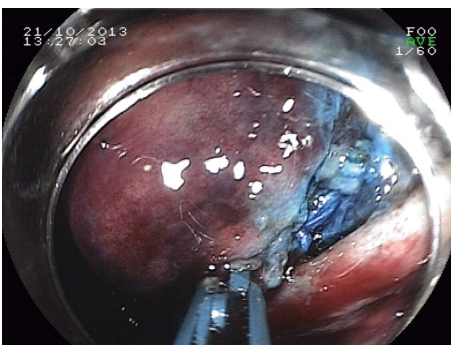


Figure 3 Endoscopic submucosal dissection of the basal area of the tumor.

mography of the epigastric zone showed irregular thickening of the gastric fundus wall adjacent to the gastric cardiac region, local nodosity, and the tumor was found to be approximately 2.4 cm in size (Figure 1). The first therapeutic choice was ESD.

Method

A hyaline cap was placed in front of the gastroscope and a hemispheroid submucosal tumor approximately 2.0 cm × 2.5 cm was seen in the gastric fundus (Figure 2). The range was marked, and a mixture of methylene blue, epinephrine, and physiological saline was injected. The mucosa was incised, the tumor was removed from the basal area using an IT knife, and the submucosa was dissected using a cut knife (Figure 3). The patient's tumor

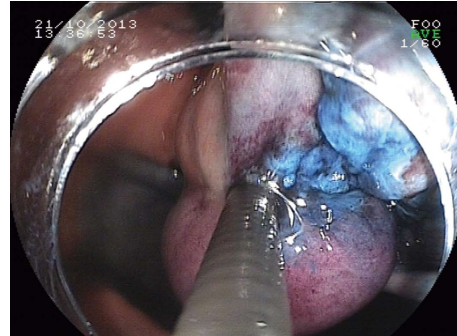


Figure 4 Blunt dissection using the spring rolling pattern.

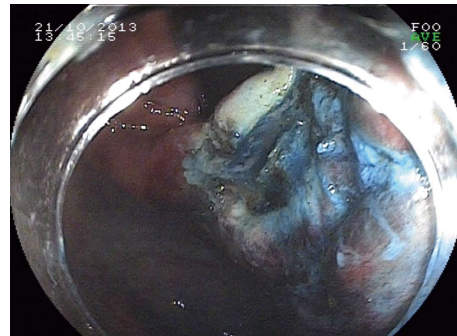


Figure 5 Base of the tumor after dissection, no perforation can be seen.

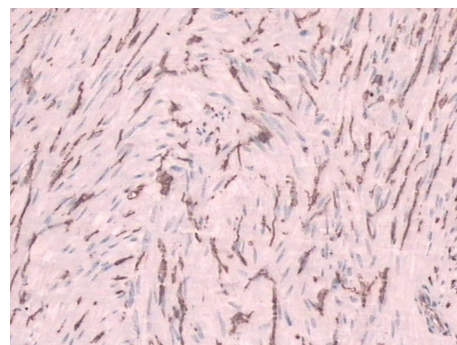


Figure 6 Immunohistochemistry confirmed the diagnosis of gastrointestinal stromal tumor.

extended to the gastric cardiac region, and it was difficult to peel from the submucosa due to space limitation. The submucosa around the tumor was injected with the above-mentioned solution, the tumor was removed using a snare which was pulled and tightened slightly around the tumor and short electronic coagulation was used during the procedure. This process was carefully repeated to avoid excessive traction. The tumor was bluntly dissected cautiously to avoid bleeding caused by mechanical cutting (Figure 4). The tumor was completely removed, the raw surface was clean with no residual tumor, bleeding or perforation (Figure 5). Pathology and immunohistochemistry results confirmed that the tumor was a gastrointestinal stromal tumor, with a low risk of malignancy (Figure 6). The patient was discharged after 5 d of observation

without bleeding, perforation or other complications.

DISCUSSION

Gastrointestinal stromal tumors are the most common mesenchymal tissue tumors in the gastrointestinal tract, and arise from the Cajal mesenchymal cells or their co-stem cells^[1]. The standard treatment is laparoscopy or laparotomy. Endoscopic therapy has been gradually developed in recent years, and the main endoscopic techniques include endoscopic band ligation, endoscopic submucosal dissection (ESD), endoscopic mucosal resection and endoscopic full-thickness resection. En bloc dissection of stromal tumors, whatever the size or shape of the tumor, is an advantage of ESD. During electromagnetic resonance, the neoplastic tissue is resected rapidly, and the endoscopist has little or no control in adjusting the plane or the margin of resection; during ESD, the endoscopist deliberately and diligently creates a plane of dissection through the submucosa, while attempting to achieve a margin that is free of neoplastic tissue^[2]. Endoscopic therapy has developed rapidly in recent years, and several methods had been modified based on ESD. BR Liu *et al.*^[3] reported an endoscopic technique known as endoscopic muscularis dissection for removing lesions located in upper gastrointestinal subepithelium. Takizawa *et al.*^[4] reported a technique to remove lesions in the colon using blunt balloon dissection.

In our patient, we examined the status of the tumor and the gastric fundus wall using computed tomography, and initially determined the possibility and risk of endoscopic therapy in this patient. ESD was the first choice in the treatment of this tumor. When dissected, the tumor body was exposed during the submucosa dissection, and it was difficult to peel the tumor tissue accurately and effectively using the cut knife due to space limitation. Following submucosa injection around the tumor, the tumor was removed at the basal area using a snare, which was slightly pulled and tightened around the tumor. Short electronic coagulation was used during the procedure. This process was carefully repeated to avoid excessive traction. The tumor was bluntly dissected cautiously, as mechanical cutting needs to be well managed to avoid excessive traction, and the tumor was completely removed. It is impossible to bluntly dissect the tumor with a snare directly without ESD. The snare may enclose too much or too little tissue, which makes it impossible to manage the range of dissection and the tissue can easily be perfo-

rated. The repair of perforated tissue is complex. In this case, the use of blunt dissection allowed peeling of the tumor, resulting in a good outcome.

COMMENTS

Case characteristics

Recurrent nausea of 3 years which worsened in the previous 2 wk. A neoplasm was found in the stomach.

Clinical diagnosis

Submucosal tumor of the gastric fundus.

Differential diagnosis

The tumor seemed to be benign from gastroscopy and computed tomography.

Laboratory diagnosis

Blood tests for tumor markers were all negative.

Imaging diagnosis

A hemispheroid submucosal tumor approximately 2.0 cm × 2.5 cm was seen in the gastric fundus by gastroscopy and computed tomography of the epigastric zone revealed irregular thickening of the gastric fundus wall adjacent to the gastric cardiac region.

Pathological diagnosis

Pathology and immunohistochemistry results confirmed that the tumor was a gastrointestinal stromal tumor, with a low risk of malignancy.

Treatment

The first treatment choice was endoscopic submucosal dissection (ESD).

Experiences and lessons

ESD of a tumor located in the gastric fundus requires considerable practice, skill, and is a difficult operation. The authors performed ESD of a gastric fundus tumor using blunt dissection, which allowed quick and safe removal of the tumor.

Peer review

This method applied blunt dissection in ESD which removed the tumor quick and safe, it still needs to be tested to prove its safety and time saving, application of gastrointestinal stromal tumor still need to explore.

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Patent (list all authors)

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

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *ν* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂, not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Instructions to authors

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kbo I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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