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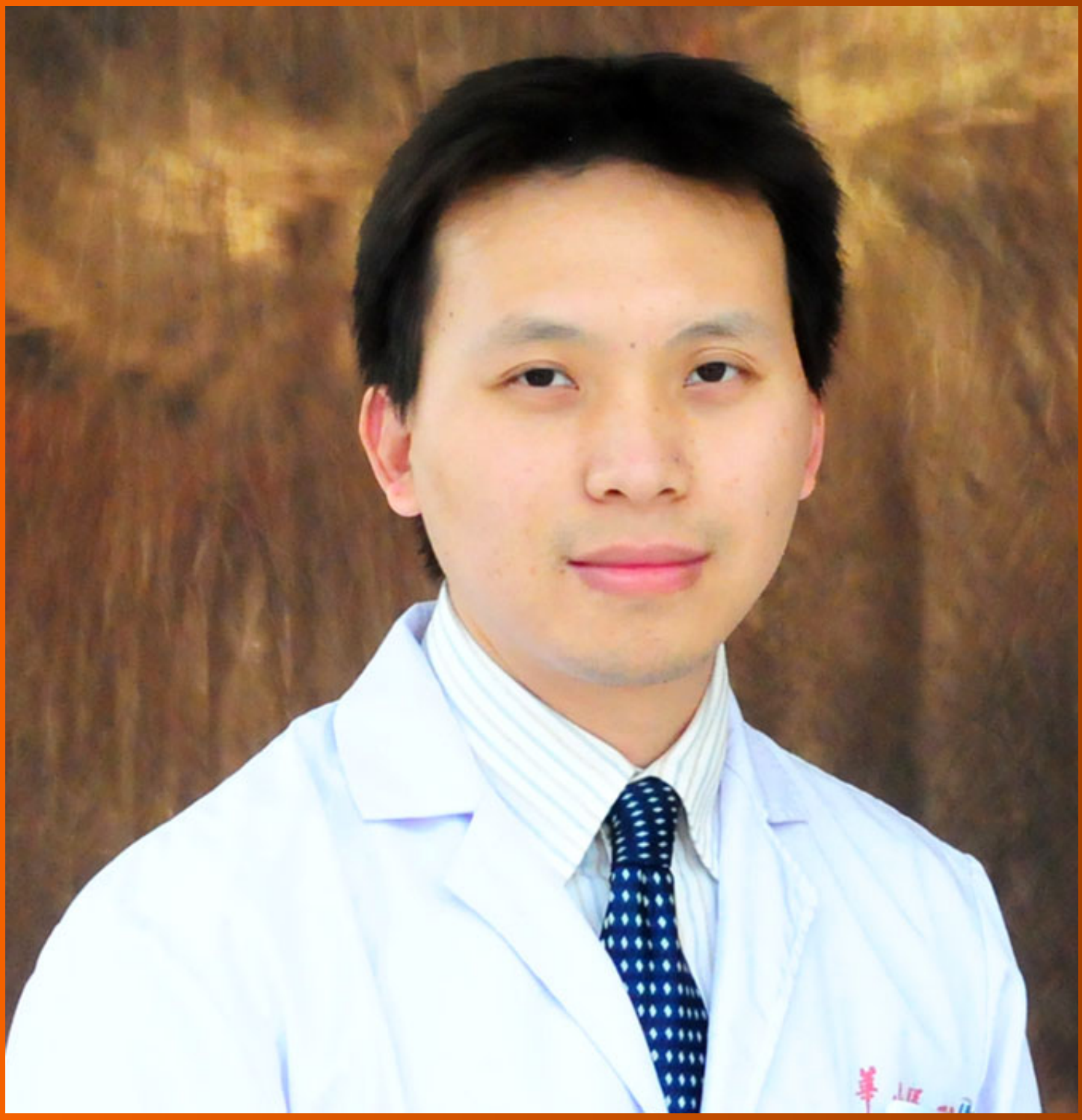

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## ABOUT COVER

Editorial board member of *World Journal of Gastroenterology*, Dr. Feng Yang is a distinguished Associate Professor at the Shanghai Medical College Affiliated to Fudan University in Shanghai, China. Having received his Bachelor's degree from Fudan University in 2003, Dr. Yang undertook his postgraduate study and surgical resident training at the Department of Surgery in Huashan Hospital Affiliated to Fudan University, receiving his PhD in 2008. He became Associate Chief Physician at the Division of Pancreatic Surgery in Huashan Hospital Affiliated to Fudan University in 2015 and has held the position since. His ongoing research interests involve basic and clinical study of metastasis and recurrence of pancreatic neoplasms, including pancreatic carcinomas, neuroendocrine tumors and solid pseudopapillary tumors. Currently, he serves as a Young Member of the Pancreatic Cancer Committee, Chinese Anti-cancer Association. He also serves as a peer reviewer for many international academic journals.

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*REVIEW*

# Impact of mobile health and medical applications on clinical practice in gastroenterology

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## Abstract

Mobile health apps (MHAs) and medical apps (MAs) are becoming increasingly popular as digital interventions in a wide range of health-related applications in almost all sectors of healthcare. The surge in demand for digital medical solutions has been accelerated by the need for new diagnostic and therapeutic methods in the current coronavirus disease 2019 pandemic. This also applies to clinical practice in gastroenterology, which has, in many respects, undergone a recent digital transformation with numerous consequences that will impact patients and health care professionals in the near future. MHAs and MAs are considered to have great potential, especially for chronic diseases, as they can support the self-management of patients in many ways. Despite the great potential associated with the application of MHAs and MAs in gastroenterology and health care in general, there are numerous challenges to be met in the future, including both the ethical and legal aspects of applying this technology. The aim of this article is to provide an overview of the current status of MHA and MA use in the field of gastroenterology, describe the future perspectives in this field and point out some of the challenges that need to be addressed.

**Key words:** Mobile health; Health applications; Medical applications; Technology; Telemedicine; Mobile applications; Smartphone; eHealth; mHealth; Digital biomarker; Electronic health records



**Core tip:** Mobile health apps (MHAs) and medical apps (MAs) are becoming popular as digital interventions in a wide range of health-related applications. This also applies to

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clinical practice in gastroenterology, which will be undergoing a digital transformation in the near future. The aim of this article is to provide an overview of the current status of MHA and MA use in the field of gastroenterology, describe the future perspectives in this field and point out some of the challenges that need to be addressed. Implications of EHR, telemedicine, smartphone apps and digital biomarkers in clinical care will be discussed.

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## INTRODUCTION

The first smartphone, Apple's iPhone, was introduced in 2007, only 13 years ago. Since then, the widespread adoption of smartphones and digital innovations, such as tablets, wearables, smartwatches and other devices, has tremendously changed everyday life and consumer behavior in many ways. The introduction of modern information and communication technologies (ICT) has been one of the most disruptive technological innovations in recent decades. The ubiquitous availability of smartphones, wearables and tablet computers and the widespread internet connectivity have led to a significant change in human-technology interaction[1]. At the same time, the exponential development of computer performance and storage capacities, cloud computing and the application and improvement of artificial intelligence (AI) methods have opened new possibilities for the design of ICT[2]. Mobile health apps (MHAs) and medical apps (MAs) are becoming increasingly popular as digital interventions in a wide range of health-related applications in almost all sectors of healthcare[3]. This also applies to clinical practice in gastroenterology, which has, in many respects, recently undergone a digital transformation that will have numerous consequences for patients and health care professionals in the near future[4-8]. The functionalities and intentions of MHAs and MAs use in gastroenterology are extremely diverse. They range from electronic health record (EHR)[9-11] and workflow management systems to specific mobile apps for the management of chronic or acute pain or the management[12-13] of specific diseases in specific settings[14-16]. MHAs and MAs are considered to have great potential, especially for chronic diseases, as they can support the self-management of patients in many ways[17,18].

During the current pandemic outbreak of the novel coronavirus caused respiratory disease (coronavirus disease 2019) the use of MHAs and MAs and telemedical solutions has tremendously increased[19]. The use of these digital technologies to set up virtual clinics, telemedical consultations, remote interpreting of data and virtual education platforms is ideal for continuing medical care during situations of local government issued curfews and shortage of specialized workforces[19]. Therefore, during the current pandemic situation in the field of gastroenterology the use of ICT has been already used for managing patients with chronic liver disease (CLD)[20] or new-onset type 1 diabetes[21] – to name just a few. Guidelines have already been published by the European Association for the Study of the Liver for telemedical management of patients with CLD[22].

Despite the great potential associated with the application of MHAs and MAs in gastroenterology and health care in general, there are numerous challenges to be met in the future, including both the ethical and legal aspects of applying this technology[23] as well as the proof of benefit in terms of evidence-based medical care[24]. The aim of this article is to provide an overview of the status quo of MHA and MA use in the field of gastroenterology, describe the future perspectives in this field and point out some of the challenges that need to be addressed.

## TECHNOLOGICAL BACKGROUND

In the context of the application of digital ICT in general and MHAs and MAs in particular, there are numerous terms used to define this field more specifically (see Table 1 for a glossary). The term eHealth is probably one of the best known terms in

**Table 1 Glossary and explanation of digital health terms**

| Term | Explanation |
|---|---|
| eHealth, electronic health | Generic term for digitalization in health care and the associated applications of ICT |
| ICT | Technologies used for communication, storage, processing and evaluation of data |
| Telemedicine/telehealth | The physical distance between the medical service provider and recipient is bridged by the use of ICT, *e.g.*, teleradiology |
| Mobile health, mHealth | Medical applications that can be accessed from mobile devices (*e.g.*, tablets, mobile phones, smart watches) |
| Electronic patient file, electronic health record[11] | The central storage of patient data, which can be accessed by different authorized persons independent of the location |
| Health apps, medicical apps | Special software programs/applications for mobile devices that serve medical issues. The transition to fitness apps is partly fluent |

ICT: Information and communication technologies.

this context. One of the most popular definitions was made by Eysenbach in 2001, who defined eHealth as "an emerging field in the intersection of medical informatics, public health and business, referring to health services and information delivered or enhanced through the internet and related technologies. In a broader sense, the term characterizes not only a technical development but also a state-of-mind, a way of thinking, an attitude, and a commitment for networked, global thinking, to improve health care locally, regionally, and worldwide, by using information and communication technology"[25]. For a more pragmatic perspective, the World Health Organization (WHO) postulated that "eHealth (electronic health) is the cost-effective and secure use of ICT for health and health-related fields". Due to the great relevance of smartphone apps in healthcare, the term mHealth has been added to the term eHealth in recent years. The term mHealth or mobile health is a component of eHealth and is defined by the WHO as "medical and public health practice supported by mobile devices, such as mobile phones, patient monitoring devices, personal digital assistants, and other wireless devices"[26]. However, general definitions of these terms have not yet been established. With regard to the properties of smartphone applications, it is important to make a distinction between the terms MHAs and MAs. MHAs, often referred to as health apps, are smartphone apps that are dedicated to consumers and are supposed to support a health-promoting lifestyle as a preventive measure. In contrast, MAs, also called smartphone apps, are subject to a medical purpose limitation and therefore have to be classified under the legal regulations for medical devices[27]. According to recent estimates, there are currently approximately 325000 smartphone apps available on health-related topics[28]. In addition, there are certainly smartphone apps that are not specifically available *via* an online platform and are not available for public download. The areas of application and functionalization of MHAs and MAs are extremely diverse and range from the management of chronic diseases, the support of health behaviors and even self-diagnostics[29]. MHAs and MAs can also provide infrastructure or support clinicians with clinical decision-making[29].

## OPPORTUNITIES

### *Digital therapeutics and diagnostics*

**Clinical care integration:** The integration of MHAs and MAs in clinical care is changing practice in gastroenterological care and other fields of healthcare (see Table 2 for examples)[30]. One example is MHAs and MAs used as a digital intervention to improve patient education in the area of preparation for a colonoscopy[31]. The usefulness of patient education before colonoscopy is well established, and optimizing preparation results improves clinical care[32]. There are many different tools and methods to provide educational material to increase adequate bowel preparation ahead of a colonoscopy. These can be booklets, cartoon-based visual aids, educational videos, short message services or social media-based interventions[33]. A major goal of digital interventions is to provide patients with essential knowledge regarding good health information about the aim of the preparation procedure to improve adherence and the overall quality of the colonoscopy[34,35]. In particular, this kind of digital intervention aims to increase the adequate cleaning of the colon before the actual

**Table 2 Examples for use of mobile health applications and medical apps**

| Type and mechanism | Example | Possible benefits or harms |
|---|---|---|
| Patient education | Teaching app for bowel preparation before colonoscopy | Improvement in results, reduction of costs |
| Telemedicine | Video or online consultation | Low barrier accessibility of specialists, patient-physician interaction is changed |
| eHealth records | EMR | Security and privacy concepts need to be addressed, interoperability issues |
| Digital biomarkers | Smartwatch, counting of steps per day | Individualized strategies for health behavior changes. So far missing standardization |

EMR: Electronic medical records.

procedure. The reason for such an intervention is that, in up to 25% of the patients who undergo colonoscopy, inadequate bowel cleansing is present[36]. Adequate bowel cleansing can increase the detection rate of polyps and reduce complications[37]. In a recently published meta-analysis[36], the authors included 6 studies out of 520 records identified in a major database. In the included studies, smartphone apps interventions were compared with standard education. The outcome was reported as adequate bowel preparation *vs* inadequate bowel preparation, measured *via* a bowel preparation scale (*e.g.*, Boston Bowel Preparation Scale)[36]. The authors pooled data from 1665 patients and concluded that – despite some limitations of the meta-analysis – app-based interventions were an effective tool for an increased improvement of bowel cleansing[36]. In addition to MHAs and MAs, which focus on improving patient education, digital interventions are often specifically designed to improve and support chronic disease management[17]. A recent trial, which included 716 patients with non-alcoholic fatty liver disease found that an internet based program with a web-based intervention was not inferior to common lifestyle programs in terms for improving clinical outcomes. At the same time participation in the web-based intervention was more suitable for people with time and job constraints[38]. In the field of gastroenterology, MHAs can also improve the support of patients with inflammatory bowel disease (IBD). Potential use cases are patient education, the management of disease monitoring, tracking of symptoms, support of medication adherence, the tracking of dietary logs and the support of patient empowerment through access to social media channels[39]. There is a large number of literature reviews that have summarized the potential of using MHAs to support patients with IBD[15,39-41]. In a recently published review from Yin *et al*[15], apps available in the official Google and Apple digital stores were investigated. Eleven MHAs and 4 MAs were identified in this review. These MHAs focused on patient education, self-monitoring of symptoms, treatment support, follow-up support after diagnosis and patient satisfaction. In a study conducted with gastroenterologic patients' willingness to use different types of health-related smartphone apps, the investigators found that most of the participants were willing to use apps up to 5 min a day. Trial participants raised concerns that location or social networking activity should not be tracked during their use of the technology[42]. Overall, patients are willing to use health-related apps to manage health problems, but they have high concerns about privacy as well as out-of-pocked payment[42].

### *Telemedicine and telehealth interventions*

Telemedicine is a digital health intervention, used in many fields of health care, that provides medical services at a distance. Telemedicine services are provided for varying conditions, such as hypertension[43], chronic heart disease[44], diabetes management[45] and mental illnesses[46]. Telemedicine and telehealth are defined by the WHO, in the global observatory for eHealth, as "The delivery of health care services, where distance is a critical factor, by all health care professionals using ICT for the exchange of valid information for diagnosis, treatment and prevention of disease and injuries, research and evaluation, and for the continuing education of health care providers, all in the interest of advancing the health of individuals and their communities"[47]. The potential for telemedicine and telehealth for the field of gastroenterology is high because of the chronic nature of many digestive diseases[48]; liver cirrhosis is an example[49]. Telemedicine is provided using different technologies,

such as smartphones, tablet computers, wearables or other medical devices. Traditional components such as monitoring of disease activities, monitoring of symptoms or teleconsultation with medical professionals are also used[50]. There are various advantages associated with telemedicine and telehealth. These are increased access to general or specialized services in healthcare and the offer of greater flexibility in scheduling appointments for health care providers and patients, saving time and money in seeking care[51]. Patients are increasingly using smartphones and the internet for more effective and efficient modalities to receive medical information and treatment descriptions[6]. Traditionally, highly specialized medical care is condensed in urban areas rather than in rural areas. Telemedicine can provide people living in rural areas with specialized care services, thus ensuring ubiquitous access to specialized treatments[52]. In the field of gastroenterology, there are numerous application scenarios for telemedical care concepts, such as general digestive disease management programs[53]. Other telemedical tools are offered for IBD[54,55], CLDs[6], liver transplant patients[56] or diabetes patients[57]. In a literature review from Serper *et al*[6], the authors illustrated different uses for telemedicine in CLDs. They included 20 published articles about telemedicine in patients with CLD. Nine of the included studies were prospective trials, three were retrospective studies, two were case reports, and six were case series. Only one of the included studies was randomized prospectively, and 10 were uncontrolled studies[6]. The authors categorized the studies into four main fields based on the aspect of CLD management in which telemedicine was used: Hepatitis C treatment, procedural or surgical management, evaluation and management of hepatocellular carcinoma and remote monitoring interventions[6]. In treatment for hepatitis C virus (HCV), many studies have investigated the use of teleconferencing for the management of HCV and reported a sustained virologic response rate in the intervention groups with telemedicine. There were low discontinuation rates and promising results for the management of side effects[6]. Generally, the satisfaction of patients who received in-person visits was high in the intervention groups with telemedicine. The authors stated that telemedicine can improve access to specialty care and can improve care of patients with liver diseases between in-person visits[6]. In addition, they emphasized that the main barriers to the widespread use of telemedicine are regulatory issues and unclear reimbursement for the provided services. Another systematic review on the use of telemedicine and mobile health technology for the management of digestive diseases included seven studies with a focus on inflammatory bowel disease, four studies with a focus on ulcerative colitis, one with a focus on Crohn's disease, six with a focus on irritable bowel syndrome, and two studies with a focus on colorectal cancer[53]. The outcomes were patient compliance, patient satisfaction, disease activity and quality of life[53]. The studies that were included were mainly pilot trials and feasibility studies, which leads to only limited generalization of the overall results[53]. In addition, only a small number of studies addressed telemedicine for gastroenterological diseases[53]. To support patients with cirrhosis, there are three main types of telemedicine: Teleconsultation, televisits and telemonitoring[49].

### *eHealth records*

Different kinds of digitally stored health information exist. The following are the most common and important: EHR[11] are managed by health care providers. These systematic, longitudinal collections of health information can refer to one person or a whole population. EHR can facilitate the sharing of stored patient data across hospitals, doctors and other health care providers and institutions. The EHR collects medical histories, laboratory test results, medications, allergy information, vital signs, age, weight, height and insurance and billing information. Primary stakeholders of EHRs are physicians, caregivers and nurses, therapists, patients, pharmacists, clinics and hospitals, laboratories, care services and nursing homes. Secondary stakeholders are insurance companies, family and the relatives of patients and employers. The tertiary stakeholders are society, research institutes, public authorities and the health care industry[58].

Electronic medical records (EMR) are created by health care practitioners for specific treatments and can be integrated into EHR[59]. Personal health records are patient managed and can contain additional information, such as data from wearables or health apps. The patient can decide who is able to see which information[11]. The exact shape of the eHealth records differs depending on the country and provider. The primary aim of electronically stored health data is to establish a record of present and future care received from the same or different practitioners. Additionally, EHR can be used to create evidence of the provided care or to improve quality of care by performance monitoring and benchmarking[60]. Other benefits are administrative uses (

*e.g.*, billing and scheduling), decision support and care management, patient support and public health research[61].

In the field of gastroenterology, various advantages result from the use of eHealth records: Elective procedures and interventions can be scheduled on the admission day as a part of just-in-time medicine. The use of eHealth records can ensure the completeness of information for pre-existing conditions[60]. Treatment of the acute abdomen is another scope for EHR; being able to have access to an entire patient's history can prevent medical malpractice in this case and others[62]. Patients are often not able to provide detailed information about prior treatments. In the case of complex diseases, multidisciplinary treatment[63]. Or if patients demand a second opinion, decisions were made based on the provided information[60]. If more extensive information is available in the EHR, decisions can be made better and faster. This is especially important for people undergoing palliative treatment and cancer care and/or patients under multidisciplinary treatment. In these cases, EHR can provide access to relevant information for all stakeholders and ensure efficient and individualized treatment[64]. In 2015, the WHO[65] conducted the third global survey on eHealth to describe the use of eHealth. Only 58% of the countries had an existing national eHealth policy or strategy at that point. A total of 66% of the countries had a national health information system policy or strategy. The study asked for the use of EHR in different health care provider/institutional groups. Primary care facilities compromised clinics and health care centers. Secondary care facilities encompassed hospitals and emergency care. Specialized care and referrals from primary or secondary care were named tertiary care. The use of EHR in health facilities differed substantially. In Finland, more than 75% of facilities were using EHR in primary, secondary and tertiary care facilities. In contrast, Jamaica had less than 25% of primary care facilities that were using EHR in 2015. Less than 25% of secondary care facilities in Panama used EHR. In Austria, less than 25% of tertiary care facilities had implemented EHR; while 50%-75% of secondary care facilities used EHR, less than 25% used EHR in primary care facilities. Apparently, there are different reasons for the slow integration of EHR. In a systematic literature review, Kruse *et al*[66]. assembled a list of barriers to EHR system adoption in the United States. The initial cost was the most frequent barrier. Technical support, technical concerns, resistance to changing work habits, maintenance, ongoing costs, training, privacy concerns and insufficient time and workflow challenges were other barriers. The most important driving force for the implementation of EHR systems was funding. Seventy-seven percent of the countries had public funding for eHealth, and 40% could confirm private or commercial funding. Donor or non-public funding was set in 63% of the countries. Forty-two percent had funding through public-private partnerships[67]. Practitioners need help to integrate EHRs into their work habits, adapt the workflow and work with EHR effectively. In the third global survey on eHealth, 74% of all countries reported that health science students were receiving pre-service training in eHealth. A total of 77% claimed that health professionals in their country were receiving in-service training in eHealth[67]. Training, money and information about use cases, problems and advantages of EHR are important to expand worldwide use.

### *Digital biomarkers*

Biological markers quantify observations that refer to an interaction between a biological system and a potential hazard[68]. Valuable biomarkers are objectively measured and change in response to changes in therapy or condition. Pulse, blood pressure and blood test outcomes are examples of objective and quantifiable biomarkers. The association between biomarkers and relevant clinical endpoints is used for research and treatment decisions[69]. In recent years, digital biomarkers have been described and measured[70]. Digital biomarkers are defined as characteristic quantifiable measurements made by means of digital devices[71]. They are objectively measured and evaluated as an indicator of normal or pathologic biological processes or biological responses to a therapeutic intervention[72]. There are various sources from which digital biomarkers can be collected, including body sensors, image processing, health platforms and EMR as well as smartphones, wearables or other digital devices[73]. Digital biomarkers are increasingly important sources of data in health care. Related to the field of neurodegenerative diseases, Kourtis *et al*[74] pointed out different reasons why digital biomarkers collected from mobile devices and wearables present a unique opportunity for collecting data. There is widespread usage of these technologies in society and immediate access to information due to our inherent connectivity. Moreover, the sensitivity and plurality of onboard sensors is increasing, and such mobile devices are uniquely equipped with sensors; thus, the burden on the health care system is low because large segments of the population are already using

such devices. From these devices, a broad range of different data can be collected actively or passively. Biomarkers measured *via* smartphones can be movements and geopositioning, speech and language or sleep patterns[74]. A systematic survey of apps listed in international curated health app libraries focused on mobile health apps using built-in smartphone sensors for diagnosis and treatment. After excluding 762 apps according to the applied inclusion and exclusion criteria, 18 apps remained. One-fourth of those apps were aligned with the diagnosis of health conditions. One half was exclusively treatment oriented. Thirty-nine percent of the apps used the camera as a mobile phone sensor. Thirty-three percent of them used the touch screen. In the identified apps, microphones, mobile phone speakers and accelerometers were used more rarely. None of the included apps used GPS[75]. These data can be correlated longitudinally and continuously to forecast critical or medically relevant situations[76]. A smartwatch measuring heart rate, for example, can be matched with a smartphone app that can alert care providers in case of conspicuous abnormalities[70]. There are different categories of digital biomarkers: Risk biomarkers indicate the disease-development potential in individuals who are not currently ill or having medical problems. Diagnostic biomarkers can detect or confirm the presence of a disease. Serial measurement by monitoring biomarkers can be used to assess the status of a disease or medical condition or to provide evidence of exposure to a medical product. If interested in the likelihood of a clinical event, prognostic biomarkers of progress or disease recurrence can be used. Some people are more likely to experience a (un)favorable effect from exposure to a medical product or the environment. To identify those people, the use of predictive biomarkers is recommended. Biological responses to exposure to medical products or environmental agents can be assessed by response biomarkers. Safety biomarkers indicate the likelihood, presence or extent of toxicity by measuring them before and after exposure to a medical product or environmental agent[70]. Platforms connecting technologies can help to raise the diagnostic and prognostic value of the information collected by using multi-sourced biomarkers. Connecting data such as height, weight and step accuracy, for example, helps to estimate information and create meaningful endpoints. Using AI, patients participate in the ongoing process of a deep learning digital health system[70]. In the case of gastroenterology, digital biomarkers play an important role, as they do, for example, in cancer patients. Digital biomarkers deliver more precise prognostic information for cancer patients than conventional survey methods[73]. This can be explained by the fact that clinicians no longer collect data only at one or a few points in time; digital biomarkers allow the continuous collection of data in a real-world setting[73]. Despite the increasing prevalence of digital devices, there is still little research on biomarkers in the field of gastroenterology.

## CHALLENGES

In the section above, we described various examples, such as the use of smartphone apps for self-management, as well as the use of EHR and telemedicine. While the benefits for such digital interventions on self-management and the management of diseases in general in the field of gastroenterology have been described, there are various challenges that have to be resolved. While the challenges are complex, we will first address an overview of topics. Then we will describe selected specific aspects of these challenges in detail. Currently, all digital interventions are highly complex, which means that both the development and the evaluation and implementation of such interventions are difficult and context dependent[77]. Gaining and accumulating evidence-based knowledge is difficult in a number of ways because the interventions are often not comparable due to the many different components that are often not evidence-based[29]. Moreover, as we mentioned before, even if a digital intervention has a profound evidence base, there is a significant regulatory ambiguity, especially for market access and reimbursement[78]. In many fields of digital health care, the current evidence of the efficacy and efficiency is limited, and research is in its infancy[5]. Furthermore, the development of digital interventions often takes place in interdisciplinary teams in the professional context of computer interaction and social sciences and medicine. Such studies are usually complicated and suffer from limitations resulting from the interdisciplinary aspects[65].

### *Quality of apps*

The main challenge regarding smartphone health apps is the disparity between their proclaimed benefits and their objectively proven and evidence-based benefits[79]. This

is a challenge for apps in all fields of health care. The limited evidence demonstrating the quality of apps has been a research topic for apps in mental health self-management of asthma[79], self-management of diabetes[80] management of postoperative pain[12] and sleep management[81]. A systematic review conducted by Vilardaga *et al*[82] on smartphone applications to support smoking cessation suggested that the majority of the studies in this field have been performed in early stages of app development, such as user-centered design studies, and the vast majority of the apps use only a limited number of theoretical mechanisms of intervention delivery. Furthermore, the study revealed that the vast majority of apps were not tested in well-designed randomized controlled trials, which leads to only limited evidence regarding possible benefit. In another review, Alessa *et al*[83] aimed to describe and assess apps in to support the management of hypertension available in different app stores. The authors included 186 apps in their analysis and identified that only a small number of the included apps were likely to be effective. This is because most of the included apps were missing an underlying theoretical foundation in behavioral theories or even basic strategies relating to self-management interventions[83]. The one major single function of most of the apps was to provide educational information, and just a few apps included comprehensive functionalities, which are probably more effective than just a single functionality[83]. Related to the field of gastroenterology, a systematic assessment of apps for the self-management of IBD identified similar problems[40]. From the 238 identified apps in the major app stores, the investigators included twenty-six apps in the final analysis of the app content. A major result was that the overwhelming majority of the apps for IBD suffered from a lack of involvement of medical and health professionals and had only limited coverage of international consensus guidelines for IBD[40]. Currently, there are no generally accepted criteria for the qualitative evaluation of apps[27]. In a systematic review to identify and summarize criteria for the assessment of the quality of apps, the authors reported large heterogeneity of different criteria for evaluating the quality of an app[84]. They identified thirty-eight classes of assessment criteria for the quality of health-related apps. Later, they were able to aggregate these thirty-eight criteria into seven main categories with thirty-seven subclasses. The seven main categories were design, information/content, usability, functionality, ethical issues, security and privacy, and user-perceived value of the app[84]. Although various methods have been developed in recent years to improve the quality of smartphone apps, these methods have not been applied in many studies[84]. One of the most widely established methods for evaluating the quality of apps is the Mobile Application Rating Scale (MARS)[85]. By using the MARS, a score is calculated with four multi-item sections: Engagement (5 items), functionality (4 items), aesthetics (3 items), and information quality (7 items); additionally, there is a subjective section (4 items)[86]. MARS is a validated scale and is now available in different languages, such as German[87] and Spanish[88].

### *Synthesis of app evidence*

One of the central research topics regarding digital health tools is the evaluation of the effectiveness and efficiency of such digital interventions. There is currently only little evidence, and only a few randomized controlled trials exist. The question is which level of evidence is necessary prior to widespread use of digital health apps[89]. A systematic literature review of the evidence-based evaluations, conducted by Enam *et al*[90], revealed that a lack of standardization of eHealth interventions is a substantial barrier to assessing the full potential of eHealth interventions. Standardization could significantly improve the quality of intervention studies and, furthermore, could also ease the implementation of eHealth interventions. To generate evidence in the field of digital interventions, it is important that trials are carried out according to standardized procedures, evaluation models and theoretical frameworks[91]. In the field of telemedicine, standardized methods are available, such as the[92] model for the assessment of telemedicine, which is an evaluation framework for telemedicine that focuses on the measurement of effectiveness as well as the quality of care[93]. The MAST includes three domains including assessment, multidisciplinary assessment and transferability of the results[94]. Kidholm *et al*[94] conducted a scoping review of studies in which the MAST was used. They included twenty-two studies and summarized that, in the predominant number of studies in which the MAST was used, a single domain was used rather than the complete framework. The authors emphasize that the MAST was developed to be used as a complete framework and to the use of single domains was not recommended[93]. The overall conclusion in the context of the MAST is that the model is not stringently used, which leads to a lack of standardization and comparability between trials on digital interventions. The discussion about the evidence base of digital interventions has intensified with the publication of the

evidence standards framework for digital health technologies from the National Institute for Health and Care Excellence[95] of the National Health Service.

### *Patient-physician relationship*

The use of mobile health applications and medical apps in clinical practice changes the relationship between patients and physicians. There are several opportunities as well as risks in the use of eHealth and the effects on patient-physician communication. One aim of eHealth services is to increase the participation of patients in their own health care. Patients and caregivers should work together in a collaborative process. Grunloh *et al*[96] evaluated the descriptions of daily physician practice on information about patient participation. All physicians reported that they focus on patient participation, but only little objective proof of this could be found. If physicians do not provide participation support, it is possible for patients to use the internet for information and to increase their participation. The attitudes towards patients who bring information from the internet to a consultation differ. Physicians who use the internet professionally and use a diverse form of media have the most positive attitude towards those patients, and an improvement of the physician-patient relationship was observed in this context. However, some physicians argue that patients are not able to differentiate between accurate and inaccurate content. Regardless of how their own internet use was described, many physicians reported that internet-informed patients are often misinformed. Physicians who were critical internet users were least likely to expect a more time-consuming consultation with patients who used the internet in advance[97]. Fifty-five percent of patients using the internet to find health information reported a change in the way they think about their health. Most of them reported that they were making subsequent health-related behavioral changes, such as asking more questions during office visits (66%), increasing their adherence to physician advice (54%). Another study discovered that patient-physician agreement on the medical situation and recommended treatment is important for patient compliance. Physician quality itself is also important for compliance[98]. This implies that internet information can help to build a new partnership between patients and physicians, where informed decisions can be made. Physicians view their role as responsible and trusted[96]. Lu *et al*[99] conducted a study about the use of online health communities (OHCs), which supports the previous information. They found that OHCs have a positive impact on patient compliance, which can be enforced even more by guiding physician-patient communication in the OHCs. The opportunity to share high-quality health information with patients and discuss the benefits, risks and costs of treatment options encourages the patients. Patients can participate in health-related decision-making, and the misunderstanding of information decreases[99]. eHealth applications not only change the way people inform themselves or track health information but also change communication. The face-to-face interaction is no longer the only way for physicians and patients to interact with each other. Telehealth and telemedicine are a part of the history of technology in healthcare, and they have the potential to increase health care for people with limited access. As we pointed out before, the use of telecommunications technologies offers people in remote locations, people with poor health and people with other limitations new ways to interact with health care professionals[100]. However, while the use of the internet for health purposes has increased[101], online communication between physicians and patients is still rare. Scheduling an appointment, requesting or renewing a prescription or asking questions are important future eHealth applications. Consultations with health professionals online are still rather uncommon in most countries, but the interest among citizens is high and increasing[102]. The physician's perspective on telemedicine has differed between studies. Less than half of responding physicians in a study in Lebanon believed that web-based apps and social media could be useful for patient-physician communication. The other half (47.5%) was strictly against the use of virtual forms of communication as they feared breaching privacy and confidentiality[103]. In another study, physicians who frequently used the internet for professional use were more likely to take a positive position towards the use of the internet for communication with patients[97]. Online consultation is a possible solution for people living in rural areas or working full time. Practitioners feared this might be more time consuming and a threat to confidentiality[103]. However, studies have reported that the length of telemedicine consultations did not differ from that of the in-person consultations[104]; in some instances, online consultations were even time saving[101]. However, trial findings indicate that telemedicine consultations are more physician centered. It happens more often that the physician controls the dialogue, while the patient is more passive than they are in in-person consultations[104]. However, patient-physician communication can still benefit from eHealth. The benefit can be even stronger if eHealth literacy helps

patients to keep control in online consultations and everyone is better informed about the limitations and security needs of eHealth.

### *eHealth literacy among patients and physicians*

In addition to the above-described aspects of a changing communication, interaction and relationship between patients and physicians as well as the ubiquitous availability of online information, there are many barriers for internet users seeking information on health-related topics. There are also barriers to the interpretation of medical jargon and inconsistencies of information found in online research across different sources[105]. Barriers arise not only from the unmanageable mass of freely available health information but also from the limited access to medical articles that are not freely available, from which information may be required[105]. Due to these manifold problems, internet users sometimes find it difficult to draw the correct conclusions and apply the information found to their individual situation. In this context, finding health-related information is centrally linked to the concept of health literacy. Patient health literacy is understood as the motivation, ability and knowledge to identify, understand and evaluate information relevant to one's own health and the ability to use this information to maintain health and to obtain support from the health system when needing assistance or treatment. From the representative HLS-GER study about the health literacy of the German population, it can be assumed that 54.3% of Germans have limited health literacy. People with low health literacy are more likely to assess their own health as wors[106]. In this context, the concept of eHealth literacy is becoming increasingly important. eHealth literacy was defined by Norman *et al*[107] as the ability to search for, find, understand and critically evaluate health-related information in electronic media in order to apply the knowledge to solve specific health problems. eHealth literacy is the most commonly used term when describing the competence of users searching for health information in the context of digital media[108]. Norman *et al*[107] understand eHealth literacy as a kind of meta-competence consisting of six different sub-competences: Basic reading and numeracy, health literacy, competencies in the use of computers, scientific literacy, and media and information literacy. eHealth literacy is not only the ability to search for good health information on the Internet but also implies a literacy and competence in the use of social networks such as Twitter Inc. (which has been dubbed "health twitteracy" by Sorensen)[109]. In the context of gastroenterology, it is known that Internet users with a high eHealth literacy are more likely to have knowledge and previous screening practices related to colorectal cancer compared to users with a low level of eHealth literacy[110]. In view of the increasing relevance of digital health information, it is important for doctors to take an in-depth look at changing information needs. In general, healthcare providers should be able to critically assess health information on the Internet to advise patients on how to deal with internet research and critically evaluate digital content[111].

### *Ethical considerations*

The implementation of new digital technologies in routine clinical practice will bring fundamental changes to the field of medicine. However, there are several ethical issues that need to be critically discussed and addressed. MHAs will have the potential to change and hopefully improve medical systems in many ways, including access to specialized medical services in rural, underserved areas and low-barrier and low-cost access to medical treatments[112]. However, at the same time, the traditional patient–physician interaction is going to change, as described in the chapter above. Therefore, several points need to be taken into account.

Apple's iOS and Google's Android system have a combined market share of more than 95%. Both systems differ in terms of data security, privacy settings, regulations and security surveillance of their app marketplaces. The development of apps should not prioritize one of the two predominant smartphone systems so that access to medical apps is available to all smartphone users and not just for those who can opt to pay for more expensive and secure devices[113].

Today, there is no possible international regulation of smartphone apps, even if they serve as medical apps and, therefore, as diagnostic or therapeutic tools. Under ethical considerations, a way to ensure that all users of health-related apps understand the apps they are using needs to be established. Users have to comprehend the positive and negative implications that the use of these digital medical tools can have on their health. As patient-physician interactions will be changing due to digital medicine, there is also a risk that real-life doctors will only be affordable for patients with adequate insurance or financial resources, while others will be predominantly treated by avatars or telemedical consultants.

Many digital innovations will be designed for chronically ill people. These patients

are typically older people with no or limited access to modern ICT devices, such as smartphones or wearables, and this population has nonexistent or only limited digital literacy. This needs to be taken into account when planning digital medical solutions.

### *Legal and data privacy considerations*

Health care sectors around the world are among the most strictly regulated markets for a good reason. Health data and medical treatment data are among the most vulnerable and sensitive existing data. This makes innovative digital interventions more difficult to establish in the medical community, as medical products need to be highly safe before widespread use and market adoption[114].

Many people and health care professionals are critical towards the implementation of (mobile) digital solutions in medicine. This is mostly due to their fear of data security and protection. There are no questions that, for digital medical products, as for any other conventional medical treatment, the same strict data security and protection regulations need to be validated. However, when correctly used, digital services can provide the highest levels of data security and protection. Correct and secure implementation of data security and integrity in digital services has been shown, for example, for secure online banking, insurance services or online retail for many years. Correctly implemented secure EHR or telemedical services will provide much higher security levels than traditionally used services, such as unsecure email or messenger correspondence. However, many health and fitness apps provide only very low or no data security for stored patient data, and this cannot easily be seen or understood by the naïve user. To date, there exists no seal or certification that makes it easy for the end user to understand which products use high industry-standard levels of security and are safe to use. The remuneration of digital medical services has also not been clearly solved in most countries. While certain regulations exist regarding payment for telemedical treatment, many issues have not yet been solved. Most health or fitness apps, for example, currently either finance themselves with advertisements or by selling data, or they have to be paid for by the individual patients[115].

### *Interoperability – technical aspects*

The health system is one of the industries with the highest level of data production and storage needs. What makes this difficult is that all of the data exist in multiple silos, which are usually not compatible with each other. For one single patient, data will exist and be stored in different electronic or analogue hospital files where the patient was treated, in different outpatient clinics or ambulatory care services, and in different pharmacies. In addition, data that the patient himself has recorded *via* wearable devices, *etc.* will exist in the future. All of the data are stored in different systems and different file formats, which makes data use and interoperability difficult. This is one of the main reasons why the introduction of the EHR, which must use common interoperable standards and interfaces, is one of the most important aspects in terms of the useful implementation of digital medical innovations[116].

## CONCLUSION

Digital interventions, such as MHAs and MAs, offer potential for diagnostic and treatment advances in the field of gastroenterology and the management of chronic diseases in general. In particular, patients with chronic diseases and health care professionals will benefit from these interventions in many different ways. Sufficient proof of benefit, however, depends on high-quality evaluation, which must be based on the standards of evidence-based medicine. This issue is complicated for digital interventions for many reasons, and to date, the specific standards for development and evaluation are generally missing. In this context, it should be clearly emphasized that frameworks of standardization, at least in many parts, can harmonize the research in the field of digital interventions. Continuous work on standardization with a clear focus on the rules of evidence-based medicine would lead to a better understanding and interpretation of the actual evidence.

Moreover, this is also necessary for the assessment of the reimbursement of such digital interventions. This would be particularly useful in guiding health care professionals in almost all health care systems worldwide to apply comparable criteria to better evaluate the reimbursement of digital interventions. Currently, the inclusion of the users concerned, in the sense of user-centered design, does not take place. In addition to the characteristics of this research field mentioned so far, no uniform quality criteria have yet been established that would allow affected users to

adequately assess the quality of a medical app. This can lead to patients using an app of insufficient quality or, in the worst case, with the potential to harm the patient and to cause damage or even death. On the basis of this, a strengthening of eHealth literacy must be a central concern of society as a whole and for persons with health-related professions in particular.

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*REVIEW*

# Endoscopic management of gastrointestinal leaks and fistulae: What option do we have?

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## Abstract

Gastrointestinal leaks and fistulae are serious, potentially life threatening conditions that may occur with a wide variety of clinical presentations. Leaks are mostly related to post-operative anastomotic defects and are responsible for an important share of surgical morbidity and mortality. Chronic leaks and long standing post-operative collections may evolve in a fistula between two epithelialized structures. Endoscopy has earned a pivotal role in the management of gastrointestinal defects both as first line and as rescue treatment. Endotherapy is a minimally invasive, effective approach with lower morbidity and mortality compared to revisional surgery. Clips and luminal stents are the pioneer of gastrointestinal (GI) defect endotherapy, whereas innovative endoscopic closure devices and techniques, such as endoscopic internal drainage, suturing system and vacuum therapy, has broadened the indications of endoscopy for the management of GI wall defect. Although several endoscopic options are currently used, a standardized evidence-based algorithm for management of GI defect is not available. Successful management of gastrointestinal leaks and fistulae requires a tailored and multidisciplinary approach based on clinical presentation, defect features (size, location and onset time), local expertise and the availability of devices. In this review, we analyze different endoscopic approaches, which we selected on the basis of the available literature and our own experience. Then, we evaluate the overall efficacy and procedural-specific strengths and weaknesses of each approach.

**Key words:** Leak; Fistula; Endotherapy; Over-the-scope clip; Suturing system; Endo-vacuum therapy; Endoscopic internal drainage; Self-expandable metal stent

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**Core tip:** Early diagnosis of gastrointestinal leaks and fistulae is associated with better outcomes. Endoscopic minimally invasive management is becoming the treatment of choice for gastrointestinal wall defects. It is more effective and safer than surgery. Several endoscopic devices and techniques are available, and they include endoclip, metal or plastic stent, tissue sealants, suturing systems and vacuum therapy. The choice of one procedure over another should depend on clinical presentation, defect features and local expertise. Early leaks have a higher rate of longstanding healing compared to late leaks and fistulae. A close collaboration between surgeons, interventional radiologists and therapeutic endoscopists is recommended to assure a favorable outcome.

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## INTRODUCTION

Gastrointestinal (GI) leaks and fistulae constitute a disruption of the GI wall. GI leaks and fistulae refer to two well distinct entities.

Leak is defined as a pathological communication between intra and extra-luminal compartments as a result of a defect in the integrity of the GI wall, which often lead to egression of luminal contents. They are mostly related to anastomotic defect after surgical procedures[1] and are associated with a high risk of morbidity and mortality. They constitute the single adverse event (AE), which is responsible for the majority of surgical mortality occurring in up to 60% of cases if the treatment is delayed[2]. Prevalence of GI leaks has increased in recent years most probably due to an increased complexity of GI surgery. Post-operative leaks after oncological surgery has been reported in 8% to 26% of cases after distal esophagectomy and in 3% to 12 % after total gastrectomy[3,4]. Leaks represent a major concern even in bariatric surgery with a prevalence of 1%-2% after sleeve gastrectomy (SG) and from 2% to 8% after Roux-Y-Gastric bypass[5,6] (Figure 1). Whereas anastomotic leakage after colorectal surgery has been observed in approximately 11% of cases with a mortality around 12%. Proctocolectomy and total mesorectal excision, followed by ileoanal or coloanal anastomosis, may reach a rate of leaks as high as 20%[7].

Fistula is defined as an abnormal communication between two epithelialized surfaces. A fistula may involve many adjacent structures: Entero-enteric, entero-bronchial/tracheal, entero-vaginal, entero-vescical, entero-cutaneous (Figure 2). Prolonged anastomotic leaks, especially if coupled with extra-luminal fluid spillage and abscess, may evolve in a chronic fistula[8] (Table 1).

The fundamental principles of GI leak and fistula management are identification of the site of defect, drainage of any leaked luminal contents and avoidance of further spillage either by diversion of luminal contents flow or by closure of the defect[9].

Mainstay of conservative management include bowel rest, adequate nutritional support and appropriate antibiotic therapy[10]. Historically, conservative management and revisional surgery with surgical drainage, defect repair or redo anastomosis, had been the mainstay treatment of GI leaks and fistulae. However, surgical interventions may be difficult and associated with a high risk of morbidity and mortality[11]. Therefore, the last decades have witnessed an increasing interest in endoscopic management. Recent advances in interventional endoscopy allowed a paradigm shift in the management of GI wall defect from surgery to minimally invasive endoscopic approaches. Endoscopy showed to be an effective and less invasive alternative to primary surgery. Several endoscopic options are available in order to re-establish GI continuity, avoid further luminal spillage thus preventing infections, drain/prevent collection and provide nutritional support. Available endoscopic treatments include: Through the scope (TTS) or over the scope (OTS) clip, stent deployment, endoscopic internal drainage (EID), suturing systems, vacuum assisted therapy (EVT) and sealants[12].

The aforementioned techniques may be applied alone or in combination, and as first line or as salvage treatment after failure of previous approaches. Unfortunately, a standardized approach that fits for all possible scenarios does not exist. Each treatment

**Table 1 Definition of leak and fistula**

| | Definition |
|---|---|
| Leak | Pathological communication between intra and extra-luminal compartments |
| Fistula | Abnormal communication between two epithelialized surfaces |

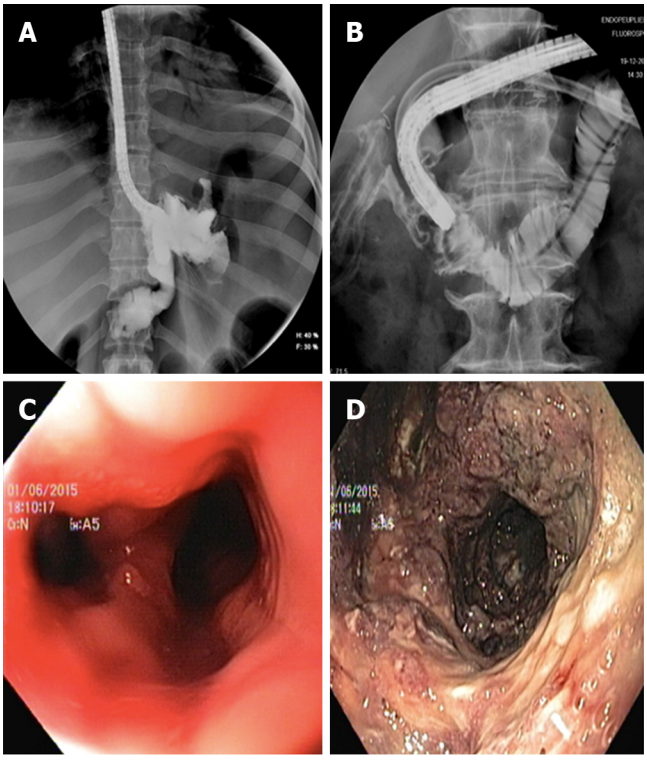


**Figure 1 Radiological evidence and endoscopic view.** A: Radiological evidence of a gastric leak after sleeve gastrectomy; B: Radiological evidence of a duodenal leak after laparoscopic right hemicolectomy; C: Endoscopic view of leak orifice after sleeve gastrectomy; and D: Endoscopic exploration of leak associated collection.

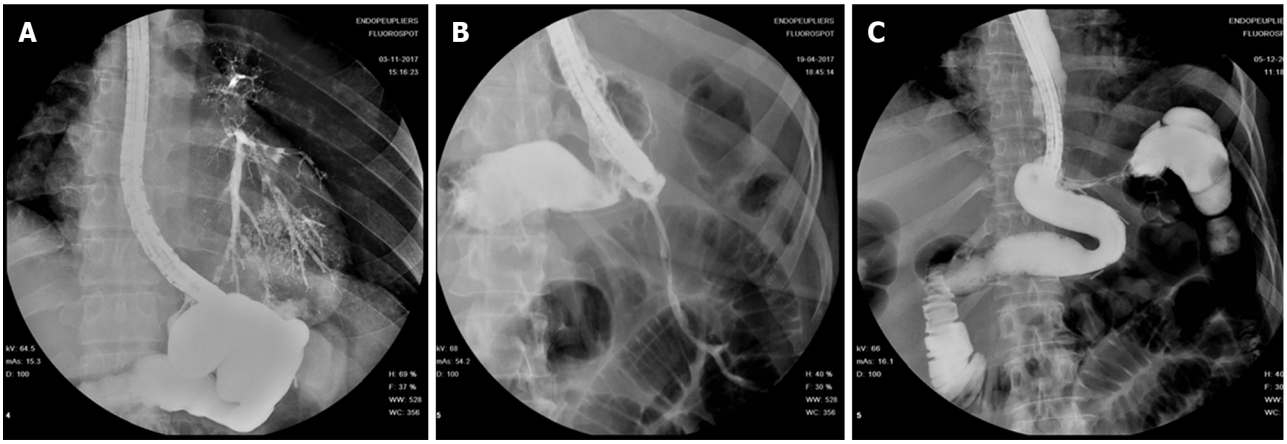


**Figure 2 A fistula may involve many adjacent structures.** A: Gastro-bronchial fistula; B: Gastro-cutaneous fistula; and C: Gastro-colic fistula.

should be tailored according to several variables, such as the clinical presentation and patient's general status, size of the defect, time of onset, defect location, endoscopic accessibility, ability to drain or avoid any associated collection and local expertise/accessories availability. In reason of technical complexity of most procedures and the relative learning curve, difficult cases should be managed in referral centers with adequate caseload, whenever possible.

Surgery as first line treatment should nowadays be reserved to patients with severe sepsis or multi organ failure. Revisional surgery plays a major role in case of generalized or extensive peritonitis because it allows to perform a complete peritoneal washout and drainage with prompt reduction of the bacterial load. Early diagnosis is of paramount importance because it is associated to better outcomes. Diagnosis should be reached based on a combination of clinical presentation, radiological findings and endoscopic evaluation. Pre-procedural assessment of defect site is mandatory in order to evaluate the feasibility of proposed endoscopic approach and features of the defect and surrounding tissue (*e.g.*, healthy, inflamed, ischemic or chronic). Defect orifice and cavity features should be assessed not only by means of intra-procedural contrast study but even, whenever possible, by means of direct endoscopic cavity exploration.

This review aims to describe the main endoscopic available techniques to manage the GI defects and to describe the pros and cons of their application in case of fistulae and leaks.

## ENDOCLIPS

Endoscopic clips are routinely used in clinical practice for a wide variety of GI conditions. Although endoclips have shown to be very effective in the management of acute intra-procedural GI perforation[13] their role in closure of chronic leak and fistula is controversial. Two main types of endoclips are available: Through-the scope clips and Over-the scope clips.

### *Through-the-scope clip*

TTS clip is a widely available accessory, routinely used in endoscopy, in different designs and sizes, and it is inserted through the operative channel of the scope. There are two main types of TTS clips: Reusable and single use clip. The first type is the most commonly used and it has a reloading manually device to load the clip onto a small hook at the end of a metal cable running through a plastic sheath. Once put in the scope, the clip arms can be aligned to the tissue that the operator wishes to grasp, by rotating the handle and cannot be reopened. Conversely, the single use clip is a preloaded accessory. This type of TTS has a wider opening then the reusable ones and its arms can be closed and reopened several times, before the definitive release of the clip. These different models make TTS clips easy to use and adaptable to different scenarios. However, clip performance in closure of chronic defects is hampered by its limited pressure applied to tissues and its "natural" tendency to dislodge spontaneously. Therefore, if necrotic or inflamed tissue is present, TTS clip may easily result in a suboptimal closure. Nonetheless, in a case series of 20 patients with anastomotic leak after gastric surgery Lee *et al*[14] reported a 95% success rate after TTS clip deployment. A mean number of 3.4 ± 1.46 clips were used. Clip deployment was coupled with fibrin glue in 14 cases whereas in 2 patients detachable snare plus clip were used.

### *Over-the-scope clip*

OTS clip is a biocompatible nitinol clip with a bear-trap shape design. It is mounted on a cap installed at the tip of the endoscope allowing full-thickness closure of GI defects up to 2 cm in size. The most common commercially available OTS clip are the over-the-scope clips (OTSC) system (OTSC, Ovesco Endoscopy AG, Tubingen, Germany) (Figure 3) and Padlock clip (Aponos Medical Corp, Kingstone, New Hampshire). OTS clips are available in different sizes and different teeth designs according to required indication.

These are the advantages of OTS over TTS clip: It consists in a clip with wider arms and it has higher mechanical tissue compression allowing long-lasting full-thickness closure[15] (Figure 4). These are the shortcomings of OTS: It requires a challenging removal procedure in case of treatment failure; it displays a high rate of fistula recurrence after initial clinical success[16] and it may cause interference with subsequent surgical procedure.

Some authors suggest to de-epithelialize the edges of the defect and surrounding mucosa with Argon Plasma Coagulation or with a cytology brush before OTS clip deployment, in order to guarantee a stronger and more durable tissue grasp. A long indwelling time of OTS reflects its correct deployment over a suitable tissue and translates into a higher long-term clinical success. Donatelli *et al*[17] reported OTS clip outcome in a retrospective study comprising 45 patients, who presented both iatrogenic acute perforation (15 pts) as well as post-surgical leak and fistula (30 pts). In

**Figure 3 Over the scope clip system (Over-the-scope clips, Ovesco Endoscopy AG, Tubingen, Germany).**

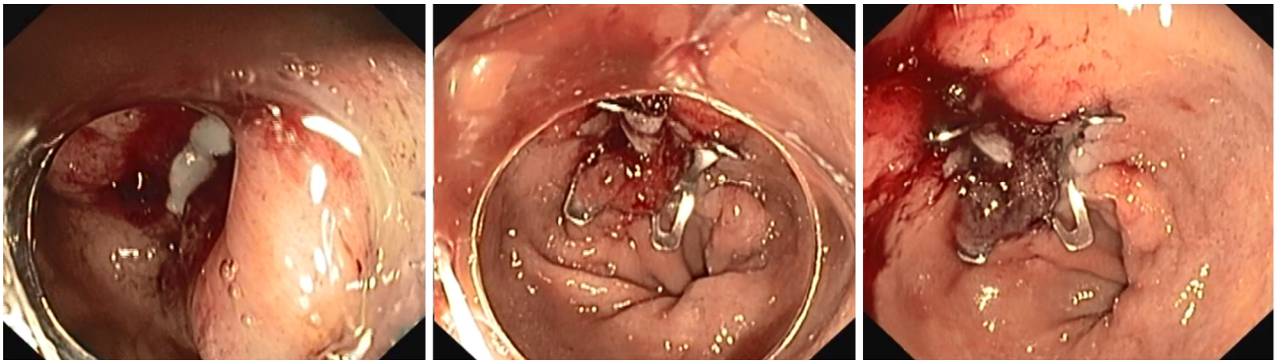

**Figure 4 Over-the-scope clips closure of a leak after sleeve gastrectomy.**

the latter group OTS clips were used as a rescue therapy after previous endoscopic treatments. Clinical success rate in the chronic setting group was significantly lower (36.6%) compared to the success rate in the acute setting group (100%). The largest multicenter series of OTS clip for management of GI wall defects highlighted a similar trend. Considering 188 patients, the rate of successful closure of perforations (90%) and leaks (73.3%) were significantly higher than that of fistulae (42.9%) ($P < 0.05$). Long-term success was significantly higher when OTSCs were applied as primary therapy (primary 69.1% *vs* rescue 46.9%; $P = 0.004$)[18]. In a recent retrospective study, Morrel *et al*[19] reported overall success rate of 64.4% in OTS deployment. Long-term success was significantly higher for leaks than for fistulae (79.6% *vs* 55.0%, $P = 0.007$) and, more patients with fistulae ultimately underwent definitive operative management (16.9% *vs* 3.9%, $P = 0.0253$). A recent systematic review, which accounted for 1517 cases retrieved from 30 studies published between 2010 and 2018, summarized OTS clip results for various GI indications. Out of 1517 cases, 388 fistulae and 97 anastomotic leaks were treated with OTS clip. The review reported an overall success rate of 51.5% in case of fistulae and 66% for anastomotic leaks[20].

## LUMINAL STENT

The use of temporary endoscopic stent has emerged has an effective and safe treatment option for the management of upper gastrointestinal leaks and fistula with acceptable morbidity and low mortality[21,22]. The rationale of stent deployment is to seal the defect and divert luminal content thus allowing mucosal wall healing. Further advantages consist in the possibility of early oral intake and reduced risk of stricture formation[23]. Complete drainage of any extra-luminal collection is mandatory before stent deployment, in order to allow successful closure and reduce septic complications[24]. Different types of stent may be used, namely: Self-expandable plastic stents (SEPS) and self-expandable metal stent (SEMS) both fully covered (FCSEMS) or partially covered (PCSEMS).

### *Self-expandable plastic stent*

SEPS are endoscopic stent made of a polyester netting fully covered with silicone. They were initially developed for the management of esophageal stricture[25] and later deployed with satisfactory results for the management of esophageal leaks[26-28].

### *Self-expandable metal stent*

SEMS may be composed either of Elgiloy, an alloy of cobalt, nickel and chromium or of Nitinol, an alloy of nickel and titanium. SEMS presents a flexible delivery system and a higher radial force compared to SEPS[29]. FCSEMS has a membrane (polyurethane, polyethylene or silicone rubber) along its full length whereas PCSEMS has uncovered distal and proximal ends.

**Comparison between SEPS and SEMS:** Presumed benefits of SEPS over SEMS are easier removability, lower costs and reduced tendency to induce hyperplastic tissue formation.

In a systematic review comprising 267 patients treated with luminal stent (FCSEMS *vs* PCSEMS *vs* SEPS) for benign esophageal rupture or leak, van Boeckel *et al*[30] showed a similar efficacy between the different stents (SEPS 84%; FCSEMS 85%; PCSEMS 86%; $P = 0.97$). These data are in accordance with other studies showing a clinical success of SEPS ranging from 66% to 100%[31-33]. However, the disadvantages of SEPS over SEMS are its large diameter, the need to mount the stent on a delivery system that may hamper its deployment if strictures or angulation are present and a high rate of migration, reaching up to 40% of cases in long term follow up[34]. Although the existing literature shows a similar efficacy of SEPS and SEMS, in recent years the use of SEMS has substantially replaced the use of SEPS. A recent expert international survey[12] on endoscopic treatment of upper gastrointestinal (UGI) leaks, identified SEMS deployment as the most frequently used technique.

**The clinical use of SEMS in upper GI tract:** Clinical success ranges in literature from 48 to 100%[35-37]. van Halsema *et al*[34] reported an overall clinical success of 76.8% (480/625) and, according to etiology 81.4% (201/247) for post-surgical leaks and 64.7% for fistulae (11/17).

A short interval time between index surgery, leak diagnosis and SEMS deployment seems to be a fundamental factor for a successful treatment[38]. Considering UGI leak, Freeman *et al*[39] identified 4 factors associated with treatment failure: Leak of the proximal cervical esophagus, stent trasversing gastroesophageal junction, esophageal rupture longer than 6 cm and anastomotic leak associated with a more distal conduit leak. Optimal stent indwelling time is not well established. Although animal studies suggested that an indwelling time of 30 d is sufficient to guarantee healing[40] a pooled analysis of 20 retrospective studies from 2013 to 2015 showed a median indwell time of 5 to 7 wk for FCSEMS and an indwell time of 7 to 10 wk for PCSEMS[37]. Lately there is a tendency to reduce the stent dwell time to 4-5 wk[12] in order to guarantee a proper time for complete closure but at the same time reduce stent related AE. Unfortunately, SEMS treatment is burdened by an AE rate that ranges in literature from 20% to 72% (Figure 5) with a stent related mortality ranging from 0 to 28%[34,35,41-44], which is lower however, than those reported after surgical management, which ranges from 12% to 50%[34].

Stent migration is a major limitation, since it is responsible for up to one third of cases needing re-intervention, thus increasing costs. Stent migration may be responsible for further AEs such as perforation or obstruction[45] and it is related to altered anatomy and absence of stenosis coupled with physiologically large diameter of GI tract. FCSEMS are more susceptible to migration than PCSEMS. A systematic review from 2011[30] reported a migration rate of 26% for FCSEMS and 13% for

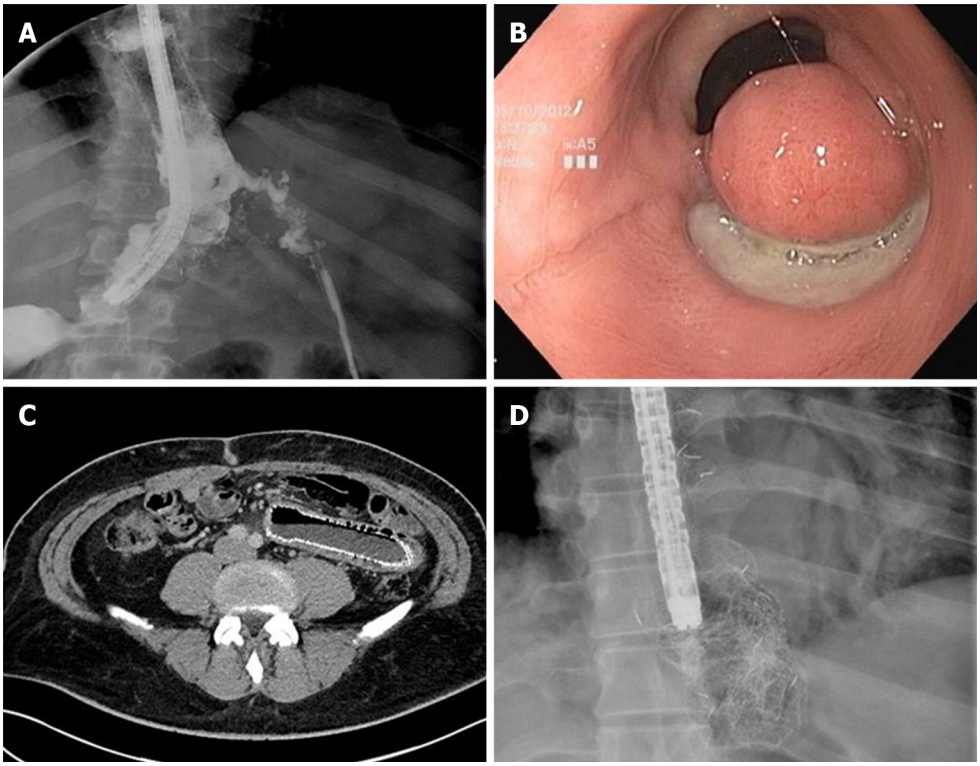


**Figure 5 Self-expandable metal stent related adverse event.** A: Proximal stent migration with leak recurrence; B: Mucosal erosion and tissue overgrowth at the distal end of the stent after fully covered self-expandable metal stent removal; C: Distal stent migration and self-expandable metal stent related perforation; and D: Stent rupture during its removal.

PCSEMS ($P \leq 0.001$). In one study endoscopic re-treatment was necessary for stent migration in 50% of cases[45]. These results suggest that in order to achieve clinical success of leaks and fistula, multiple stent deployment may be necessary.

Fixating the proximal flange of the stent to the esophageal wall by means of through-the scope (TTS) clips, OTSC or endosuturing devices has been proposed[46-50]. Fixation techniques are used in 80% of expert centers, particularly in case of previous stent migration, when incomplete sealing between stent and esophageal wall is present or when stents are placed across jejunal anastomoses[12]. In a multicenter retrospective study, Ngamruengphong *et al*[51] evaluated 74 patients underwent to stent deployment for benign UGI conditions (strictures, leaks, fistulae and perforations). All subjects were treated either with PCSEMS (28 pts) or with FCSEMS sutured to the esophageal wall with the Overstitch suturing device (Apollo Endosurgery, Austin, TX, United States). The study detected no statistically significant difference in stent migration rate between the 2 techniques (adjusted odds ratio 0.56; 95%CI 0.15-2.00; $P = 0.37$). However, the rate of other stent-related AEs was higher in the PCSEMS group (46% *vs* 21%; $P = 0.37$)[51].

Tissue hyperplasia within the mesh (ingrowth) or at stent edges (overgrowth) has been reported as high as 41% to 53% after PCSEMS deployment[52,53]. Granulation tissue may hamper stent removal or induce stricture formation. Different methods to remove partially embedded PCSEMS has been described. The most common one is the so called "stent-in-stent" technique in which a second stent is deployed inside the embedded one in order to induce pressure necrosis of hyperplastic tissue thus allowing stent removal. Swinnen *et al*[54] demonstrated a successful rate of 97.8% for stent removal after SEPS deployment for 6 to 10 wk. Use of Argon Plasma Coagulation in order to ablate the ingrowing tissue has been proposed as well[55]. Nonetheless, hemorrhage and esophageal rupture have been described after stent removal[23].

The literature describes the following stent related AEs as well: Stent rupture, food impaction, severe pain, mucosal erosion with fistulae formation or massive bleeding due to erosion into major vessels[56].

**The clinical use of SEMS in bariatric surgery:** Specifically designed SEMS have been recently developed for the management of leaks after bariatric surgery. The most common used are: Mega Stent (Taewoong medical, Seoul, South Korea) a fully covered ultra large and long (18-24 cm) stent with a design studied to reduce migration and to

give additional flexibility to better adapt to post sleeve gastrectomy anatomy (Figure 6) and Niti-S-Beta stent (Taewoong medical, Seoul, South Korea) a fully covered stent with a proximal flange and a double-bump in the proximal third in order to reduce migration. Nonetheless, data from literature showed a similar success rate without statistically significant differences in migration rate[57,58]. Moreover, special attention should be taken when placing a stent across gastro-jejunal anastomosis after Roux-Y-Gastric bypass because its migration in the small bowel may hamper endoscopic removal causing obstruction or perforations. In similar scenario, if stent management is decided, proximal fixation is advised to reduce the risk of migration.

**The clinical use of SEMS in lower GI tract:** The role of SEMS has been investigated even in the management of colorectal leaks and fistulae. A meta-analysis considering 17 studies including 68 patients treated with SEMS showed a success rate in approximately 75% of cases[59]. A case series considering 22 patients treated for anastomotic leakage (at least 30% of circumference) reported a healing rate with diverting stoma reversal of 84%[60]. However, due to vigorous motility and luminal diameter, stent migration may occur in approximately 40% of cases, reaching up to 80% of cases if a concomitant stricture is not present[61]. The following general consideration must be kept in mind if SEMS treatment is decided: Mandatory use of FCSEMS, avoid use of stent closer than 1 cm from the anal verge due to patient discomfort, prior drainage of any nearby collection and avoid if sepsis is present[62].

## ENDOSCOPIC INTERNAL DRAINAGE

In recent years, endoscopic management of leak and fistula after bariatric surgery started to shift from stent deployment to EID. Nonetheless, SEMS remains the most used technique although it is associated with significant rate of AE. Moreover, long term success after stent management may not be reached in more than 70% of cases, independently from the type of stent or combination of different endoscopic approaches[63,64].

Pequignot *et al*[65] in 2012 described for the first time the use of double pigtail stent or naso-biliary drain across leak orifice in order to guide drainage toward GI lumen and promote healing while favoring leak orifice closure. In their case series, 25 patients presenting with gastric leak after SG were treated either with SEMS deployment or EID. EID was mainly used in case of late onset of gastric leak and after failure of the other techniques. In their study EID was more effective and safer than SEMS. The authors reported that pigtail stents were better tolerated, requiring less procedures per patient with a shorter healing time, lower morbidity and mortality.

The rationale of EID with deployment of one or more pigtail plastic stents across leak orifice is to internally drain any fluid collection, obstruct the leak orifice thus allowing early oral intake and to induce mechanical re-epithelization of the fistula tract[66]. According to Donatelli *et al*[67] pigtail stents acting as a foreign body promotes re-epithelialization while guarantying internal drainage. Moreover, stents allow in most cases early removal of surgical drainage, thus reducing the risk of chronic fistula formation along drainage tract[67]. Before deciding the number, length and diameter of pigtail stent, it is of paramount importance to adequately assess orifice and cavity features not only by means of intra-procedural contrast study but even, whenever possible, by means of direct endoscopic cavity exploration. Donatelli *et al*[68], differently from other authors, advises enteral nutrition by means of feeding tube placed in the third part of the duodenum for the first 4 wk in order to allow hyper-alimentation. Systematic endoscopic review is advisable after 4 to 6 wk to avoid stent obstruction and to induce fistula traumatism (Figure 7). Lorenzo *et al*[69] in 2018 published a study comparing the outcomes of internal drainage versus closure (SEMS, glue or OTSC) for the management of fistula after SG in 100 patients. The efficacy of EID was significantly higher than that in the closure group (86% *vs* 64%; $P = 0.55$) and the mean (± SD) number of endoscopic sessions needed were 3.7 ± 3.4 per patient. The authors identified, in accordance with previous studies, the following risk factors associated to treatment failure: Delay of more than 21 d between diagnosis and treatment, large fistula, late patient referral, sepsis, presence of gastro-bronchial fistula, previous OTSC deployment. In the largest series of patients treated solely with EID consisting of 67 patients, clinical success was achieved in 78.2% of cases, after a mean time of 57.5 d (10-206) and an average of 3.14 sessions (2-16), whereas 9 patients were still under treatment at the end of the study after an average of 36 d of treatment. Clinical failure was observed in 5 patients (7.8%), all with a chronic fistula, whereas 6 patients

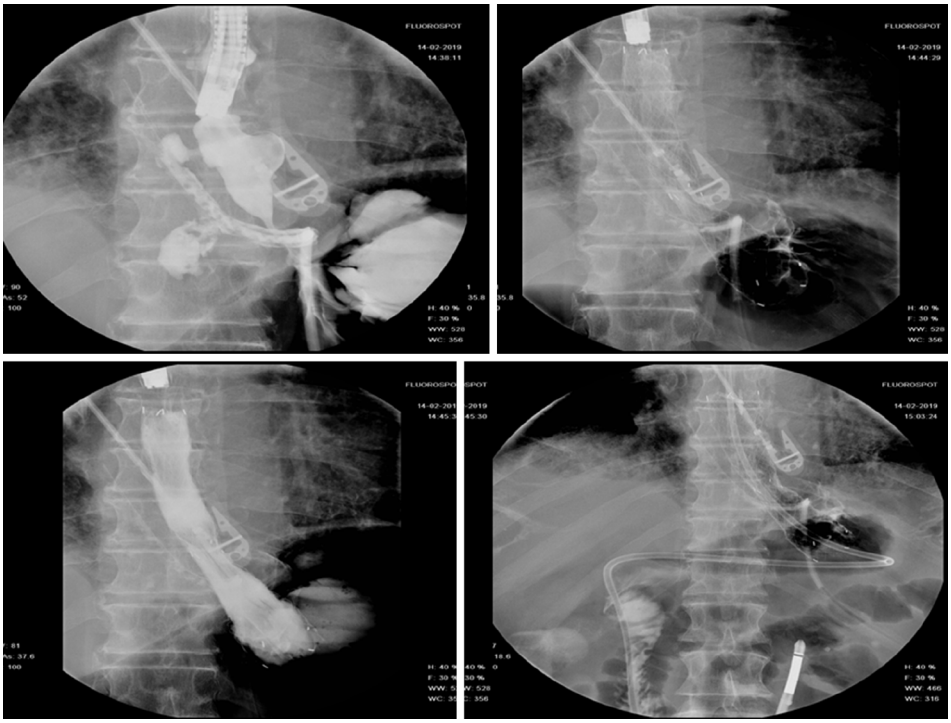

**Figure 6 Niti-S-Beta stent.** (Taewoong medical, Seoul, South Korea) deployment for the management of an early leak after sleeve gastrectomy.

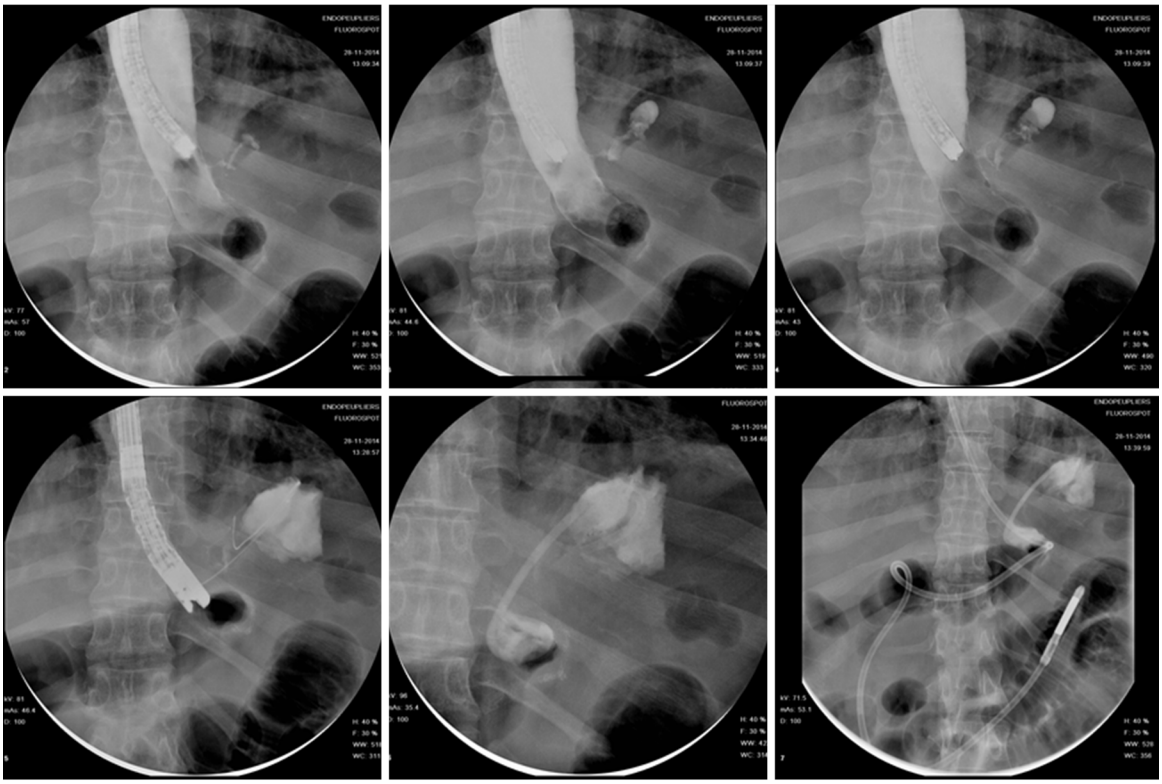

**Figure 7 Endoscopic internal drainage coupled with enteral nutrition for the management of a late leak following sleeve gastrectomy.**

presented a stricture after a mean period of 36 d from the end of the treatment. They were thus successfully treated with endoscopic dilation[67]. In a case series of 11 patients, Donatelli *et al*[70] proposed EID as first line treatment for fistula following GI surgery different from bariatric procedures. Leaks were as follow: 4 duodenal leaks (biliopancreatic cancer), 2 colonic leaks (colorectal surgery) and 5 esophagogastric-jejunal fistulas (foregut surgery). The overall clinical success was achieved in 9 patients

(82%) after an average of 44 d (28-90) and a median of 2.3 endoscopic session (2-4).

## SUTURING SYSTEM

In the past two decades several suturing systems have been developed for full-thickness closure of GI defect. However, most of them have shown major limitations preventing their widespread clinical use.

Currently OverStitch[71] (Apollo Endosurgery, TX, United States) has become the main endoscopic suturing platform enabling single operator surgical suturing with a flexible endoscope. The original Overstitch is a single use disposable platform that is mounted on a double therapeutic channel endoscope (Olympus only), allowing full-thickness uninterrupted or continuous suturing with both non-absorbable or re-absorbable stitches. The main components of the platform are: The needle driver handle, the cap mounted on top of the endoscope and an anchor exchange catheter. Grasping forceps or tissue retracting helix device may be used to aid tissue apposition. An important innovation was carried out with the recent introduction of Overstich SX device (Apollo Endosurgery, TX, United States) that can be mounted on single channel endoscope and it is compatible with over 20 single-channel endoscopes and 4 platforms (Figure 8). Nonetheless, Overstitch requires expertise and a specific training limiting its use to tertiary centers only. Sutures may be particularly demanding when endoluminal space is tight and suturing site is tangential; moreover, especially in case of large defect, similarly to surgical sutures, a robust and healthy tissue is necessary for successful primary closure[72]. The overstitch system has been successfully used for a growing variety of indications, including sleeve gastroplasty in obese patients, trans-oral outlet reduction after bariatric surgery, stent anchorage, and closure of mucosal defects after endoscopic resections[73-76]. However only a small amount of literature evaluated the role OverStitch for primary closure of GI leaks and fistula.

In a multicenter retrospective study Sharaiha *et al*[77] analyzed the results of endoscopic suturing in 122 patients. Among these, 40 fistulae (32.7%) and 15 leaks (12.3%) were treated. Although high technical success was reported, long term clinical success was obtained in respectively 80% and 27% of the cases. Mukewar *et al*[78], in the largest series of endoscopic suturing management for a wide variety of GI fistula (51.8% gastro-gastric fistulae), showed an immediate success rate of 100% and a sustained clinical success for nearly 40% of patients, with 13 patients requiring an additional endoscopic procedure. Despite multiple endoscopic attempts, the fistula of many patients (26 out of 56; 46%) failed to close or surgical treatment was required.

Before attempting endoscopic closure of an epithelialized fistula is of paramount importance to de-epithelialize it in order to guarantee a liable closure. Coagulation of the defect perimeter by means of deployment is the most common technique followed by mechanical abrasion of the fistula tract[79] by means of brush catheter. Modified endoscopic submucosal dissection technique to completely ablate the mucosa of the fistula or multiple endoscopic mucosal resections around the fistula opening has been described[80,81] as well. Granata *et al*[82], in a recent case series of 20 patients with post-operative leaks, described an interesting multimodality approach. The therapeutic approach was stratified in 3 groups based on structural condition of the wall defect layers (tissue status and suture feasibility). The study proposed the following strategies: Pure endoscopic direct suture (Group A: Healthy tissue and feasible suture), combined therapy with endoscopic direct suture + FC-SEMS placement + anchoring (Group B: Unhealthy tissue and feasible sutures) and FC-SEMS placement + anchoring (Group C: Unhealthy tissue and suture not feasible). The overall long-term clinical success was 80% (16/20 patients). Considering the results in each group success rate was 77% (7/9) in group A, 85% (6/7) in group B and 75% (3/4) in group C. AEs occurred in 4 cases consisting in short strictures of the distal esophagus.

In conclusion, literature shows that OverStitch is a minimally invasive endoscopic technique with interesting results in the management of leak and fistula, since it allows true full-thickness closure. However, it is a complex procedure and it is required a high level of expertise and a proper training. Hence, its use is still limited to referral center. Moreover, even though most studies so far show a high technical success rate, further prospective studies are needed to determine its long-term efficacy and safety.

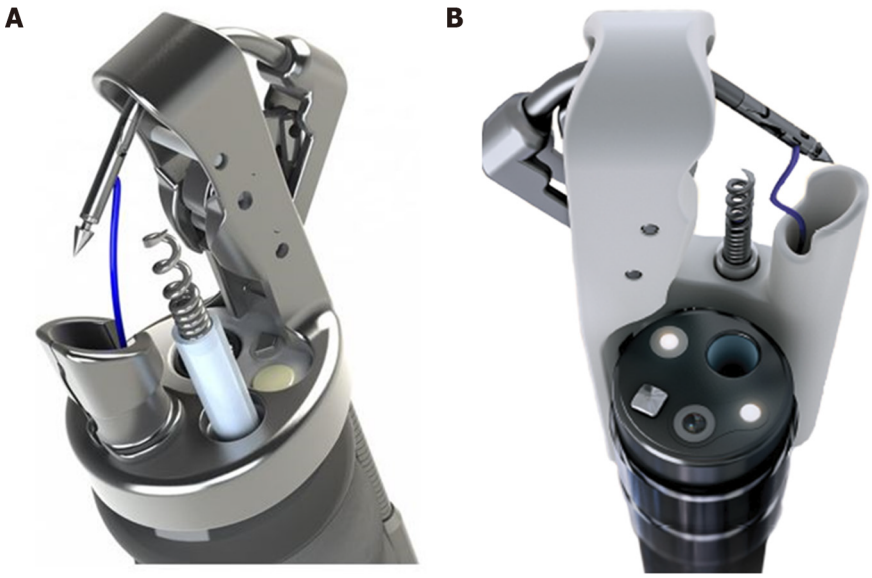


**Figure 8 Overstich SX device.** A: OverStitch device (Apollo Endosurgery, Texas, United States); and B: Overstich SX device (Apollo Endosurgery, Texas, United States).

## ENDOSCOPIC VACUUM ASSISTED SYSTEM

EVT is a minimally invasive technique for the management of anastomotic leakage, especially following rectal and esophageal surgery. EVT is an open-pored polyurethane foam connected by a suction tube to a wound drainage system producing a continuous endo-luminal vacuum therapy (Figure 9). It ensures continuous drainage, promotes granulation tissue formation and re-epithelialization, thus inducing second intention closure of the defect/cavity. Negative pressure within the defect allows mechanical cleaning of the wound from microorganism and interstitial edema reduction by improvement of microcirculation. The system needs to be changed every 3-4 d until wound cavity is healed.

### *The use of EVT in lower GI tract*

In colon-rectum, the ultimate goal of EVT is to allow early closure of defunctioning ileostomy and to avoid Hartmann's procedure. It has shown to be effective, well tolerated and safe, especially if offered at early stage in case of distal leakages in patients with a de-functioning stoma and without sepsis (Figure 10).

In detail, the first use of EVT for leaks following colorectal surgery has been proposed in 2004 but it was only until 2008 that the first large series was published by Weidenhagen *et al*[83]. The authors described EVT in 29 patients achieving definitive closure in 97% of cases. In a recent systematic review, analyzing 17 studies for a total of 276 patients treated with EVT for various colorectal pathologies (209/276 anastomotic leakage), a weighted mean success rate of 85.3 was highlighted with 25 patients (9.1%) requiring additional treatment and 38 (13.8%) developing procedure related AEs[21]. Similar results were confirmed by Popivanov *et al*[84] reporting in their review a success rate of 85.4% (range 80%-91%) with ileostomy closure achieved in 72.6% of cases. A median of 7 sponges (2-34) were required for a median period of treatment of 31 d (14-217). AEs were observed in 19% of cases with abscess being the most frequent (11.5%) followed by anastomotic stenosis (4.4%). From literature, factors associated with EVT failure are late start of EVT, neoadjuvant therapy, lack of protective stoma, age over 60 years and male sex. Interestingly most aforementioned conditions are also known risk factors for anastomotic leakage after surgery[85].

A study compared 21 patients, treated with EVT for anastomotic colorectal leakage, and a historical cohort of 41 patients, receiving conventional treatment. EVT showed, at intention-to-treat analysis, a significantly higher success rate over the conventional treatment (95.2% *vs* 65.9%; $P = 0.011$). Moreover, EVT was associated with preservation of intestinal continuity in a significant higher percentage of patients (86.7% *vs* 37.5%; $P = 0.001$)[86]. In a study from 2014 analyzing management of 103 leaks after colorectal surgery, non-operative management (drainage and antibiotics) was successful in 57% of patients with extra-peritoneal leak, whereas surgical revision (diverting ileostomy,

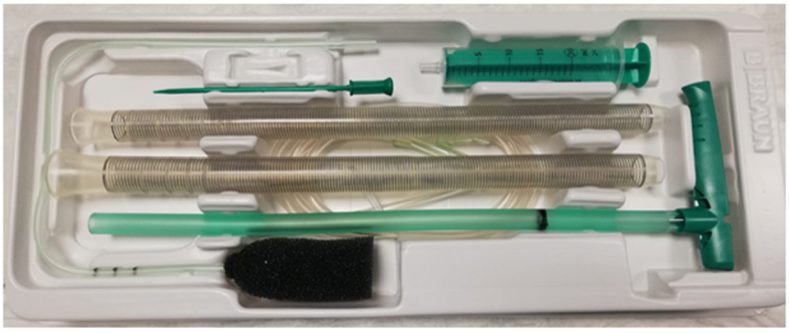

**Figure 9 Endo-SPONGE® (B.** Braun Medical B.V., Melsungen, Germany).

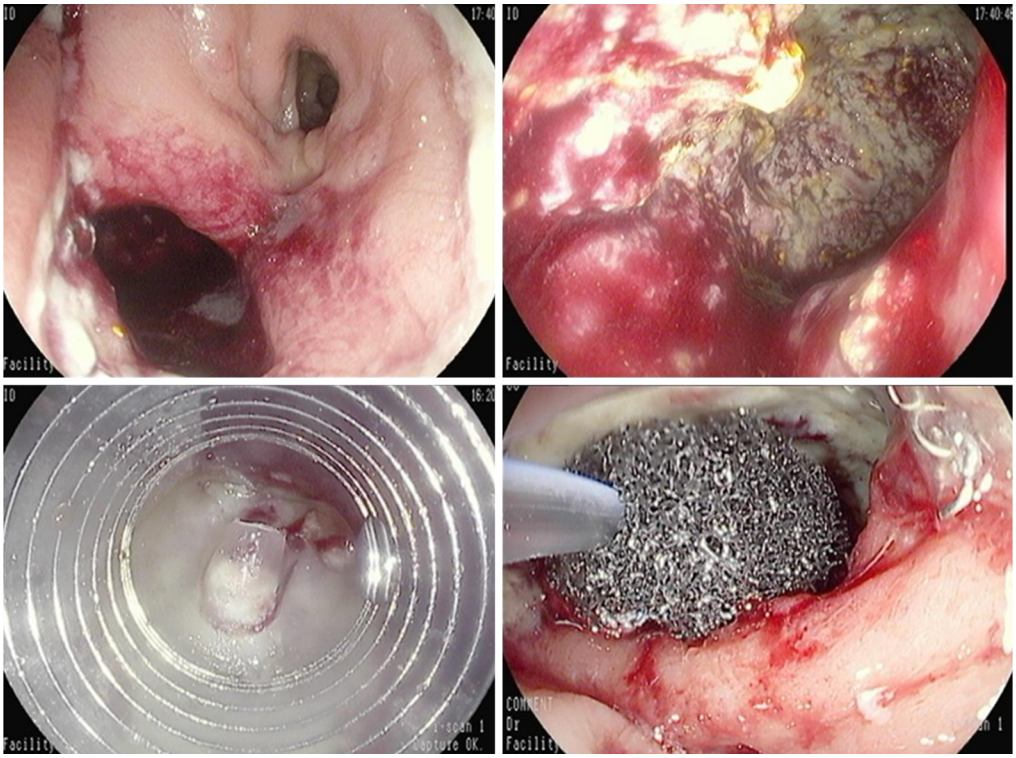

**Figure 10 Endoscopic vacuum assisted therapy for the management of an anastomotic leak after low anterior rectal resection.**

Hartmann's procedure and redo anastomosis) was successful in 41% of patients[87].

### *The use of EVT in upper GI tract*

EVT has been subsequently proposed as a viable treatment for UGI defects as well. In UGI the use EVT has been described both inside the cavity (intra-cavitary) in case of large sized leaks or within the esophageal lumen (intra-luminal) in case of small defects.

Yim *et al*[25] reported their experience of EVT in 77 patients. 59 of these patients presented post-operative leakages (36 after Ivor-Lewis esophagectomy, 15 after gastrectomy and 8 other procedures). In most cases, EVT was placed intraluminal (68/77) rather than intra-cavitary (12/77). Considering the leakage subgroup only, the authors reported a success rate of 77.9% (46/59) and a median treatment period of 11 d (1-65) with a median of 2.75 (1-9) sponges per patient. In 2017, Kuehn *et al*[88] published a systematic review comprising more than 200 patients treated with EVT for management of UGI defects. Analyzing all published series with more than 5 patients, the study highlighted a success rate of 90% (range 70%-100%), with low incidence of AE: Stricture (7.6%) and anecdotally bleeding after intra-cavitary sponge deployment. Although RCT are not available, the authors evaluated 4 retrospective studies[89-92] and

reported higher success rate, lower mortality and lower incidence of AEs for EVT compared to stent therapy.

Presumed advantages of EVT over SEMS are continuous drainage of septic locus, ability of a regular endoscopic evaluation of the defect and the possibility to deploy the sponge in all esophageal region (*e.g.*, cricopharyngeal).

Low quality evidence (retrospective studies)[88-91,93-97] showed advantages of EVT over surgical revisions for patients with sepsis or major esophageal defects in particular.

### Other EVT clinical use

Other proposed indication for EVT are leakages after bariatric and pancreatic surgery and duodenal perforation after ERCP[95,97-101]. However, up to now, a systematic approach has not yet been defined.

## TISSUE SEALANT

Tissue sealants have been successfully used in the management of anastomotic leak and low output fistula[102]. The 2 most common tissue sealants are fibrin glue and cyanoacrylate.

### Fibrin Glue

Fibrin glue consists of two components: Human fibrinogen reconstituted with aprotronin and human thrombin reconstituted with calcium chloride. The glue is applied with a double lumen catheter forming an absorbable flexible fibrin cloth mimicking the early stage of blood coagulation and wound healing. Fibrin glue acts more efficiently in dry areas; therefore, it is advisable to remove all purulent material and to ablate the surrounding mucosa before its application.

Ramón Rábago *et al*[103] reported their experience in fistula closure with fibrin glue in a case series of 30 patients, refractory to standard conservative treatment. Complete sealing of fistulas was achieved in 75% of cases (80% in low-output, 25% in high-output and 55.5% in internal fistulas). Healing time was 17 d (4-90) with a mean of 2.8 sessions per patient (1-5). Lippert *et al*[104] published in 2011 the largest series on fibrin glue management of GI leak and fistulae. The author reported in their retrospective study on 52 patients a durable closure with fibrin glue as sole endoscopic option in 36.5% of cases ($n$ = 19) and in 55.7% of patients ($n$ = 29) when fibrin glue was coupled with others endoscopic techniques (cyanoacrylate, clip or stent). From 2 to 81 mL fibrin glue (median 8.5) was used in 1-40 sessions (median 4). Nonetheless endoscopic treatment, surgical intervention became necessary in 23.1% ($n$ = 12).

### Cyanoacrylate

Cyanoacrylate (N-butyl-2-cyanoacrylate) is a synthetic glue that polymerizes after contact with moisture, causing tissue necrosis and inflammatory reaction acting as a foreign body, thus inducing tissue healing. Cyanoacrylate presents high adhesive properties that are not affected by gastric or pancreatic juice. Moreover, its antibacterial properties make its use suitable for infected areas[105]. The efficacy of cyanoacrylate was summarized in a systematic review in 2015 comprising 13 studies (prospective and retrospective case series) for a total of 203 patients, which presented foregut, midgut and hindgut fistulae. Cumulative success rate was 81% (range 0% to 100%) and 3 out of 203 patients (1%) developed minor AEs[106].

### Surgisis® anal fistula plug

Although data from literature shows satisfactory results, the use of tissue sealants as the sole endoscopic treatment, it should be limited to small low-output leaks or fistulae only. Fibrin glue and cyanoacrylate may play a useful role for the management of GI defects in combination with other endoscopic techniques[107].

Darrien *et al*[108] proposed an interesting approach for closure of refractory entero-cutaneous fistulae with Surgisis® anal fistula plug (Cook Surgical, Bloomington, United States). The Surgisis® anal fistula plug is an advanced tissue repair graft made from porcine submucosa developed for the management of perineal fistula. It serves as a scaffold for host cells to replace and repair damaged tissue. The acellular matrix promotes fistula closure without foreign body inflammatory reaction. Surgisis® has been used for management of fistulae after bariatric surgery as well. In a case series of 25 patients with gastro-cutaneous fistula after Roux-en-Y-gastric Bypass strip-shaped Surgisis was used for 20 patients and cone-shaped Surgisis in 5 patients[109]. Using the strip-shaped biomaterial, success rates were approximately 75% after two or three

sessions, whereas using cone-shaped matrix fistula closure was accomplished after a single session in all patients.

## CONCLUSION

Endoscopy is emerging as first line approach over surgery for the management of Gastrointestinal leaks and fistulae. The steadfast advancements of interventional endoscopy in the last decades allowed for new endoscopic closure devices and techniques, which provide a minimally invasive and more effective therapeutic option than surgery. A single therapy, or even a combination of different techniques, can integrate the use of different endoscopic options (Table 2). Comparison between different approaches is difficult due to heterogeneous populations, prevalence of retrospective studies, lack of uniform definitions and lack of comparative studies. Therefore, it is difficult to establish a standardized therapeutic algorithm. Each treatment should be tailored to the single patient, by taking into account the several variables that may at the end influence the outcome. Endoscopic management of leaks and fistulae requires a personalized and multidisciplinary approach, comprising a close collaboration between surgeon, interventional radiologist and endoscopist, allowing Gastrointestinal wall defect management with high clinical success rate and low rate of morbidity and mortality.

**Table 2 Main features of the different endoscopic approaches to leak and fistula**

| | Mechanism of action | Advantages | Disadvantages | Cost | N° of sessions | Expertise needed |
|---|---|---|---|---|---|---|
| Endoclips | Direct closure | More effective in acute setting | Less effective in chronic setting; Need of external drainage | + | + | + |
| Stent | Defect sealing | Early oral intake; Reduce stricture formation | Stent migration; Tissue ingrowth/overgrowth pain; Need of externaldrainage | ++ | + | ++ |
| EID | Second intention closure | Early oral intake; Internal drainage; More effective in acute setting | Stricture | + | ++ | ++ |
| Suturing system | Direct closure | True full-thickness closure; Single operator (Overstich®) | On healthy tissue; More difficult in tight endoluminal space and tangential suturing site | +++ | + | +++ |
| EVT | Second intention closure | Continuous drainage; More effective in early stage | Limited to rectal/esophageal site; Need of de-functioning stoma; Less effective if late diagnosis | + | +++ | + |
| Tissue sealant | Miscellaneous | Antibacterial (cyanoacrylate); Used in combination; No inflammatory reaction (Surgisis®) | On dry areas (fibrin glue); Inflammatory reaction (cyanoacrylate/fibrin glue) | + | ++ | + |

EID: Endoscopic internal drainage; EVT: Endoluminal vacuum therap.

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*REVIEW*

# Watch and wait approach in rectal cancer: Current controversies and future directions

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## Abstract

According to the main international clinical guidelines, the recommended treatment for locally-advanced rectal cancer is neoadjuvant chemoradiotherapy followed by surgery. However, doubts have been raised about the appropriate definition of clinical complete response (cCR) after neoadjuvant therapy and the role of surgery in patients who achieve a cCR. Surgical resection is associated with significant morbidity and decreased quality of life (QoL), which is especially

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relevant given the favourable prognosis in this patient subset. Accordingly, there has been a growing interest in alternative approaches with less morbidity, including the organ-preserving watch and wait strategy, in which surgery is omitted in patients who have achieved a cCR. These patients are managed with a specific follow-up protocol to ensure adequate cancer control, including the early identification of recurrent disease. However, there are several open questions about this strategy, including patient selection, the clinical and radiological criteria to accurately determine cCR, the duration of neoadjuvant treatment, the role of dose intensification (chemotherapy and/or radiotherapy), optimal follow-up protocols, and the future perspectives of this approach. In the present review, we summarize the available evidence on the watch and wait strategy in this clinical scenario, including ongoing clinical trials, QoL in these patients, and the controversies surrounding this treatment approach.

**Key words:** Watch and wait; Rectal cancer; Clinical complete response; Organ preservation; Dose intensification



**Core tip:** The Watch and wait strategy in selected patients with locally-advanced rectal cancer is associated with lower morbidity and better quality of life than conventional treatment, with good cancer control. Given the growing relevance of this strategy, which is increasingly being used at international centres of reference, a comprehensive review of the available data is needed. In addition, there are several open questions and controversies about this strategy that can only be resolved by an in-depth analysis and consensus among the specialists involved in treating these patients.

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## INTRODUCTION

According to the most recent GLOBOCAN data (2018), colorectal cancer is the 4th most common cancer worldwide, with an annual incidence of more than 700000 cases and the 3rd highest mortality rate[1]. In patients with locally-advanced rectal cancer (LARC), the most effective treatment, in terms of efficacy and toxicity, is long-course neoadjuvant chemoradiotherapy (CRT) followed by total mesorectal excision (TME)[2]. An important disadvantage of this approach is a high risk of surgical complications, with a postoperative mortality rate at 6-months ranging from 2%-8%, and as high as 30% in older patients (> 85 years)[3].

Given this context, in recent years there has been a growing awareness of the need to strike a balance between curative treatment and quality of life (QoL). As a result, the application of radical surgery in all patients diagnosed with LARC is increasingly being questioned. The rising interest in organ preservation strategies reflects the need to prevent, whenever possible, the significant postoperative morbidity (intestinal, urinary and sexual dysfunction) associated with TME. The risk of postoperative dysfunction is particularly evident in surgical procedures such as abdominoperineal resection, which requires a permanent ostomy, which has a severe negative impact on QoL.

According to the available data, from 10%-25% of patients with LARC achieve a pathologic complete response (pCR) - defined as the absence of viable residual tumour cells in the surgical specimen - after neoadjuvant treatment[4]. The response rate is higher in patients who receive high-dose radiotherapy[5] and/or optimized chemotherapy[6]. Research is currently underway to identify predictors of pCR after standard neoadjuvant treatment in order to improve response rates. In this context, the organ-preserving treatment approach that has come to be known as "watch and wait",

in which surgery is omitted after CRT, has become increasingly relevant.

The watch and wait strategy was originally proposed by Dr. Habr-Gama and her group, who have supported the non-surgical treatment of LARC for nearly two decades in patients who achieve a complete clinical response (cCR), defined as the absence of clinically-detectable residual tumour, after neoadjuvant therapy. The findings of the studies conducted by this group[7-11] suggest that overall survival (OS) rates in selected patients who undergo observation with regular follow-up after neoadjuvant treatment are comparable to those obtained in patients who achieve a pCR after radical surgery. The main advantage of the watch and wait approach is that it avoids all of the significant morbidity and mortality risks associated with abdominoperineal resection.

Subsequent studies carried out by other groups support these data, as shown in a recent systematic review[12] that evaluated a total of 23 studies (867 patients), concluding that there are no significant differences in OS and local recurrence between surgically-treated patients and those managed with the watch and wait protocol. However, larger prospective studies are needed to confirm long-term outcomes and to resolve controversies surrounding the selection of candidates for watch and wait, the accurate determination of cCR, and the optimal follow-up protocols.

## DIAGNOSIS AND REASSESSMENT

Imaging studies in patients with a recent diagnosis of rectal cancer are primarily performed for TNM staging to select the optimal therapeutic strategy, whereas the main aim of imaging after neoadjuvant therapy is to evaluate treatment response and to identify areas of tumour infiltration for surgical planning. These same images are used to determine eligibility for the watch and wait approach[13].

Although several different imaging modalities are available for locoregional staging of rectal cancer, the standard technique is magnetic resonance imaging (MRI), which provides visualization of the entire pelvis as well and offers the best assessment of the circumferential resection margin and other prognostic factors[14-17]. In previously-treated patients, MRI can differentiate between foci of tumour persistence (residual disease) and changes secondary to treatment, an important advantage over other imaging techniques[18-20]. Endorectal ultrasound also provides good results, but its efficacy is limited by a loss of resolution at depth, and difficulties associated with stenotic, bulky or localized rectal tumours[15,21-23]. Notwithstanding these disadvantages, endorectal ultrasound remains the technique of choice to differentiate between early stage tumour (T1 *vs* T2), where its diagnostic accuracy is superior to MRI[14-16,24]. In cases in which MRI is contraindicated (due to a pacemaker or non-MRI-compatible metal implants), ultrasound is the technique of choice[25,26]. Other imaging modalities such as positron-emission tomography (PET) have also shown good results, but these are either not recommended for routine use (*e.g.*, PET) or not yet commercially available, as is the case with specific MRI contrast agents such as ultra-small superparamagnetic particles of iron oxide (USPIO) and gadofosveset[27-29].

### *MRI: Techniques and sequences*

The MRI protocol for primary staging and post-treatment follow-up is the same, despite the different aims[13,16]. MRI scanners of at least 1.5 Tesla with 8-32 channel coils are recommended[30-32]. Endorectal gel can be administered to increase distension, which may facilitate detection of polypoidal or small lesions[16,31,33-35]; however, the use of these gels is controversial because displacement secondary to the compression of the mesorectal fat could theoretically induce false positives (invasion of the mesorectal fascia) or impede the accurate assessment of nodal disease[30,33,36,37]. Nonetheless, this has not been demonstrated[35]. The use of spasmolytics such as glucagon and butylscopolamine is highly variable, although decreased intestinal peristalsis may be useful in assessing tumours located in the upper rectum or when using 3T MRI, which is more sensitive to motion artefacts[16,31,33,36]. The optimal interval between completion of neoadjuvant therapy and follow-up MRI remains controversial, although recent data appear to support an interval of approximately 8 wk[16,17].

Oblique T2-weighted sequences are recommended to locate pelvic lesions. High-resolution T2 imaging should be obtained in different planes with respect to the longitudinal axis of the tumour, with a maximum slice of 3 mm. At present, the T2-weighted sequence is the most commonly used in staging rectal cancer[13,17,38,39]. The use of T1-weighted imaging with intravenous contrast administration is not considered necessary, although some authors suggest that it could facilitate the detection of

tumour foci or vascular involvement[13,16,40-42].

The value of diffusion-weighted imaging (DWI) for rectal cancer is also unclear, as no definitive conclusions can be made due to the heterogeneity of the available studies[43,44]. Currently, it is thought that combining DWI with high-resolution T2 imaging could facilitate assessment of the primary tumour after neoadjuvant therapy, especially to help differentiate between partial and complete response[15,16,21]. However, in tumours with mucinous differentiation, this capacity may be limited due to the difficulty of distinguishing between residual tumour and mucin foci[13,17,45]. Some authors have suggested that the quantitative evaluation of the apparent diffusion coefficient (ADC) could be beneficial; however, the results to date have been variable and-given the overlap between benign and malignant ADC values and the complex extrapolation between MRI scanners - no clear recommendations can be made at present[16,46-48]. Although ADC has other potential uses (primary staging, assessment of nodal disease and extramural vascular infiltration) the current evidence base is insufficient to draw any definitive conclusions; that said, some authors have suggested that ADC may be useful in certain well-defined cases[16,49,50].

### *MRI in watch and wait*

Many recent studies of MRI in rectal cancer have focused on its role in watch and wait strategies, with the following findings considered to indicate complete response of the primary tumour after neoadjuvant therapy: Normalization of the rectal wall, with good differentiation between mucosa and muscular layers without significant thickening. The presence of hypointense residual foci is indicative of fibrosis[16,17,51]. De Jong *et al*[52] conducted a meta-analysis to assess the utility of MRI to detect complete response, reported a pooled accuracy of 75%, sensitivity and specificity of 95% and 31%, and positive and negative predictive values of 83% and 47%, respectively. These findings suggest that MRI may be more useful to rule out complete response rather than to confirm it. In this regard, DWI-MRI is especially promising, as it provides a functional assessment of the tissues and improves the diagnostic accuracy of complete response (defined as the absence of residual hyperintensity)[16,17,51,53-55]. One study found that DWI-MRI increased sensitivity (response prediction) from 50% to 84%[43]; however, the heterogeneous designs of the studies that have evaluated this imaging tool - some of which do not use high resolution imaging - do not allow us to make any definitive conclusions[51,53,56].

The greatest challenge in MRI-based rectal staging is the assessment of regional nodes[17]. In general, MRI is considered to be more efficacious for follow-up staging after neoadjuvant therapy[17,57]. In a meta-analysis carried out by van der Paardt *et al*[43], the mean sensitivity and specificity rates for determining nodal stage (per patient) were 76.5% and 59.8%, respectively, and 91.7% and 73% per lesion. Only patients with a confirmed lack of nodal involvement should be considered candidates for watch and wait[58]; in this regard, a negative predictive value of 95% has been described in patients with stage ypN0 disease[57]. Based on published data, up to 16% of lymph nodes remain positive after neoadjuvant therapy, even in cases in which the primary tumour shows a complete clinical response[59-61]. Similarly, cases of recurrent nodal disease with apparent negativization have been documented, raising doubts about our ability to ensure all residual nodal disease has been eliminated[62].

A wide range of criteria have been used to define malignant lymph nodes, including size, morphology, and signal intensity, among others factors. However, due to the highly variable results the optimal criteria remain unclear[21,30-32,63]. The utility of morphological criteria after neoadjuvant therapy is limited because negative nodes may show irregular borders or heterogeneity secondary to residual fibrosis or mucinous degeneration[19,64,65]. Nonetheless, in patients treated with radiotherapy, node size decreases in up to 84% of cases; crucially, nodes that remain enlarged are more likely to be malignant[66-68]. Accordingly, a recent consensus statement recommended using nodal size for follow-up assessment after neoadjuvant therapy (with nodes whose short axis diameter is < 5 mm considered benign), given the absence of other reliable criteria[16]. However, several studies have reported the presence of small groups of residual cancerous cells in a significant number of small nodes (up to 3 mm), a finding that limits the sensitivity of this criterion[67,69,70]. Some authors have suggested that these foci could show a late response to treatment, but this hypothesis is unconfirmed and controversial[10,71,72].

Other authors have suggested applying mixed size and morphology criteria, similar to those recommended for primary staging; however, the evidence to support this approach remains insufficient[26,73,74]. Although MRI-DWI improves node localisation, there is no evidence that this imaging modality is more accurate than other approaches in determining malignancy[75,76]. In any case, caution is recommended when trying to

establish a possible complete response based solely on MRI data in patients managed with a watch and wait strategy[16,52,77,78] given the less than optimal results obtained to date[55,73,78] (Figure 1).

The value of radiomics in assessing rectal cancer by MRI is currently being investigated through the application of tools to perform multifactorial quantitative analysis of digital images[79,80]. Highly promising results have been reported identifying complete response using several different parameters in both T2 and DWI sequences, including changes in the relative signal intensity pre- and post-neoadjuvant therapy, texture analysis, kurtosis, and/or volumetry[47,59,79,81-87]. Some studies have even found that the application of these analyses to pre-treatment staging MRI can predict responders[88,89].

## PATIENT SELECTION

One of the most important obstacles in assessing the published findings of watch and wait strategies is the heterogeneity in data quality, mainly due to inadequate staging techniques or insufficient clinical data, which limits our capacity to interpret these findings adequately and to define the clinical characteristics of the patients most likely to benefit from this strategy. It is also difficult to determine the patient profile most likely to achieve a cCR; similarly, it is hard to know the true correlation between clinical and pathological complete response.

Tumour location is an important factor in patient selection, as tumours located in the middle and lower third of the rectum (close to the anal verge) require definitive stoma. Up to 90% of patients who undergo TME develop low anterior resection syndrome (LARS), and 33% and 50% of patients develop, respectively, urinary and sexual dysfunction[3]. Unsurprisingly, these patients generally experience a significant deterioration in QoL. Due to these adverse effects, the main candidates for watch and wait are patients with tumours in these areas of the rectum (in whom TME is indicated) but who successfully achieve a cCR after neoadjuvant therapy, or patients with multiple comorbidities and/or those not considered candidates for surgery. With regard to this latter group, this is considered a different clinical entity and should be excluded from any watch and wait analysis given that surgery is not possible even if indicated.

A significant proportion of the cases included in retrospective watch and wait series are patients who refuse surgery, even though this is not contraindicated. The clinical characteristics of this subset of patients are highly variable, the quality of the data is poor, and there is only limited follow-up data. For these reasons, the highest levels of evidence for watch and wait comes from other patient groups.

### *Patients with low risk of local recurrence*

The standard treatment in patients with early stage LARC without associated poor prognostic factors is surgery without neoadjuvant therapy. If LARC is histopathologically confirmed, no additional treatments are indicated. Surgical resection yields exceptional results in terms of both local and distant control. In this clinical scenario, a watch and wait strategy can only be applied by disregarding existing multidisciplinary protocols, or in the context of a clinical trial. Nevertheless, this approach is increasingly considered a viable option in well-selected patients, especially those who rejected surgery and those with tumours located in the lower third of the rectum[90], knowing that is not yet a standard treatment. At the moment, neoadjuvant treatment-related toxicity is considered to be an important limitation when evaluating watch and wait strategies in patients with low risk of local recurrence as well as the lack of high level of evidence in a clinical scenario where the oncological results of the standard treatment with surgery are excellent.

### *Patients with a high risk of tumour recurrence*

The standard of care in patients with LARC and poor prognostic factors is neoadjuvant therapy followed by TME[91]. These patients have the highest risk of residual tumour persistence after the initial treatment, and treatment intensification is important to achieve a safe surgical plane to ensure complete resection of all cancerous tissue; otherwise, more aggressive interventions-with the associated morbidity, especially in tumours located in the lower rectum-could be necessary[92,93]. Patients who achieve a complete or near-complete clinical response may be excellent candidates for the watch and wait approach, given that LARS is presented in up to 90% of these patients after TME[94]. Nonetheless, the most suitable subgroup for this approach remains unclear

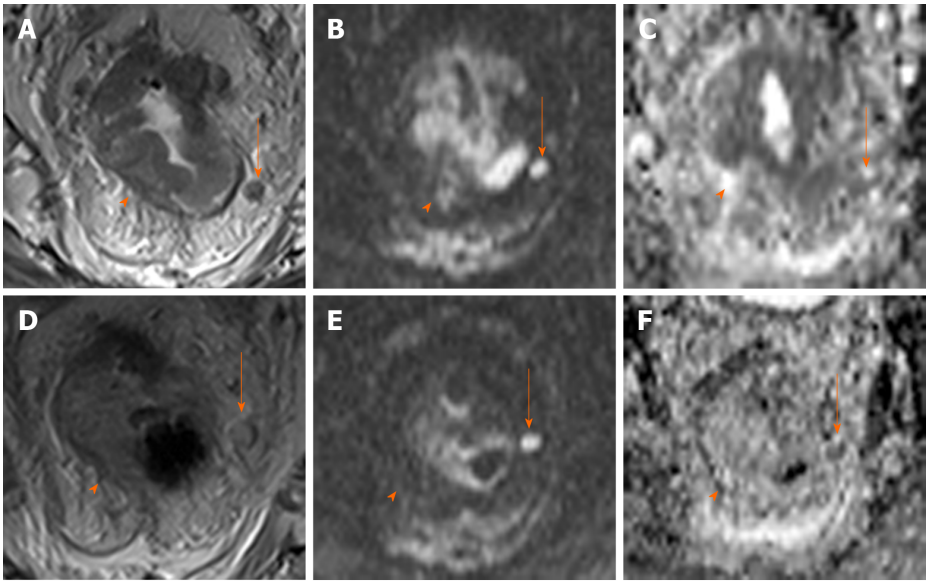


**Figure 1 Discrepancies in magnetic resonance sequences in follow-up imaging after neoadjuvant therapy.** Magnetic resonance imaging (MRI) of the rectum for initial staging (upper row): High-resolution T2 sequences (A), high b value diffusion-weighted imaging (DWI) (B) and apparent diffusion coefficient (ADC) map (C). Protuberant wall thickening (arrow) with an adjacent enlarged, heterogeneous lymph node (arrow); signs of restricted diffusion are observed in both sequences (hyperintensity in DWI and hypointensity in ADC). MRI after neoadjuvant treatment in the same patient (bottom row) reveals near complete resolution of the main mass on the various imaging sequences. In the affected node, fewer morphological alterations are visible, but with no decrease in size (D) and with signs of restricted diffusion (E and F), suggesting persistent malignancy. No evidence of nodal malignancy is evidenced on the histological analysis of the surgical specimen.

because most of the available evidence comes from patients with distal tumours, patients with proximal tumours have been excluded from most clinical trials due to the difficulty of performing digital rectal examination (DRE), which is important to evaluate response and to monitor the course of disease, limiting the possibility of generalizing the use of this strategy in this setting. Regarding surgical treatment, one could argue that salvage surgery might potentially be more challenging than an upfront procedure. In patients previously treated with CRT, salvage surgery has a higher risk of complications[95,96] due to the increased fibrosis in the pelvis and the greater technical difficulty of the surgical procedure, both of which are relevant factors that must be considered as an important limitation in treatment selection for this approach.

On the other hand, long-term outcomes in patients managed with the watch and wait strategy are excellent, with 5-year OS rates ranging from 91% to 96%, as follows: Habr-Gama *et al*[97] (91%), Martens *et al*[98] (97%), Appelt *et al*[5] (100% at 2 years), and Renehan *et al*[99] (96%). Importantly, this approach does not appear to be associated with worse outcomes in patients who develop locally-recurrent disease during follow-up[12]. In recent years, some studies have found that patients with locally-recurrent disease present more distant metastases[100,101]. van der Valk *et al*[102] found that OS was lower in patients who achieved a cCR compared to a retrospective cohort with pCR. Notwithstanding those findings, the results must be interpreted in the context of the study limitations: Retrospective study design, differences among patients in clinical characteristics and treatments; lack of MRI assessment in most case, and the moderate correlation between cCR and pCR[103].

## TREATMENT DURATION AND INTENSIFICATION

### *Standard chemoradiotherapy. Dose escalation*

Numerous efforts have been made to improve cCR rates by modifying the neoadjuvant therapy scheme to lower the risk of local recurrence in well-selected patients, with promising results[5,58,104]. However, it is difficult to establish a standardized approach due to the diversity of approaches utilized, which include radiation dose escalation - highly conformal external beam radiotherapy (*e.g.*, intensity-modulated radiotherapy; IMRT) or brachytherapy - as well as induction and/or consolidation chemotherapy[5,58,104]. Another approach used in elderly patients who are not candidates for chemotherapy is a short cycle of radiation (25 Gy in 5

sessions) followed by a watch and wait strategy[105].

In 2004, Habr-Gama *et al*[8] reported the first results of the watch and wait strategy in 71 patients with LARC who achieved a cCR after standard neoadjuvant therapy (50.4 Gy to the pelvic volume plus fluoropyrimidine-based chemotherapy), with a local recurrence rate of only 2.8%. However, subsequent studies were unable to replicate those results, with reported local recurrence rates ranging from 5% to 60%[104]. This variability is likely due to patient selection bias; for example, Habr-Gama *et al*[8] only evaluated patients who showed no evidence of recurrent disease 12 mo after neoadjuvant therapy.

With regard to radiation dose escalation, Appelt *et al*[5] conducted a prospective, observational study in patients with tumours located ≤ 6 cm from the anal verge (stage T2-3,N0-N1) received high dose radiotherapy (50 Gy) to the pelvic volume (1.6 Gy/session), 30 Gy (2 Gy/session) to the tumour, and a brachytherapy boost (5 Gy). The patients also received concomitant oral tegafur [300 mg/(m$^2$·d)]. At six weeks, response was assessed with CT, MRI, endoscopy, and four biopsies from the initial tumour site (previously ink-marked). Although Maas *et al*[58] were only able to include 11% of their patients in the watch and wait strategy after standard treatment, 78% of patients achieved a cCR (35% stage T2N0). The local recurrence rate at 12 months was 15.5%, with 69% of patients presenting good anal sphincter function; grade 3 diarrhea was observed in 8%. In terms of long terms toxicity, the main adverse effect was grade 3 rectal bleeding, affecting 7% of the patients, a finding that led the authors to reconsider the application of the brachytherapy boost.

In another study, Habr-Gama *et al*[106] found that dose escalation (54 Gy plus six cycles of type 5-FU-LV chemotherapy) resulted in better cCR rates (57%). However, the 2-year local recurrence rate was 27%, probably due to the disease stage (T2N0); in these patients the standard treatment was surgery, which has a higher probability of achieving a cCR after high dose CRT. In this regard, this CRT scheme proposed by Habr-Gama *et al*[107] should be performed in a clinical trial. In another study, the same authors retrospectively compared dose-intensified CRT to conventional treatment. At 5 years, patients in the experimental arm presented a significantly higher cCR rate (67% *vs* 30%; $P$ = 0.001). However, there were no differences in surgery-free survival among the patients who achieved a cCR. By contrast, a study[108] based on data from the National Cancer Database found no benefit to radiation dose escalation, although it is worth noting that most of the patients in that study who received higher doses were older, had more comorbidities, and were more likely to be medically inoperable.

A wide range of neoadjuvant therapies have been described in the studies that have evaluated watch and wait strategies. In general, the reported cCR rates are high, especially in patients who receive intensified neoadjuvant therapy, although treatment-related toxicity is also higher[7-11,106]. Habr Gama *et al*[106] retrospectively evaluated patients with stage cT2N0 tumours located < 7 cm from the anal verge, reporting a cCR rate of 56.6% with standard treatment versus 85.7% in the dose escalated (54 Gy) group ($P$ < 0.001), with a 5 years surgery-free survival rate of 78%[106].

### *Contact X-ray brachytherapy - High-dose rate brachytherapy*

Brachytherapy can also be used to escalate radiation doses. The value of this technique is that it permits local application of a higher dose directly to the tumour, thus preserving the surrounding healthy tissue. The brachytherapy dose is delivered either by contact X-ray applicators (CXB) or with endorectal or perineal intraluminal applicators, using high-dose rate brachytherapy (HDR-BT). Sun Myint *et al*[109] evaluated inoperable patients (stage cT2-T3) treated with dose-escalated CRT (45 Gy at 1.8 Gy/fr) plus a 90 Gy boost with CXB (30 Gy/fr to the rectal surface), finding a cCR of 63.8% in patients with residual tumour < 3 cm. The local recurrence rate at 2.5 years was 11.3%. Gérard *et al*[110] treated patients with stage cT2-T3 rectal cancer with 50 Gy CRT (2Gy/fr) plus a 90 Gy boost of CXB (except for tumours < 3.5 cm, in which CXB was performed before radiotherapy), reporting a cCR rate of 86% and a local recurrence rate at 3 years of 10%. In both series, the most common toxicity was grade 1-2 proctitis, with grade 3 proctitis described in 0-9% of cases. Garant *et al*[111] evaluated dose escalation with HDR-BT in patients with inoperable stage cT2-T3 rectal cancer, finding a cCR rate of 86.6% in patients who received radiotherapy alone (40 Gy; 2.5 Gy/fr) plus HDR-BT (3 fractions of 10 Gy). The 3-year local control rate was 67.1%, with a local recurrence rate of 21.9%. The most common adverse effect was rectal toxicity, with nearly all patients experiencing grade 1-3 proctitis, and 12.8%-13% developing grade 3 proctitis. Urogenital and cutaneous toxicities were also observed in this group, but not in those who underwent CXB. A retrospective study performed in the United Kingdom by Smith *et al*[112] evaluated radiation dose escalation in 14 patients, who were treated with CXB or HDR-BT. In that study, a complete or partial

clinical response was observed in 79% of cases, with colostomy-free survival of 93%.

### *Short-course radiotherapy*

Rupinski *et al*[113] evaluated neoadjuvant short-course radiotherapy (SCRT) in a small series ($n$ = 30) of older patients (> age 70) who received 5 sessions of radiotherapy at 5 Gy/session. Of these patients, 20% achieved a cCR and were kept under observation. Of the 30 patients, three were stage T2N0 and three T3N0. Tumour regrowth was observed in 16.6% of patients. The authors concluded that watch and wait is feasible after SCRT without associated chemotherapy[113].

The available evidence suggests that, due to technological advances in EBRT techniques, radiation doses can be safely elevated to increase the cCR rate and the number of patients eligible for conservative strategies. Most of the studies published to date have included a high percentage of patients with early-stage disease. Given that we still lack data from randomized controlled trials, dose escalation cannot yet be considered a standard approach. Although the addition of a brachytherapy boost has been shown to improve cCR rates, prospective studies are needed to better define the role of brachytherapy in organ preservation strategies. Similarly, consensus-based guidelines are needed to define and describe the main technical aspects of endorectal brachytherapy (*e.g.*, technique, dose, point of prescription, volume delimitation, and constraints). Such studies would also help to better determine which patients would truly benefit from this approach.

### *Consolidation chemotherapy and induction chemotherapy*

Optimization of chemotherapy schemes and agents could improve the cCR rate, although these chemotherapy regimens are normally reserved for patients with poor prognostic factors. Various chemotherapy schemes are available, such as induction chemotherapy (ICT) and consolidation chemotherapy (CCT), including active regimens that include a combination of agents, However, due to the heterogeneity of the available studies, no firm conclusions can be drawn at present.

## CONSOLIDATION CHEMOTHERAPY

The pCR rate can be increased by extending the interval between neoadjuvant CRT and surgery (without additional treatment), but this strategy also increases the risk of distant progression. The addition of chemotherapy during this time period could prevent distant spread and help to downstage the primary tumour.

García-Aguilar *et al*[6] conducted a non-randomized, multicenter study to evaluate 256 patients with stage 2 or 3 rectal cancer. One arm received standard chemoradiation followed by surgery 6-8 wk later, with a pCR of 18%. In the others arm, CCT was added to the treatment protocol to extend the interval between CRT and surgery, leading to a significant increase in the pCR rate, as follows: 25% for a 12-wk interval (two cycles of mFOLFOX6), 30% for a 16-wk interval (four cycles of mFOLFOX6), and 38% for a 20-wk interval (six cycles of mFOLFOX6) ($P$ = 0.004). However, it is not clear the extent to which these differences are attributable to patient selection bias and/or the delay in evaluating treatment response, rather than to the direct effects of treatment.

CCT after SCRT is an interesting therapeutic strategy that has been explored in other studies[114-116]. In a phase 3 clinical trial in Poland[115], this approach improved 3-year OS outcomes versus standard treatment (73% *vs* 65%, $P$ = 0.046), with less acute toxicity. The ongoing RAPIDO study[116], which is currently comparing SCRT followed by 6 cycles of CAPOX to long-cycle CRT with capecitabine, will better define the role of consolidation chemotherapy as a standard of care in these patients. Nevertheless, it is worth emphasizing that Habr-Gama *et al*[97,107,117] have previously reported good results using CCT as part of a treatment intensification strategy followed by watch and wait.

## INDUCTION CHEMOTHERAPY

Administration of all chemotherapy treatments prior to CRT [total neoadjuvant therapy (TNT)] may increase adherence, an approach which has been investigated in several studies. The Spanish Group of Rectal Cancer[118,119] randomized 108 patients with LARC to receive either concurrent CRT with CAPOX followed by surgery plus

postoperative adjuvant chemotherapy (4 cycles of CAPOX), or induction chemotherapy (4 cycles of CAPOX) followed by the same treatment combination used in the other arm (*i.e.*, CRT followed by surgery). Treatment adherence was higher in the ICT arm, with a lower proportion of patients developing severe (grade 3-4) chemotherapy-related adverse effects. Between-group differences in pCR (13% *vs* 14%) were not clinically significant.

Other studies-including the EXPERT, EXPERT-C[120], AVACROSS[121] trials-have reported higher R0 resection rates with ICT, although without any improvement in pCR. In the EXPERT-C and AVACROSS studies, there was no benefit to adding targeted therapies to induction chemotherapy in this clinical scenario.

Given the limited available evidence, it is not possible to reach definitive conclusions regarding which of the two treatment options (CCT *vs* ICT) has better adherence, nor which approach induces greater primary tumour regression.

## TIMING OF ASSESSMENT

Several strategies have been shown to improve cCR rates. The simplest-but not least important-approach is to extend the time between completion of neoadjuvant therapy and reassessment. Several retrospective studies in patients with LARC have shown that extending the interval between CRT and surgery increases tumour regression and improves pCR rates[122-124]. The optimal time interval is 8 wk, as studies show that this yields the best pCR outcomes[125,126]. Reassessment before 8 wk is not recommended, as the results could be interpreted as a false incomplete res-ponse[97,107,117].

In the studies conducted to date to evaluate the watch and wait strategy[5,58,97-99,117,127-132], cCR has been assessed at various time points, ranging from 4 to 20 wk after completion of neoadjuvant therapy (Table 1). Consequently, the optimal time to assess cCR remains undefined.

Given these findings, it appears that assessment of treatment response to determine the cCR should be performed sometime around week 8 after completion of CRT. However, this criterion may need to be adjusted according to the patient's initial tumour stage, since more advanced tumours require a longer time interval to reach a cCR. Nonetheless, the initial reassessment should not be excessively delayed given the importance of early determination of poor response to neoadjuvant therapy to avoid delaying surgery unnecessarily.

## FOLLOW-UP PROTOCOLS

The watch and wait strategy in rectal cancer has several important drawbacks, including the lack of a consensus-based definition of treatment response and follow-up protocols, as well as the poor reliability of the current predictors of response.

In patients managed with a watch and wait strategy, the main recommendation given by specialised centres is close monitoring through frequent follow-up visits. However, these recommendations are probably not practical in routine clinical practice at most centres[133]. In general, the initial assessment of treatment response should be performed 6-10 wk after completion of neoadjuvant therapy, with intensive surveillance during the first two years and longer follow-up intervals thereafter[58,107,123,127,129,134].

In the absence of prospective controlled trials, at present is not possible to provide well-defined, evidence-based guidelines on the optimal follow-up protocols to improve prognosis[12,105]. While endoscopy is the main tool for follow-up evaluation, the use of MRI is increasing. MRI findings should correlate with the combined findings of DRE and endoscopy, the combination that offers the best diagnostic accuracy for the evaluation of complete response[16,59,135] and for initial disease staging[16,61,136]. Most protocols also recommend determination of CEA levels after neoadjuvant therapy since normalization (< 5 ng/dL) of this biomarker in patients with elevated levels prior to treatment appears to predict treatment response[137-139].

The following endoscopic findings were first defined by Habr-Gama *et al*[140] as predictors of response: Complete elimination of the rectal tumour, replaced by a flat, regular, whitish scar, with telangiectatic vessels on its surface. These findings have been shown to have a high negative predictive value[141]. Other endoscopic findings, such as the presence of ulcerations, mucous irregularities, nodules, stenosis, or persistence of rectal masses indicate incomplete response. Nonetheless, none of these findings are reliable predictors of response, as measured by sensitivity and (especially)

**Table 1 Time between completion of neoadjuvant therapy and first reassessment in watch and wait clinical studies**

| Study | Patients (*n*) | Neoadyuvant therapy | | Timing of assessment after CRT |
|---|---|---|---|---|
| | | Radiotherapy schedule | Chemotherapy regimen | |
| Habr-Gama *et al*[107], 2013 | 70 | 54Gy/30 | CRT: 5-FU/LV CNCT: 5-FU/LV x3 | 10 wk |
| Araujo *et al*[128], 2015 | 51 | 45 Gy/25 or 50, 40 Gy/28 | CRT: 5-FU or capecitabine | NS |
| Smith *et al*[129], 2012 | 32 | 50,4 Gy/28 | CRT: 5-FU or capecitabine | 4-10 wk |
| Dalton *et al*[127], 2012 | 12 | 45 Gy/25 | CRT: capecitabine | 8 wk |
| Renehan *et al*[99], 2016 | 259 | 45 Gy/25 | CRT: 5-FU or capecitabine | ≥ 8 wk |
| Appelt *et al*[5], 2015 | 51 | 60 Gy/30 to tumor + 50 Gy/30 to LNs | Tegafur-uracil (UFT) | 6 wk |
| Vaccaro *et al*[130], 2016 | 204 | 50.4 Gy/28 | CRT: 5-FU/LV | 8-12 wk |
| Lai *et al*[131], 2016 | 267 | 45 Gy/25 or 54 Gy/30 | CRT: 5-FU/LV | 8-12 wk |
| Martens *et al*[98], 2016 | 141 | 50.4 Gy/28 or 5 Gy/5 | CRT: 5-FU | 8-20 wk |
| Creavin *et al*[132], | 362 | 50-54 Gy/30 | CRT: 5-FU | 6-8 wk |

CRT: Chemoradiation therapy; CNCT: Consolidation chemotherapy; NS: Not stated; 5-FU: 5-fluorouracil; LV: Leucovorin.

specificity[8,142-144]. In other words, these signs of remission are not always present in patients with a pCR, only presenting in 25% to 77% of cases, depending on the series[104,140,145,146]. Similarly, certain mucous abnormalities, particularly flat, regular ulcerations, are common in patients with complete remission[8,141,144]. In case of uncertainty, a second early reassessment, performed 6-12 wk after treatment, could be justified to identify tumours that are likely to respond eventually[147]. The persistence of large, anfractuous masses or ulcers indicates - to a high degree of certainty - a lack of response. (Figure 2 and Figure 3)

The utility of performing additional biopsies is highly controversial, as biopsies do not appear to be superior to optical diagnosis by the endoscopist[141]. Moreover, biopsy has such a high false negative rate that it is impossible to reliably rule out the presence of residual disease, nor can biopsy examination be used to determine the degree of invasiveness[8,142,143]. Therefore, despite the widespread use of this procedure, its use cannot be recommended[133]. Similarly, endorectal ultrasound has not demonstrated sufficient diagnostic accuracy to provide any real utility in follow-up, despite the fact that it is routinely used in experienced centres[133,148-155].

## OUTCOMES AND MANAGEMENT OF TUMOUR REGROWTH

Some authors have investigated alternative strategies to reduce the morbidity and mortality associated with conventional treatment, especially in tumours located in the lower third of the rectum. One such strategy is transanal resection before or after neoadjuvant therapy, mainly in cases with cT2 disease[156,157]. Other strategies include local resection of cT2 tumours followed by CRT, an approach that yields excellent results, as evidenced by the study carried out by the American College of Surgeons Oncology Group (ACOSOG Z6041). That study included 72 patients, finding 3-year disease-free survival (DFS) and OS rates of 87% and 96%, respectively, at a median follow-up of 4.2 years[156].

Conventional treatment (neoadjuvant therapy followed by TME) has been compared to local resection in several randomized trials, including the trial performed by Lezoche *et al*[157], as well as the GRECCAR (2017)[158] and Dutch CARTS study (2018)[159]. None of those trials found any significant between-group differences in DFS. In the Lezoche trial, the DFS rates were 89% and 94%, respectively, for local resection *vs* TME ($P$ = 0.609). It is worth noting, however, that 36% of the patients in the local resection arm later required TME, which increased treatment-related morbidity. As a result, there were no clear benefits for local resection compared to standard treatment. These findings were later confirmed in the GRECCAR and CART studies[158,159].

In the management of tumour regrowth with the watch and wait strategy, the main difficulty in attempting to draw firm conclusions from the current evidence base is that most of the available studies are retrospective, often comprised of small, highly

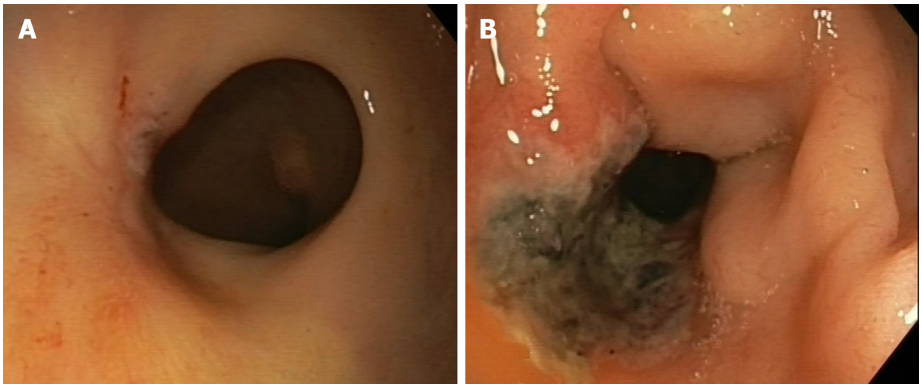


**Figure 2 Clinical incomplete response.** A: Endoscopic evaluation after 9 wk of chemoradiotherapy completion, detecting a small, but irregular, residual ulcer. B: Regrowth is more evident 12 wk later, as a deep, irregular and necrotic ulcer.

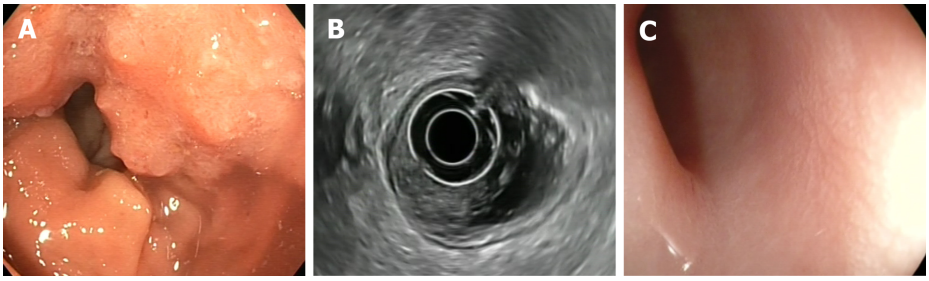


**Figure 3 Clinical complete response.** A: Endoscopic view of a rectal tumor prior to the neoadjuvant chemoradiotherapy; B: Endoscopic ultrasound with radial probe, showing that the tumor (T) is located within the mucosa, submucosa and muscular layers (uT2N0); C: Flat scar 10 wk after treatment completion: An endoscopic response feature.

heterogeneous samples with wide variety in the characteristics of the patients, the tumour types, and even treatment regimens. Approximately 30% of patients who achieve a cCR after neoadjuvant therapy experience local regrowth[105], especially in the first two years. At some point during follow-up, most of these patients will be candidates for salvage surgery, either local excision, low anterior resection, or abdominoperineal excision. Although some authors currently favour local resection[160], TME remains the treatment of choice after local regrowth[107]; however, in 2%-3% of these patients, salvage therapy may not be feasible due to an unresectable local invasion, concomitant non-curative systemic recurrence, or the presence of significant medical comorbidities[161]. Surgery for local regrowth is known as "salvage surgery" or "regrowth deferred surgery".

In the OnCoRe project[104], 88% of patients with non-metastatic local regrowths were salvaged, a slightly higher rate than reported by Kong *et al*[162] (83.8%) and Smith *et al*[163] (85%), and well above the 68.4% rate described by On *et al*[164] and the 69% rate reported in the International Watch and Wait Database[107]. Moreover, the salvage rate in the OnCoRe study were close to those described by Chadi *et al*[165] (89%) and by the Habr-Gama group (90%)[161] (Table 2).

According to Smith *et al*[163], treatment outcomes (OS and DFS) in patients who undergo salvage surgery are comparable to those achieved in patients who undergo conventional surgery. That said, most of the reported survival outcomes are based on only 3 years of follow-up. Nasir *et al*[160] presented similar short-term results. In the longer term, the Habr-Gama group reported a 5-year OS of 63.3% in patients who underwent salvage surgery[166], substantially less than the 85% reported in the International Watch and Wait Database[107]. On *et al*[164] found no significant differences in survival rates between salvage and upfront surgery (92.3% *vs* 92.9%, respectively) (Table 2).

Deferred surgery for local regrowth has shown promising short-term oncological and surgical results. However, the risk of distant metastases in patients managed with the watch and wait strategy remains undefined and this will need to be assessed through randomized controlled trials. The emergence of local regrowth in a patient managed with the watch and wait strategy should not be considered equivalent to local recurrence in a patient treated with radical surgery or transanal excision[103,111].

**Table 2 Tumor regrowth and salvage surgery in watch and wait clinical studies**

| Study | Patients (*n*) | Regrowth | Salvage surgery | Distant metastasis | Survival |
|---|---|---|---|---|---|
| Habr-Gama *et al*[161] | 90 | 27 (31%) | 93% | 13 (14%) | 3 yr (88%) |
| Renehan *et al*[99] | 129 | 44 (34%) | 84% | 5 (4%) | 3 yr (96%) |
| Kong *et al*[162] | 370 | 105 (28.4%) | 83.80% | | |
| van der Valk *et al*[102] | 1000 | 250 (25%) | 86% | 80 (8%) | 5 yr (85%) |
| Chadi *et al*[165] | 602 | 168 (28%) | 89% | 60 (10%) | 5 yr (87%) |
| Dattani *et al*[100] | 692 | 149 (21.6%) | 88% | 56 (8.2%) | 3 yr (93.5%) |
| On *et al*[164] | 248 | 37 (15.3%) | 68.40% | 8 (21%) | 92.30% |
| Nasir *et al*[160] | 78 | 23 (29.5%) | 100% | 1 (4.35%) | 3 yr (96%) |

Local recurrence after surgery indicates a failure of definitive therapy; consequently, the potential for successful salvage is low, with only 20%-30% of patients with locally-recurrent rectal cancer ultimately undergoing a potentially-curative R0 resection[167].

## QoL

QoL is a crucial aspect when considering the treatment strategy in patients with LARC. QoL is particularly relevant for sphincter preservation. Studies have shown a clear improvement in QoL in patients managed with a watch and wait approach versus surgical patients with a postoperative pCR, with a lower Wexner incontinence score (0.8 *vs* 3.5) ($P$ = 0.182) and defecation frequency (1.8 times/d *vs* 2.8 times/d) ($P$ = 0.323)[58].

Renehan *et al*[99] compared 3-year colostomy-free survival (CFS) rates in patients who had achieved a cCR with the watch and wait strategy versus a control group who underwent surgical resection after failing to achieve a cCR. The CFS was significantly higher in the watch and wait group (74% *vs* 47%; hazard ratio, 0.445; $P$ < 0.0001), with a 26% absolute difference at 3-years in the percentage of patients without a permanent colostomy. Another study found a high sphincter preservation rate at one year (72%), with no faecal incontinence in 69% of patients at 2 years, and a median Wexner score of 0 (IQR, 0-0) at all timepoints[5].

The comparative QoL study by Hupkens *et al*[168] merits mention due to the better outcomes in the watch and wait arm on physical and emotional function (36-item short form) and better physical function, and functional and cognitive capacity outcomes on the European Organization for the Cancer Research and Treatment questionnaire (QLQ-C30).

## FUTURE PERSPECTIVES

Despite the substantial increase in recent years in the number of published studies on the watch and wait approach-a direct result of the growing interest in this strategy, together with an increase in follow-up data-several aspects surrounding the optimal management of patients with LARC. There is a clear need to determine which patients would most benefit from the watch and wait approach, as this would permit us to individualize treatment in accordance with individual risk profiles.

Multiple clinical trials (Table 3) are current underway to evaluate different strategies to improve complete clinical response rates. One such strategy is radiotherapy dose escalation, an approach that is supported by the findings of prospective multicenter studies in patients with early stage rectal cancer (NCT00952926 and NCT02438839), demonstrating high organ-preservation rates[5]. That said, we still do not know whether the excellent results reported in those studies are more attributable to the tumour stage or to the higher radiation doses. Intensification of chemotherapy is also being assessed, as exemplified by the phase 3 randomized trial underway at the Memorial Sloan Kettering Cancer Center (MSKCC) (NCT02008656)[169]. In that trial, indication chemotherapy is compared to consolidation chemotherapy in patients with a cCR, offering them the option of non-surgical management with organ preservation.

**Table 3 Selected ongoing clinicals trials in patients with rectal cancer in a watch-and-wait program**

| Clinicaltrials.gov identifier (NCT number) | Study type | Neoadjuvant schedule | Primary outcome | Planned enrollment (*n*) | Recruitment status |
|---|---|---|---|---|---|
| NCT03402477 | Observational<br>Prospective | Radiotherapy or chemo-radiotherapy (at least 40 Gy) or short-course radiotherapy combined with chemotherapy | Local relapse rate | 100 | Recruiting |
| NCT03125343 | Interventional<br>Non-randomized | According to the Swedish National Program for rectal cancer | 3-yr disease free survival | 200 | Recruiting |
| NCT03846726 | Observational<br>Retrospective | Neoadjuvant chemoradiotherapy | Disease free survival | 513 | Active, not recruiting |
| NCT03064646 | Interventional<br>Non-randomized | Neoadjuvant chemoradiotherapy or neoadjuvant radiotherapy associated or not with induction chemotherapy | Local relapse rate | 30 | Recruiting |
| NCT03426397 | Observational<br>Prospective | Short course of radiation or neoadjuvant chemoradiotherapy | 2-yr non-regrowth disease free survival | 220 | Recruiting |
| NCT04009876 | Interventional<br>Non-randomized | 5-FU/LV + Oxaliplatin + nal-IRI for 8 cycles followed by standard chemoradiation (5 wk) | Clinical complete response rate | 30 | Recruiting |
| NCT03001362 | Interventional<br>Non-randomized | 54 Gy in 30fx with radiosensitizing chemotherapy as per institutional standard | Local relapse rate | 48 | Recruiting |
| NCT02704520 | Interventional<br>Randomized | Experimental arm: 45Gy-55Gy long course radiotherapy with radiosensitizing chemotherapy as per institutional standard | Feasibility phase: To assess the rate of patient recruitment<br>Phase III trial: 3-years disease free suvival | 98 | Recruiting |
| NCT04095299 | Interventional<br>Randomized | Experimental arm: 62 Gy to the clinical tumor volume and 50.4 Gy to the elective volume with capecitabine | 2-yr rectal preservation | 111 | Recruiting |

An advanced search of ClinicalTrials.gov was performed in March 2020 for "wath and wait in rectal cancer" (retrieved 10 records). These were reviewed and selected based on the status of the study. nal-IRI: Liposomal irinotecan; 5-FU: 5-fluorouracil; LV: Leucovorin.

The results will provide crucial data on the risk of distant metastases in patients selected for watch and wait who receive intensified systemic treatment.

Patients with multiple comorbidities are routinely excluded from clinical trials. Consequently, virtually all of the available data on these patients come from retrospective or non-randomized studies. Accordingly, these data must be interpreted cautiously given the potential for bias, as these patients are often dissuaded from surgery and directed towards watch and wait. As a consequence, OS outcomes in these patients tend to be worse than would otherwise occur if comparisons were made between similar groups with comparable clinical characteristics.

Alternative approaches are currently being explored in an effort to reduce the morbidity and mortality associated with TME for LARC. The TAU-TEM (NCT01308190)[170] and STAR-TREC trials (NCT02945566)[171] are both evaluating the viability of less aggressive surgical approaches in these patients. The results of these trials are expected to provide data comparing this alternative surgical approach to standard treatment and watch and wait.

In the absence of randomized clinical trials, the International Watch and Wait Database (IWATCH-AND-WAITD), created in 2014 (http://watch-and-waitw.iwatch-and-waitd.org), has the largest number of patients managed with a watch and wait strategy[107]. That database includes both retrospective and prospective data and the evidence base for watch and wait will increase substantially when long-term outcomes in these patients become available.

## CONCLUSION

There are clear short-term advantages-mainly reduced morbidity and better quality of life-to omitting surgery in patients with locally-advanced rectal cancer who have successfully achieved a complete clinical response after neoadjuvant therapy. In this clinical scenario, numerous studies have been conducted to date. However, many questions remain, including: (1) The optimal intensity and duration of clinical, radiological, and pathological follow-up; (2) Whether neoadjuvant therapy should be intensified based on the initial clinical stage; and (3) The need to identify strategies to reliably diagnose the greatest number of patients with cCR.

Based on the current data, the watch and wait strategy appears to be safe option in patients with LARC who have achieved a cCR after neoadjuvant therapy and who either present a high surgical risk or refuse surgical treatment. However, data from prospective multicentre studies are needed to confirm the non-inferiority of this approach in terms of cancer control versus standard treatment before this strategy can be more widely offered.

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*REVIEW*

# Role of long noncoding RNA-mediated competing endogenous RNA regulatory network in hepatocellular carcinoma

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## Abstract

Long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) are noncoding RNAs (ncRNAs) that occupy over 90% of the human genome, and their main function is to directly or indirectly regulate messenger RNA (mRNA) expression and participate in the tumorigenesis and progression of malignances. In particular, some lncRNAs can interact with miRNAs as competing endogenous RNAs (ceRNAs) to modulate mRNA expression. Accordingly, these RNA molecules are interrelated and coordinate to form a dynamic lncRNA-mediated ceRNA regulatory network. Mounting evidence has revealed that lncRNAs that act as ceRNAs are closely related to tumorigenesis. To date, numerous studies have established many different regulatory networks in hepatocellular carcinoma (HCC), and perturbations in these ceRNA interactions may result in the initiation and progression of HCC. Herein, we emphasize recent advances concerning the biological function of lncRNAs as ceRNAs in HCC, with the aim of elucidating the molecular mechanism underlying these HCC-related RNA molecules and providing novel insights into the diagnosis and treatment of HCC.

**Key words:** Hepatocellular carcinoma; Long noncoding RNA; MicroRNA; Competing endogenous RNA; Function; Mechanism

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**Core tip:** Mounting evidence has revealed that long noncoding RNA (lncRNA)-mediated competitive endogenous RNA (ceRNA) regulatory network plays a crucial role in tumorigenesis. To date, numerous studies have established many different regulatory networks in hepatocellular carcinoma (HCC), and perturbations in these ceRNA interactions may result in the initiation and progression of HCC. Herein, we emphasize recent advances concerning the biological function of lncRNAs as ceRNAs in HCC, with the aim of elucidating the molecular mechanism underlying these HCC-related RNA molecules and providing novel insights into the diagnosis and treatment of HCC.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is a malignant tumor with high morbidity and mortality worldwide[1]. However, the pathogenesis of HCC remains elusive. Although great progress has been made in the diagnosis and treatment of HCC in recent years, the overall and long-term effects of treatment in patients with advanced HCC are poor. Therefore, in-depth studies are needed to explore the mechanisms underlying HCC occurrence and development, which will contribute to the development of effective diagnostic biomarkers and therapeutic targets for HCC.

Protein-coding genes account for less than 2% of the human genome, while most of the genome is composed of genes that are transcribed into noncoding RNAs (ncRNAs)[2]. NcRNAs are divided into long noncoding RNAs (lncRNAs), small ncRNAs, and intermediate-sized ncRNAs by length[3]. LncRNAs have been identified as key regulators of transcription and translation and are involved in a variety of biological processes by regulating gene expression[4]. MicroRNAs (miRNAs) are small ncRNAs that interact with the 3′-untranslated region (3′-UTR) of target mRNAs to facilitate their degradation or inhibit their translation. MiRNAs play a critical role in tumorigenesis and tumor cell proliferation, migration, and invasion[5]. LncRNAs and miRNAs are regulatory ncRNAs, and dysregulation of lncRNAs or miRNAs is involved in tumor initiation and progression either *via* the activation or inhibition of target genes[6].

Existing evidence indicates that there are interactions among RNA molecules, such as lncRNAs and miRNAs[7], miRNAs and mRNAs[8], and lncRNAs and mRNAs[9]; these RNA molecules are interrelated and collaborate to form a dynamic regulatory network of lncRNAs acting as competitive endogenous RNAs (ceRNAs)[10]. The ceRNA mechanism is one of the important ways by which an lncRNA exerts its posttranscriptional gene regulation in the cytoplasm, and perturbations in these ceRNA interactions contribute to tumor initiation and progression. Currently, the identified ceRNAs include protein-coding RNAs (mRNAs) and ncRNAs, such as lncRNAs, pseudogene transcripts, viral RNAs, and circular RNAs (circRNAs). LncRNAs are the main component of the ceRNA network, as they regulate mRNA expression by acting as miRNA sponges. To date, numerous different regulatory networks of lncRNAs acting as ceRNAs in HCC have been established. Accumulating evidence has revealed that lncRNAs acting as ceRNAs play pivotal roles in HCC initiation and progression[11,12]. Herein, we emphasize recent advances concerning the biological function of lncRNAs acting as ceRNAs in HCC, with the aim of elucidating the molecular mechanism underlying these HCC-related RNA molecules and providing novel insights into the diagnosis and treatment of HCC.

## MECHANISM OF ACTION OF LNCRNAS INVOLVED IN THE CERNA REGULATORY NETWORK

Theoretically, any RNA molecule with a miRNA binding site can bind to a miRNA to form an intricate ceRNA regulatory network. RNA transcripts in the ceRNA network

are in a state of equilibrium under physiological conditions; once perturbed, this will lead to the occurrence of disease[13,14]. In the ceRNA regulatory network, miRNA acts as a bridge between ncRNA and mRNA and negatively regulates the expression of its target mRNA[15]. There is growing evidence that each miRNA can regulate many transcripts. In turn, RNA transcripts with different miRNA response elements (MREs) may also be targets of multiple miRNAs[16]. The multiplicity of targets allows RNA and RNA to interact with each other by competitively binding to a common MRE, and the same MRE is the structural basis for the binding of different RNAs[16].

In addition to directly regulating mRNAs, lncRNAs can also indirectly affect the expression of target genes by sponging miRNAs[10]. Structurally, most lncRNAs are similar to mRNAs, which makes their patterns of gene regulation more diverse and extensive and unaffected by translation[17]. This may be the reason why many lncRNAs can act as ceRNAs to sponge miRNAs to inhibit miRNAs from degrading their target mRNAs. In general, the more miRNA binding sites there are on an lncRNA, the stronger the competition[18]. When lncRNAs are expressed at low levels, they can bind only a few miRNAs, and the remaining miRNAs interact with mRNAs to promote their degradation. In contrast, when lncRNAs are expressed at high levels, they can combine with more miRNAs, thus relieving the inhibitory effect of miRNAs on their target mRNAs. The new regulatory pattern of lncRNA-miRNA-mRNA is an extension of the traditional miRNA-mRNA regulatory model[10].

## ROLE OF LNCRNAS AS CERNAS IN HCC

To date, mounting evidence indicates that oncogenic or tumor suppressive lncRNAs can regulate their target genes by acting as ceRNAs to sponge miRNAs[19,20], thereby affecting glucose metabolism, immune escape, autophagy, angiogenesis, liver cancer stem cells (LCSCs), proliferation, apoptosis, epithelial-mesenchymal transition (EMT), migration, invasion, metastasis, chemoresistance, and radioresistance in HCC (Figure 1). Specifically, the majority of the identified lncRNAs exhibit oncogenic properties that function as ceRNAs for tumor suppressive miRNAs, thereby activating the expression of oncogenic mRNAs to promote HCC occurrence and progression (Figure 2A). In addition, some lncRNAs exhibit tumor suppressive properties, acting as ceRNAs for oncogenic miRNAs (oncomiRs), thus upregulating the expression of tumor suppressive targets to inhibit HCC occurrence and progression (Figure 2B). Here, we elucidate the functions of some lncRNA-mediated ceRNA regulatory networks in HCC (Table 1). Note that there are many abbreviations in this paper, so they are listed and expanded in Table 1 below.

### *Glucose metabolism*

The "reprogramming" of glucose metabolism is regarded as a prominent characteristic of cancer cells. A large amount of lactic acid produced by glycolysis forms an inflammatory microenvironment around the tumor, which contributes to tumor cell proliferation, EMT, invasion, metastasis, immune escape, and resistance to chemotherapy and radiotherapy. Existing evidence indicates that aberrant glucose metabolism plays a pivotal role in the invasion and metastasis of HCC[21,22]. The mechanism of abnormal activation of glycolysis in cancer cells is complex, and many studies have confirmed that lncRNAs play a significant role in modulating glycolysis by sponging miRNAs in HCC, among which oncogenic lncRNAs that act as ceRNAs can promote glycolysis. For instance, lactate dehydrogenase isoform A (LDHA), a glycolytic enzyme, can mediate aerobic glycolysis in cancer cells[23]. The lncRNA *RAET1K*, as a miR-100-5p sponge, can enhance LDHA expression and facilitate hypoxia-induced glycolysis, thereby promoting HCC progression[24]. In addition, hemikinase 2 (HK2) is another glycolytic enzyme related to glycolysis in cancer cells[25], and the lncRNA *TUG1* induces glycolysis and promotes HCC metastasis by acting as a ceRNA to enhance HK2 expression by sponging miR-455-3p[26]. Additionally, hypoxia-inducible factor (HIF) 1 has been confirmed to promote aerobic glycolysis in cancer[27], and the lncRNA *HOTAIR* promotes glycolysis by acting as a ceRNA for miR-130a-3p to increase HIF1 expression in HCC cells[28]. By contrast, tumor suppressive lncRNAs that act as ceRNAs can inhibit glycolysis in HCC. For example, endoplasmic reticulum protein 29 (ERp29), an endoplasmic reticulum protein, and the lncRNA *MEG3* are downregulated in high-glucose (HG) HCC cells, while miR-483-3p is upregulated in HG HCC cells. Mechanistically, the overexpression of *MEG3* inhibits glycolysis by sponging miR-483-3p to increase ERp29 expression in HCC[29]. Currently, antitumor drugs that target glucose metabolism are being researched and developed;

**Table 1 Long noncoding RNA-mediated competitive endogenous RNA network in hepatocellular carcinoma**

| LncRNA | Dysregulation | SpongedmiRNA(s) | Affected mRNA(s)/ signaling pathway(s) | Biological functions | Ref. |
|---|---|---|---|---|---|
| *RAET1K* | Up-regulated | miR-100-5p | LDHA | Enhances HCC glycolysis and progression | Zhou *et al*[24] |
| *TUG1* | Up-regulated | miR-455-3p | HK2 | Promotes HCC glycolysis and metastasis | Lin *et al*[26] |
| *HOTAIR* | Up-regulated | miR-130a-3p | HIF1 | Promotes glycolysis | Hu *et al*[28] |
| | | miR-218 | Bmi-1 | Promotes HCC cell proliferation | Fu *et al*[74] |
| | | miR-1 | FOXC1 | Promotes HCC cell proliferation, migration, and invasion | Su *et al*[75] |
| | | miR-214-3p | FLOT1 | Promotes HCC cell proliferation, migration, and invasion | Liu *et al*[77] |
| | | miR-23b-3p | ZEB1 | Promotes HCC invasion and metastasis | Yang *et al*[78] |
| *MEG3* | Down-regulated | miR-483-3p | ERp29 | Inhibits glycolysis | Li *et al*[29] |
| | | miR-9-5p | SOX11 | Promotes HCC cell apoptosis and inhibits cell growth | Liu *et al*[93] |
| | | miRNA-10a-5p | PTEN | Inhibits HCC cell proliferation, migration, and invasion | Zhang *et al*[94] |
| *NEAT1* | Up-regulated | miR-155 | Tim-3 | Facilitates CD8+T cells-mediated immune escape | Yan *et al*[35] |
| | | miR-335 | c-Met-Akt pathway | Enhances HCC resistance to sorafenib | Chen *et al*[130] |
| *FENDRR* | Down-regulated | miR-423-5p | *GADD45β* | Suppresses Treg-mediated immune escape | Yu *et al*[38] |
| *PVT1* | Up-regulated | miR-365 | ATG3 | Promotes autophagy | Yang *et al*[44] |
| *HNF1A-AS1* | Up-regulated | hsa-miR-30b-5p | ATG5 | Stimulates autophagy | Liu *et al*[45] |
| *CCAT1* | Up-regulated | miR-181a-5p | ATG7 | Induces autophagy | Guo *et al*[46] |
| | | Let-7 | HMGA2 and c-Myc | Promotes HCC cell proliferation and migration | Deng *et al*[81] |
| | | miR-490-3p | CDK1 | Promotes HCC cell proliferation and invasion | Dou *et al*[82] |
| | | miR-30c-2-3p | CCNE1 | Promotes HCC cell proliferation | Zhang *et al*[83] |
| *HCG11* | Up-regulated | miR-26a-5p | ATG12 | Promotes autophagy | Li *et al*[47] |
| *LINC00665* | Up-regulated | miR-186-5p | MAP4K3 | Facilitates autophagy | Shan *et al*[49] |
| *HULC* | Up-regulated | miR-107 | SPHK1 | Promotes angiogenesis | Lu *et al*[52] |
| | | miR-200a-3p | ZEB1 | Enhances EMT and promotes HCC growth and metastasis | Li *et al*[65] |
| | | miR-2052 | MET | Promotes HCC cell proliferation, migration, and invasion | Zhang *et al*[66] |
| | | miR-186 | HMGA2 | Promotes HCC growth and metastasis | Wang *et al*[68] |
| | | miR-372-3p | Rab11a | Promotes HCC growth and metastasis | Cao *et al*[71] |
| | | miR-6825-5p, miR- | USP22 | Increases HCC resistance | Xiong *et al*[142] |

| | | | | | |
|---|---|---|---|---|---|
| | | 6845-5p, and miR-6886-3p | | to oxaliplatin | |
| *MALAT1* | Up-regulated | miR-3064-5p | FOXA1/CD24/Src pathway | Promotes angiogenesis | Zhang *et al*[53] |
| | | miR-140 | VEGF-A | Promotes angiogenesis | Hou *et al*[54] |
| | | miR-124 | PI3K/Akt pathway | Enhances HBx-induced CSC properties | He *et al*[59] |
| | | miR-216b | HIF-2α | Enhances HCC resistance to 5-FU | Yuan *et al*[155] |
| *LINC00488* | Up-regulated | miR-330-5p | TLN1 | Facilitates angiogenesis | Gao *et al*[55] |
| *DANCR* | Up-regulated | miR-214, miR-320a and miR-199a | CTNNB1 | Enhances the stemness of HCC cells | Yuan *et al*[61] |
| *ANRIL* | Up-regulated | miR-384 | STAT3 | Promotes HCC cell proliferation, migration, and invasion | Ji *et al*[85] |
| | | miR-122-5p | N/A | Promotes HCC cell proliferation, metastasis, and invasion | Ma *et al*[86] |
| | | miR-144 | PBX3 | Promotes HCC cell growth, migration, and invasion | Ma *et al*[87] |
| *GAS5* | Down-regulated | miR-21 | PDCD4 and PTEN | Suppresses HCC cell migration and invasion | Hu *et al*[89] |
| | | miR-135b | RECK | Inhibits HCC invasion | Yang *et al*[90] |
| | | miR-1323 | TP53INP1 | Inhibits HCC cell proliferation and invasion and promotes apoptosis | Zhang *et al*[91] |
| | | miR-222 | N/A | Increases HCC sensitivity to cisplatin | Zhao *et al*[151] |
| *ASB16-AS1* | Up-regulated | miR-1827 | FZD4 Wnt/β-catenin pathway | Promotes HCC growth and invasion | Yao *et al*[97] |
| *DSCR8* | Up-regulated | miR-485-5p | FZD7 Wnt/β-catenin pathway | Promotes HCC cell proliferation and cell cycle | Wang *et al*[99] |
| *LINC00662* | Up-regulated | miR-15a, miR-16, and miR-107 | WNT3A Wnt/β-catenin pathway | Promotes HCC growth and metastasis | Tian *et al*[101] |
| *SNHG5* | Up-regulated | miR-26a-5p | GSK3β Wnt/β-catenin pathway | Promotes HCC metastasis and EMT | Li *et al*[103] |
| *SOX9-AS1* | Up-regulated | miR-5590-3p | SOX9 Wnt/β-catenin pathway | Facilitates HCC growth and metastasis | Zhang *et al*[105] |
| *DLGAP1-AS1* | Up-regulated | miR-26a-5p and miR-26b-5p | IL-6 JAK2/STAT3 pathway and CDK8/LRP6 Wnt/β-catenin pathway | Facilitates HCC EMT and progression | Lin *et al*[109] |
| *MIR22HG* | Down-regulated | miR-10a-5p | NCoR2 Wnt/β-catenin pathway | Inhibits HCC growth, migration, and invasion | Wu *et al*[111] |
| *PTTG3P* | Up-regulated | miR-383 | CCND1/PARP2 and PI3K/Akt pathway | Promotes HCC cell proliferation, migration, and invasion and inhibits apoptosis | Zhou *et al*[115] |
| *DLEU1* | Up-regulated | miR-133a | IGF-1R PI3K/AKT pathway | Promotes HCC cell proliferation, migration, and invasion | Zhang *et al*[116] |
| *TCL6* | Down-regulated | miR-106a-5p | PTEN PI3K/AKT pathway | Inhibits HCC cell proliferation, migration, and invasion | Luo *et al*[117] |
| *CDKN2B-AS1* | Up-regulated | let-7c-5p | NAP1L1 PI3K/AKT/ mTOR pathway | Promote HCC growth and metastasis | Huang *et al*[118] |
| *GAS6-AS2* | Up-regulated | miR-493-5p | OTUB1 PI3K-AKT- FoxO3a pathway | Promotes HCC cell proliferation, migration, and invasion | Liang *et al*[119] |

| | | | | | |
|---|---|---|---|---|---|
| *SNHG12* | Up-regulated | miR-199a/b-5p | MLK3 NF-κB pathway | Promotes HCC cell proliferation and tumorigenicity | Lan *et al*[125] |
| *CASC2* | Down-regulated | miR-362-5p | CYLD NF-κB pathway | Inhibits HCC cell migration and invasion | Zhao *et al*[127]; Ni *et al*[128] |
| | | miR-222 | N/A | Enhances HCC sensitivity to cisplatin | Liu *et al*[150] |
| *LINC-ROR* | Up-regulated | miR-876-5p | FOXM1 | Increases HCC resistance to sorafenib | Zhi *et al*[134] |
| | | miR-145 | RAD18 | Enhances radiation resistance of HCC cells | Chen *et al*[163] |
| *LINC00160* | Up-regulated | miR-132 | PIK3R3 | Promotes HCC resistance to sorafenib | Zhang *et al*[136] |
| *FOXD2-AS1* | Up-regulated | miR-150-5p | TMEM9 | Facilitates HCC resistance to sorafenib | Sui *et al*[138] |
| *SNHG3* | Up-regulated | miR-128 | CD151 | Promotes HCC resistance to sorafenib | Zhang *et al*[140] |
| *SNHG16* | Up-regulated | miR-140-5p | N/A | Increases HCC resistance to sorafenib | Ye *et al*[141] |
| | | let-7b-5p | N/A | Enhances HCC resistance to oxaliplatin | Li *et al*[149] |
| *NR2F1-AS1* | Up-regulated | miR-363 | ABCC1 | Enhances HCC resistance to oxaliplatin | Huang *et al*[144] |
| *KCNQ1OT1* | Up-regulated | miR-7-5p | ABCC1 | Increases HCC resistance to oxaliplatin | Hu *et al*[145] |
| *NRAL* | Up-regulated | miR-340-5p | Nrf2 | Facilitates HCC resistance to cisplatin | Wu *et al*[147] |
| *LINC01234* | Up-regulated | miR-31-5p | MAGEA3 | Promotes HCC resistance to cisplatin | Chen *et al*[148] |
| *CRNDE* | Up-regulated | miR-33a | HMGA2 | Promotes HCC resistance to 5-FU | Han *et al*[152] |
| *KRAL* | Down-regulated | miR-141 | Keap1 | Reverses HCC resistance to 5-FU | Wu *et al*[158] |
| *NEAT1_2* | Up-regulated | miR-101-3p | WEE1 | Enhances radio-resistance of HCC cells | Chen *et al*[160] |
| *LINC00473* | Up-regulated | miR-345-5p | FOXP1 | Promotes radio-resistance of HCC cells | Zhang *et al*[165] |

HCC: Hepatocellular carcinoma; LncRNAs: Long noncoding RNAs; ceRNA: Competitive endogenous RNA; RAET1K: Retinoic acid early transcript 1K; LDHA: Lactate dehydrogenase isoform A; TUG1: Taurine up-regulation gene 1; HK2: Hemikinase 2; HOTAIR: Homeobox transcript antisense RNA; HIF1: Hypoxia-inducible factor 1; Bmi-1:B lymphoma moloney murine leukemia virus insertion region 1; FOXC1: Forkhead box C1; FLOT1: Flotillin 1; ZEB1: Zinc finger E-box binding homeobox 1; MEG3: Maternally expressed gene 3; ERp29: ER protein 29; SOX11: SRY-related HMG-box transcription factor 11; PTEN: Phosphatase and tensin homolog; NEAT1: Nuclear enriched abundant transcript 1; Tim-3: T cell immunoglobulin mucin-3; FENDRR: Fetal-lethal noncoding developmental regulatory RNA; GADD45β: Growth arrest and DNA damage-inducible beta; PVT1: Plasmacytoma variant translocation 1; ATG3: Autophagy related genes 3; HNF1A-AS1: HNF1A antisense RNA 1; ATG5: Autophagy related genes 5; CCAT1: Colon cancer associated transcript 1; ATG7: Autophagy related genes 7; HMGA2: High mobility group AT-hook 2; CDK1: Cyclin-dependent kinase 1; CCNE1: Cyclin E1; HCG11: HLA complex group 11; ATG12: Autophagy related genes 12; LINC00665: Long intergenic non-protein coding RNA 665; MAP4K3: Mitogen-activated protein kinase kinase kinase kinase 3; HULC: Highly upregulated in liver cancer; SPHK1: Sphingosine kinase 1; MET: Hepatocyte growth factor receptor; Rab11a: Member RAS oncogene family; USP22: Ubiquitin-specific peptidase 22; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; FOXA1: Forkhead box A1; VEGF-A: Vascular endothelial growth factor A; PI3K/Akt pathway: Phosphoinositide-3-kinase/protein kinase B; CSC: Liver cancer stem cell; HIF-2α: Hypoxia-inducible factor 2α; TLN1: Talin 1; DANCR: Differentiation antagonizing non-protein coding RNA; CTNNB1: Catenin beta-1; ANRIL: Antisense noncoding RNA in the INK4 locus; STAT3: Signal transducer and activator of transcription 3; N/A: Not available.; PBX3: Pre-B-cell leukemia homeobox 3; GAS5: Growth arrest-specific 5; PDCD4: Programmed cell death 4; RECK: Cysteine-rich protein with Kazal motifs; TP53INP1:Tumor protein p53-induced nuclear protein 1;ASB16-AS1:ASB16 antisense RNA 1; FZD4: Frizzled 4; DSCR8: Down syndrome critical region 8; FZD7: Frizzled-7; WNT3A:Wingless-type MMTV integration site family 3A; SNHG5: Small nucleolar RNA host gene 5; GSK3β: Glycogen synthase kinase 3β; EMT: Epithelial-mesenchymal transition; SOX9-AS1: SOX9 antisense RNA 1; SOX9: Sex determining region Y-box 9; DLGAP1-AS1: Long noncoding RNA DLGAP1 antisense RNA 1; IL-6: Interleukin- 6; JAK2: Janus kinase 2; CDK8: Cyclin-dependent kinase 8; LRP6: Low-density lipoprotein receptor-related protein 6; MIR22HG: MIR22 host gene; NCoR2: Nuclear receptor corepressor 2; PTTG3P: Pituitary tumor-transforming 3; CCND1: Cyclin D1; PARP2: Poly ADP-ribose polymerase 2; DLEU1: Deleted in lymphocytic leukaemia 1; IGF-1R : Insulin-like growth factor 1 receptor; TCL6: T cell leukemia/lymphoma 6; CDKN2B-AS1: CDKN2B antisense RNA 1; NAP1L1: Nucleosome assembly protein 1 like 1; mTOR: Mammalian rapamycin; GAS6-

AS2: Growth arrest specific 6 antisense RNA 2; OTUB1: OTU domain-containing ubiquitin aldehyde-binding protein 1; FOXO3a: Forkhead Box O3a; SNHG12: Small nucleolar RNA host gene 12; MLK3: Mixed-lineage kinase 3; NF-kB: Nuclear factor kappa-B; CASC2: Cancer susceptibility candidate 2; CYLD: Cylindromatosis; LINC-ROR: Intergenic non-protein coding RNA, regulator of reprogramming; FoxM1: Forkhead box M1; RAD18: A RING-type ubiquitin ligase E3; PIK3R3: Phosphoinositide-3-kinase regulatory subunit 3; FOXD2-AS1: FOXD2 adjacent opposite strand RNA 1; TMEM9: Transmembrane protein 9; SNHG3: Small nucleolar RNA host gene 3; SNHG16: Small nucleolar RNA host gene 16; NR2F1-AS1: NR2F1 antisense RNA 1; ABCC1: Multidrug resistance-associated protein 1; KCNQ1OT1: KCNQ1 overlapping transcript 1; NRAL: Nrf2 regulation-associated lncRNA; Nrf2: Nuclear factor erythroid-2-related factor 2; MAGEA3: Melanoma-associated antigen A3; CRNDE: Colorectal neoplasia differentially expressed; KRAL: Keap1 regulation-associated lncRNA; Keap1: Kelch-like ECH-associated protein 1; NEAT1_2: Nuclear enriched abundant transcript 1_2; WEE1: WEE1 G2 checkpoint kinase; FOXP1: Forkhead box protein P1.

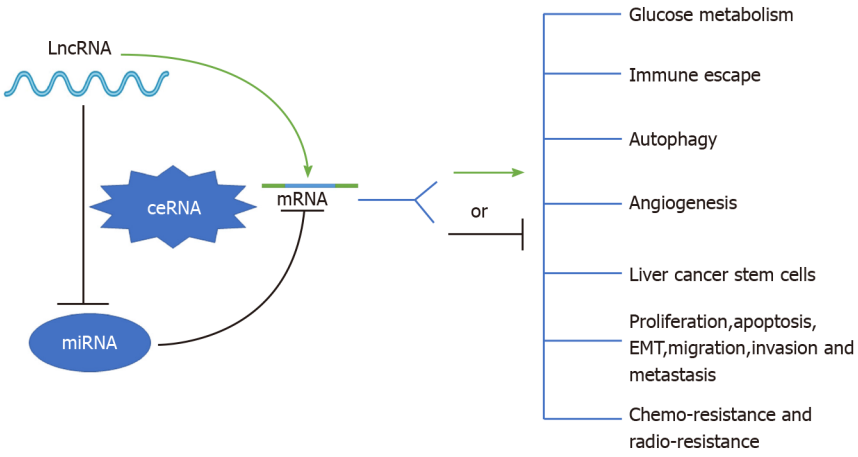


**Figure 1 Schematic diagram of the role of long noncoding RNA-mediated competitive endogenous RNA regulatory network in hepatocellular carcinoma.** See the text for details. LncRNA: Long noncoding RNA; ceRNA: Competing endogenous RNA; miRNA: MicroRNA; mRNA: Messenger RNA; EMT: Epithelial-mesenchymal transition.

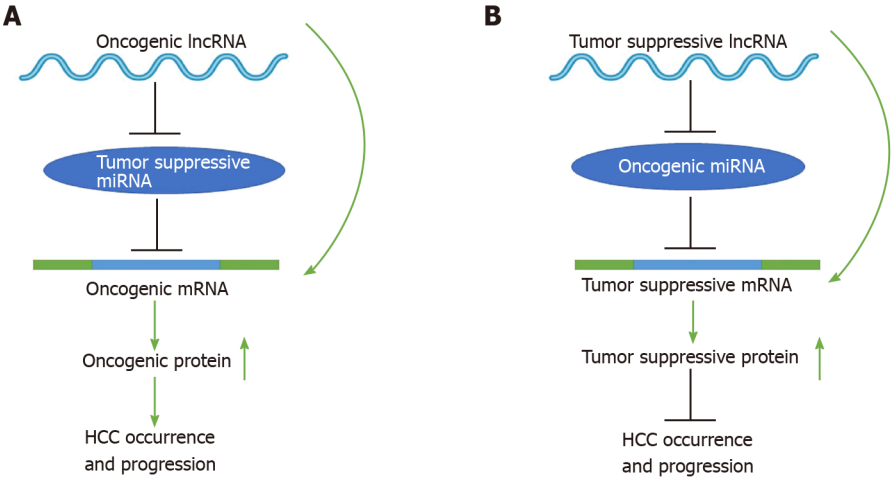


**Figure 2 Schematic diagrams of long noncoding RNA-mediated competitive endogenous RNA regulatory network that mediates the occurrence and progression of hepatocellular carcinoma.** See the text for details. LncRNA: Long noncoding RNA; miRNA: MicroRNAs; mRNA: Messenger RNA.

the above findings suggest that the lncRNA-mediated ceRNA network could provide new ideas for inhibiting glycolysis in HCC.

### *Immune escape*

Tumor immune escape refers to the phenomenon that tumor cells can survive and proliferate by escaping immune system-mediated recognition and attack by changing themselves or their tumor microenvironment[30]. Currently, the effectiveness of immunotherapy is limited by tumor immune escape. Thus, an in-depth exploration of the mechanisms of tumor immune escape may provide novel insights into tumor

immunotherapy. Current studies have shown that lncRNAs can modulate immune escape in HCC by acting as ceRNAs of miRNAs, among which oncogenic lncRNAs that function as ceRNAs can promote the immune escape of HCC cells. For example, *NEAT1*, a newly discovered oncogenic lncRNA, is specifically localized in nuclear paraspeckles and participates in paraspeckle formation and the transcriptional regulation of many genes[31,32]. T cell immunoglobulin mucin-3 (Tim-3), an immune checkpoint molecule, can suppress the immune response[33], and the increased expression of Tim-3 within the tumor can inactivate killer T cells, thus preventing the death of tumor cells[34]. Mechanistically, *NEAT1* facilitates the CD8+ T cell-mediated immune escape of HCC cells by acting as a ceRNA for miR-155 to enhance Tim-3 expression[35]. Conversely, tumor suppressive lncRNAs that act as ceRNAs can inhibit the immune escape of HCC cells. For instance, *GADD45β*, a tumor suppressor, is associated with antitumor immune responses[36], and $CD4^+$ T cells lacking *GADD45β* are less responsive to the stimulation of T cell receptors or inflammatory cytokines[37]. *FENDRR*, a tumor suppressor lncRNA, upregulates *GADD45β* by sponging miR-423-5p, thereby suppressing the immune escape of HCC cells[38]. These findings suggest that the lncRNA-mediated ceRNA network is involved in mediating immune evasion in HCC and thus may be a promising therapeutic target for HCC immunotherapy.

### Autophagy

Autophagy is closely associated with the development of malignant tumors and can promote tumor survival and proliferation by regulating interactions between the tumor and tumor microenvironment[39]. In HCC, autophagy plays a vital role in tumor immunity, oxidative stress, and the maintenance of hepatic homeostasis and thus participates in HCC initiation and progression and resistance to chemotherapy drugs[40]. Identification of the mechanisms by which autophagy is activated in HCC will help clarify HCC pathogenesis and reveal novel treatments for HCC patients. Numerous investigations have indicated that oncogenic lncRNAs that function as ceRNAs are required for promoting autophagy in HCC. For example, autophagy-related genes 3, 5, 7, and 12 (*ATG3*, *ATG5*, *ATG7*, and *ATG12*, respectively) are major regulators of the induction of autophagosome formation[41-43]. The lncRNA *PVT1* promotes autophagy in HCC by enhancing *ATG3* expression by sponging miR-365[44]. The lncRNA *HNF1A-AS1* upregulates the expression of *ATG5* in HCC by acting as a sponge of hsa-miR-30b-5p, thus stimulating autophagy[45]. The lncRNA *CCAT1* serves as a ceRNA for miR-181a-5p to induce autophagy in HCC by enhancing *ATG7* expression[46]. The lncRNA *HCG11* promotes autophagy in HCC by enhancing *ATG12* expression by sponging miR-26a-5p[47]. In addition, mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3), an upstream kinase of the MAPK pathway, is a key node in the regulation of autophagy[48]. The lncRNA *LINC00665* facilitates autophagy by sponging miR-186-5p to enhance MAP4K3 expression[49]. Collectively, these results suggest that the lncRNA-mediated ceRNA network could provide novel treatments for HCC patients.

### Angiogenesis

Angiogenesis is responsible for HCC growth, proliferation, invasion, and metastasis[50]. The mechanisms underlying HCC angiogenesis are complex, and exploration of the factors involved in regulating HCC angiogenesis is of great significance for improving antiangiogenic treatments for HCC. Emerging evidence indicates that oncogenic lncRNAs that act as ceRNAs are tightly linked to HCC angiogenesis. For instance, sphingosine kinase 1 (SPHK1), a key metabolic enzyme, is correlated with tumor angiogenesis[51]. A study found that the lncRNA *HULC* promotes angiogenesis by upregulating SPHK1 in HCC; *HULC* acts as a ceRNA to increase the expression of transcription factor E2F1 by competitively binding to miR-107 and subsequently results in the activation of the *SPHK1* promoter, thus promoting HCC angiogenesis *in vivo*[52]. In addition, the lncRNA *MALAT1* can promote HCC angiogenesis by sponging miR-3064-5p to activate the forkhead box A1 (FOXA1)/CD24/Src pathway[53] or by functioning as a miR-140 sponge to enhance vascular endothelial growth factor A expression[54]. Similarly, *LINC00488*, another lncRNA, upregulates the expression of talin 1 to facilitate HCC angiogenesis by sponging miR-330-5p[55]. These findings suggest that the lncRNA-mediated ceRNA network may be a promising target for antiangiogenic therapies for HCC.

### Liver cancer stem cells

LCSCs exhibit high proliferation, self-renewal, high tumorigenicity, chemoresistance, and radioresistance[56-58], and their abundance is positively associated with the degree

of HCC malignancy. Elucidation of the regulatory mechanisms of LCSCs will contribute to our understanding of the pathogenesis of HCC and the identification of novel therapeutic strategies. Existing evidence has shown that oncogenic lncRNAs help sustain cancer stem cell (CSC) traits by acting as ceRNAs for miRNAs to initiate HCC development. For instance, the lncRNA *MALAT1* activates the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway by acting as a miR-124 sponge to enhance HBx-induced CSC properties[59]. In addition, catenin beta-1 (CTNNB1)/β-catenin sustains the stemness properties of LCSCs[60]. In another recent study, it was found that the lncRNA *DANCR* was highly expressed in HCC tissues and stem-like HCC cells; *DANCR* can act as a ceRNA to enhance CTNNB1 expression by sponging miR-214, miR-320a, and miR-199a, thereby enhancing the stemness of HCC cells[61]. Thus, the lncRNA-mediated ceRNA network may serve as a potential therapeutic target for LCSCs.

### *Proliferation, migration, EMT, invasion, and metastasis*

**The lncRNA-mediated ceRNA regulatory network functions by regulating miRNA target genes:** LncRNAs acting as ceRNAs can participate in cell proliferation, apoptosis, migration, EMT, invasion, and metastasis by modulating mRNAs in HCC. Currently, there are many lncRNA-miRNA-miRNA target gene (mRNA) networks reported in HCC. In this review, we provide only a few examples of lncRNA-miRNA-mRNA networks, including those involving oncogenic lncRNAs such as *HULC, HOTAIR, CCAT*, and *ANRIL* and tumor suppressive lncRNAs such as *GAS5* and *MEG3*.

**LncRNA HULC-miRNA-mRNA:** *HULC* has been identified as a specifically highly expressed lncRNA in HCC[62]. In a recent study, high *HULC* expression in HCC was significantly connected to increased lymph node metastasis and advanced TNM stage[63]. This finding indicates that *HULC* can facilitate the proliferation, migration, invasion, and metastasis of HCC cells, leading to the malignant development of HCC. Thus far, it has been reported that *HULC* may exert its oncogenic function in HCC through diverse molecular mechanisms, of which the *HULC*-mediated ceRNA network is important. For instance, zinc finger E-box binding homeobox 1 (ZEB1), a key regulator of EMT, contributes to HCC cell invasion and metastasis[64]. As a ceRNA of miR-200a-3p, *HULC* increases the expression of ZEB1, thereby enhancing EMT and promoting HCC growth and metastasis[65]. In addition, *HULC* can enhance the expression of hepatocyte growth factor receptor (MET) by sponging miR-2052, thus promoting HCC cell proliferation, migration, and invasion[66]. High mobility group AT-hook 2 (*HMGA2*), an oncogene, has been shown to be closely associated with cancer progression and metastasis[67]. *HULC* promotes HCC growth and metastasis by enhancing HMGA2 expression by acting as a ceRNA of miR-186[68]. Rablla, a central regulatory protein, promotes exosome secretion, and exosomes significantly promote HCC progression[69,70]. A mechanistic investigation revealed that *HULC* increases RAS oncogene family member expression to induce exosome secretion by sponging miR-372-3p, contributing to HCC growth and metastasis[71].

**LncRNA HOTAIR-miRNA-mRNA:** The lncRNA *HOTAIR* has been reported to exert an oncogenic role in a variety of malignances[72,73]. Emerging evidence suggests that *HOTAIR* functions as a ceRNA and facilitates HCC cell proliferation, migration, invasion, and metastasis. For instance, a study confirmed that *HOTAIR* promotes HCC cell proliferation and tumorigenicity by competitively binding to miR-218 to activate B lymphoma Moloney murine leukemia virus insertion region 1 expression and inactivate $P16^{Ink4a}$ and $P14^{ARF}$[74]. Another study also demonstrated that Forkhead box C1-activated *HOTAIR* promotes HCC cell proliferation, migration, and invasion by acting as a sponge of miR-1[75]. In addition, flotillin 1 (FLOT1), a marker of lipid rafts, is highly expressed in HCC and contributes to aggressive tumor characteristics[76]. *HOTAIR* enhances FLOT1 expression by sponging miR-214-3p, thereby promoting HCC cell proliferation, migration, and invasion[77]. Additionally, *HOTAIR* promotes HCC cell invasion and metastasis by sponging miR-23b-3p to upregulate ZEB1 expression[78].

**LncRNA CCAT-miRNA-mRNA:** The lncRNA *CCAT1* is located on chromosome 8q24.21 and plays vital roles in promoting HCC cell proliferation and metastasis[79]. *CCAT1* has been shown to upregulate the expression of its downstream gene *c-Myc*, thereby promoting tumorigenesis[80]. Subsequently, many studies have explored the potential mechanism by which *CCAT1* upregulates *c-Myc* to promote tumorigenesis. In HCC, *CCAT1* functions as a ceRNA of miRNA let-7, thus counteracting the inhibitory effect of Let-7 on its target genes, *HMGA2* and *c-Myc*, which upregulates the

expression of *HMGA2* and *c-Myc* and ultimately facilitates HCC proliferation and migration[81]. In addition, *CCAT1* upregulates cyclin-dependent kinase 1 expression by acting as a miR-490-3p sponge, thereby promoting HCC cell proliferation and invasion[82]. Furthermore, *CCAT1* acts as a sponge of miR-30c-2-3p to upregulate the expression of cyclin E1, leading to HCC cell proliferation[83].

**LncRNA ANRIL-miRNA-mRNA:** The lncRNA *ANRIL* is located on chromosome 9p21 and plays an oncogenic role in tumorigenesis[84]. *ANRIL* functions as a ceRNA to sponge miRNAs, thereby regulating gene expression in HCC. The high expression of *ANRIL* in HCC is related to HCC cell proliferation, migration, and invasion; mechanistically, *ANRIL* exerts its biological action in HCC by sponging miR-384 to upregulate signal transducer and activator of transcription 3 (STAT3) expression[85]. *ANRIL* can also promote HCC cell proliferation, metastasis, and invasion by acting as a ceRNA of miR-122-5p[86]. In addition, ANRIL can upregulate the expression of pre-B-cell leukemia homeobox 3 by sponging miR-144 to facilitate HCC cell growth, migration, and invasion[87].

**LncRNA GAS5-miRNA-mRNA:** The lncRNA *GAS5* is downregulated in diverse malignancies, including HCC[88]. Increasing evidence indicates that the *GAS5*-mediated ceRNA network may be one of the important mechanisms by which *GAS5* exerts its biological functions in HCC. For example, *GAS5* restrains HCC cell migration and invasion by sponging miR-21 to upregulate its targets, programmed cell death 4 and phosphatase and tensin homolog (PTEN)[89]. In addition, *GAS5* suppresses HCC invasion by sponging miR-135b to enhance cysteine-rich protein with Kazal motifs expression[90]. *GAS5* also functions as a miR-1323 sponge to upregulate tumor protein p53-induced nuclear protein 1 expression, thus inhibiting HCC cell proliferation and invasion and promoting apoptosis[91].

**LncRNA MEG3-miRNA-mRNA:** *MEG3* is an imprinted gene and a tumor suppressive lncRNA. *MEG3* is inversely related to tumorigenesis and plays an inhibitory role in many malignancies[92]. Acting as a ceRNA against miRNA is an important mechanism of action of *MEG3* in HCC. For example, the overexpression of *MEG3* can promote cell apoptosis and inhibit HCC growth by upregulating SRY-related HMG-box transcription factor 11 expression by acting as an miR-9-5p sponge[93]. In addition, *MEG3* enhances the expression of PTEN to restrain HCC cell proliferation, migration, and invasion by sponging miRNA-10a-5p[94].

**The lncRNA-mediated ceRNA regulatory network functions by modulating signaling pathways:** LncRNAs acting as ceRNAs can also participate in HCC cell proliferation, migration, EMT, invasion, and metastasis by modulating various signaling pathways in HCC, including the Wnt/β-catenin pathway, PI3K/AKT pathway, and nuclear factor kappa-B (NF-kB) pathway.

**Wnt/β-catenin pathway:** Abnormal activation of the Wnt/β-catenin pathway, a key event implicated in HCC carcinogenesis, is believed to be a key target for the clinical diagnosis and treatment of HCC[95]. Thus, elucidation of the regulatory mechanisms of the Wnt/β-catenin pathway will provide new insights into a new anticancer therapy for HCC. Extensive evidence to date has indicated that lncRNAs can mediate the Wnt/β-catenin pathway by acting as ceRNAs of miRNAs, thereby modulating HCC cell proliferation and invasion; oncogenic lncRNAs that act as ceRNAs can perform their biological actions by activating the Wnt/β-catenin pathway in HCC. For example, frizzled (FZD) 4, a Wnt receptor, can activate the Wnt/β-catenin pathway in HCC[96], and the lncRNA *ASB16-AS1* enhances FZD4 expression to activate the Wnt/β-catenin pathway by acting as a miR-1827 sponge and subsequently facilitates HCC growth and invasion[97]. Likewise, another Wnt receptor, FZD7, can also activate the Wnt/β-catenin pathway in HCC[98]. The lncRNA *DSCR8* activates the Wnt/β-catenin pathway by enhancing FZD7 expression by acting as a miR-485-5-p sponge to facilitate HCC cell proliferation and the cell cycle[99]. Wingless-type MMTV integration site family 3A (WNT3A) is one of the crucial components of the Wnt/β-catenin pathway related to HCC progression[100], and the lncRNA *LINC00662* activates the Wnt/β-catenin pathway by enhancing WNT3A expression *via* the competitive sponging of miR-15a, miR-16, and miR-107, thereby promoting HCC growth and metastasis[101]. Glycogen synthase kinase 3β (GSK3β) is a pivotal regulator of β-catenin signaling[102], and the lncRNA *SNHG5* acts as a miR-26a-5p sponge to enhance GSK3β expression, thereby activating the Wnt/β-catenin pathway to facilitate HCC metastasis and EMT[103]. In addition, sex determining region Y-box (SOX) 9 can activate the Wnt/β-catenin pathway in HCC[104], and the lncRNA *SOX9-AS1* facilitates HCC growth and

metastasis by increasing SOX9 expression to activate the Wnt/β-catenin pathway by acting as a miR-5590-3p sponge[105]. In HCC, interleukin (IL)-6 is associated with the activation of Janus kinase 2 (JAK2)/STAT3 signaling[106]; cyclin-dependent kinase (CDK) 8 and low-density lipoprotein receptor-related protein 6 (LRP6) are associated with the activation of Wnt/β-catenin signaling[107,108], and the lncRNA *DLGAP1-AS1* increases the expression of IL-6 and CDK8/LRP6 by functioning as a sponge of miR-26a-5p and miR-26b-5p, thereby activating JAK2/STAT3 and Wnt/β-catenin signaling to facilitate EMT and the progression of HCC, respectively[109]. Instead, tumor suppressive lncRNAs that act as ceRNAs function by inactivating Wnt/β-catenin signaling in HCC. For example, in HCC, nuclear receptor corepressor 2 is associated with inhibition of the activation of Wnt/β-catenin signaling[110]. *MIR22HG*, a tumor suppressive lncRNA, increases NCOR2 expression by sponging miR-10a-5p, thereby inactivating Wnt/β-catenin signaling to inhibit HCC cell growth, migration, and invasion[111]. The abovementioned findings suggest that different lncRNA-mediated ceRNA networks can exert their biological functions in HCC by mediating the Wnt/β-catenin pathway; these networks may become effective therapeutic targets for treating HCC patients.

**PI3K/AKT pathway:** PI3K/AKT, a highly activated pathway in HCC, is implicated in HCC carcinogenesis and chemoresistance[112-114]. At present, emerging evidence indicates that multiple lncRNA-mediated ceRNA networks can exert their biological functions by modulating the PI3K/AKT pathway, among which oncogenic lncRNAs that act as ceRNAs exert their biological function by activating the PI3K/AKT pathway in HCC. For instance, the lncRNA *PTTG3P* facilitates the proliferation, migration, and invasion and inhibits the apoptosis of HCC cells by increasing the expression of cyclin D1/poly ADP-ribose polymerase 2 and activating the PI3K/Akt pathway by acting as a ceRNA of miR-383[115]. Similarly, the lncRNA *DLEU1* activates the PI3K/Akt pathway by increasing insulin-like growth factor 1 receptor-1R expression by sponging miR-133a, thereby facilitating HCC cell proliferation, migration and invasion[116]. By contrast, tumor suppressive lncRNAs that act as ceRNAs can exert their biological function by inactivating the PI3K/Akt pathway in HCC. For instance, *TCL6*, a tumor suppressive lncRNA, upregulates PTEN expression by sponging miR-106a-5p to suppress the PI3K/AKT pathway, thereby inhibiting HCC cell proliferation, migration, and invasion[117]. Intriguingly, several oncogenic lncRNAs that act as ceRNAs have been reported to exert their biological functions by activating the PI3K/AKT/mammalian rapamycin (mTOR) or PI3K/AKT/FoxO3a pathway in HCC. For example, the lncRNA *CDKN2B-AS1*, an oncogenic lncRNA, enhances nucleosome assembly protein 1 like 1 expression by acting as a ceRNA of let-7c-5p, thus activating the PI3K/AKT/mTOR pathway to promote HCC cell growth and metastasis[118]. In addition, the lncRNA *GAS6-AS2* activates the PI3K/AKT /FoxO3a pathway by upregulating OTU domain-containing ubiquitin aldehyde-binding protein 1 expression by sponging miR-493-5p, which promotes HCC cell proliferation, migration, and invasion[119]. In short, the lncRNA-miRNA-PI3K/AKT, PI3K/AKT/mTOR or PI3K-AKT-FoxO3a regulatory network is expected to be a potential therapeutic target for the treatment of HCC patients.

**NF-κB pathway:** Numerous studies have shown that abnormal activation of the NF-kB pathway is related to HCC growth, EMT, and invasion[120-122]. Existing evidence suggests that lncRNAs can act as miRNA sponges and exert their biological function by mediating the NF-κB pathway in HCC, among which oncogenic lncRNAs that act as ceRNAs can exert their biological function by activating the NF-κB pathway in HCC. For example, in the NF-κB pathway, mixed-lineage kinase (MLK) 3 contributes to cancer migration, invasion, and metastasis[123,124], and the lncRNA *SNHG12* enhances MLK3 expression by competitively sponging miR-199a/b-5p, thereby activating the NF-κB pathway to promote HCC proliferation and tumorigenicity[125]. By contrast, a tumor suppressive lncRNA that acts as a ceRNA can exert its biological function by inactivating the NF-κB pathway in HCC. For example, CYLD, a tumor suppressor, can negatively regulate the NF-κB pathway in HCC[126], and the lncRNA *CASC2*, a tumor suppressive lncRNA, suppresses the NF-κB pathway by enhancing CYLD expression by sponging miR-362-5p, thereby inhibiting HCC cell migration and invasion[127,128]. These findings indicate that the lncRNA-miRNA-NF-κB pathway network may serve as a therapeutic target for patients with HCC.

### *Chemoresistance and radioresistance*

Although current chemotherapy and radiotherapy regimens can prolong the survival of HCC patients, tumor recurrence and metastasis due to chemoresistance and

radioresistance lead to unsatisfactory long-term efficacy. The underlying mechanisms of therapeutic resistance in HCC are still unclear, and the exploration of such mechanisms will help improve the current treatment of HCC. Emerging evidence suggests that lncRNAs play a critical role in mediating chemoresistance and radioresistance by acting as ceRNAs of miRNAs in HCC.

Currently, the lncRNA-mediated ceRNA network has been proven to mediate HCC resistance to chemotherapy drugs, including sorafenib, oxaliplatin, cisplatin, and 5-fluorouracil (5-FU). Exploration of the resistance mechanisms to chemotherapy drugs in the treatment of HCC will provide new insights into overcoming chemoresistance.

Sorafenib has been approved for treating advanced HCC; however, the emergence of sorafenib resistance has affected the efficacy of HCC treatment. Existing studies have shown that the lncRNA-mediated ceRNA network is responsible for HCC resistance to sorafenib. Specifically, oncogenic lncRNAs that act as ceRNAs can enhance HCC resistance to sorafenib. For example, activation of the c-Met-Akt pathway can promote sorafenib resistance in HCC cells[129], and the lncRNA *NEAT1* activates the c-Met-Akt pathway by sponging miR-335 to enhance sorafenib resistance in HCC cells[130]. In addition, recent studies have suggested that the abnormal activation or expression of forkhead box M1 (FoxM1) contributes to chemotherapy resistance in various cancer cells[131,132]. In HCC cells, FoxM1 knockout sensitizes drug-resistant HCC cells to sorafenib[133], and the lncRNA *LINC-ROR* increases sorafenib resistance in HCC cells by elevating FOXM1 expression by sponging miR-876-5p[134]. Phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3), a regulatory subunit of PI3K, activates the PI3K/AKT pathway to enhance the resistance of HCC cells to sorafenib-induced apoptosis[135], and the lncRNA *LINC00160* acts as a miR-132 sponge to promote sorafenib resistance by increasing PIK3R3 expression in HCC cells[136]. Transmembrane protein 9 (TMEM9) plays a vital role in HCC cell growth[137], and the lncRNA *FOXD2-AS1* upregulates TMEM9 expression by sponging miR-150-5p to facilitate the resistance of HCC cells to sorafenib[138]. The tetraspanin protein CD151 has been shown to attenuate drug-induced apoptosis in cancer cell lines[139], and the lncRNA *SNHG3* enhances CD151 expression by acting as a ceRNA for miR-128 to promote HCC resistance to sorafenib[140]. Additionally, the lncRNA *SNHG16* is upregulated in sorafenib-resistant HCC cells, and *SNHG16* increases sorafenib resistance partly by competitively sponging miR-140-5p[141].

Oxaliplatin has been approved for the treatment of patients with locally advanced and metastatic HCC who are not eligible for surgical resection or local treatment; however, oxaliplatin resistance affects the efficacy of HCC treatment. The lncRNA-mediated ceRNA network has been confirmed to modulate oxaliplatin resistance in HCC. In particular, oncogenic lncRNAs that act as ceRNAs can enhance HCC resistance to oxaliplatin. For instance, *HULC* can upregulate the expression of the ubiquitin-specific peptidase 22 (USP22) protein by suppressing miR-6825-5p, miR-6845-5p, and miR-6886-3p at the epigenetic or transcriptional level in HCC cells; USP22 enhances the *HULC*-induced deubiquitination of Sirt1 and stabilizes it, and Sirt1 stability induces the autophagy of HCC cells, thus increasing the resistance of HCC cells to oxaliplatin[142]. Multidrug resistance-associated protein 1 (ABCC1) is indicative of chemotherapy resistance[143], and the lncRNA *NR2F1-AS1* elevates ABCC1 expression by sponging miR-363 to enhance oxaliplatin resistance in HCC cells[144]. Similarly, the lncRNA *KCNQ1OT1* increases oxaliplatin resistance in HCC cells by sponging miR-7-5p to elevate ABCC1 expression[145].

The antitumor efficacy of cisplatin in the treatment of advanced HCC patients is unsatisfactory due to drug resistance. The lncRNA-mediated ceRNA network has been demonstrated to modulate cisplatin resistance in HCC, among which oncogenic lncRNAs that act as ceRNAs can enhance HCC resistance to cisplatin. For example, nuclear factor erythroid-2-related factor 2 (Nrf2) is upregulated in HepG2/cisplatin cells and mediates the chemoresistance of HCC cells to cisplatin[146]. The lncRNA *NRAL* increases Nrf2 expression by sponging miR-340-5p, thereby facilitating cisplatin resistance in HCC cells[147]. Melanoma-associated antigen A3 (MAGEA3) enhances chemoresistance to cisplatin in HepG2 cells, and the lncRNA *LINC01234* enhances MAGEA3 expression by sponging miR-31-5p to promote cisplatin resistance in HCC[148]. The lncRNA *SNHG16* enhances cisplatin resistance in HCC cells by sponging let-7b-5p[149]. Conversely, tumor suppressive lncRNAs that act as ceRNAs can reduce HCC resistance to cisplatin. For example, the overexpression of *CASC2*, a tumor suppressor lncRNA, strengthens cisplatin sensitivity in HCC cells by sponging miR-222[150]. In addition, the overexpression of *GAS5*, another tumor suppressor lncRNA, enhances the sensitivity of HCC cells to cisplatin by sponging miR-222[151].

The inhibitory efficacy of 5-FU on HCC cells is limited by chemical resistance. Emerging evidence indicates that the lncRNA-mediated ceRNA network is correlated

with 5-FU resistance in HCC cells, among which oncogenic lncRNAs that act as ceRNAs can enhance HCC resistance to 5-FU. For example, the lncRNA *CRNDE* acts as a ceRNA of miR-33a in HCC to enhance HMGA2 expression, thereby promoting chemoresistance to 5-FU in HCC cells[152]. HIHIF-2α is related to the resistance of HCC cells to doxorubicin and sorafenib[153,154], and the lncRNA *MALAT1* acts as a ceRNA to increase HIF-2α expression by competitively sponging miR-216b, leading to the enhanced chemoresistance of HCC cells to 5-FU[155]. In contrast, tumor suppressive lncRNAs that act as ceRNAs can reduce HCC resistance to 5-FU. For example, Kelch-like ECH-associated protein 1 (Keap1) inactivation enhances the resistance of HCC cells to chemotherapy drugs such as sorafenib[156,157], and the overexpression of *KRAL*, a tumor suppressive lncRNA, enhances Keap1 expression by functioning as a ceRNA for miR-141 to reverse the resistance to 5-FU in HCC cell lines[158].

Currently, an oncogenic lncRNA-mediated ceRNA network has been demonstrated to enhance HCC resistance to radiation therapy. For instance, AZD1775, an inhibitor of WEE1, has been reported to sensitize HCC cells to radiation[159], suggesting that WEE1 can enhance radioresistance in HCC. The lncRNA *NEAT1_2* upregulates WEE1 expression by acting as a ceRNA for miR-101-3p to reduce the radiosensitivity of HCC[160]. A RING-type ubiquitin ligase E3 (RAD18), an E3 ubiquitin-linked enzyme, can induce radiation resistance in glioma cells[161,162]. The lncRNA *LINC-ROR* competes with sponge miR-145 to increase RAD18 expression, thereby enhancing the radiation resistance of HCC cells[163]. Forkhead box protein P1 (FOXP1), a transcription factor, attenuates radioresistance in cervical cancer[164]. The lncRNA *LINC00473* promotes radioresistance in HCC by increasing FOXP1 expression by sponging miR-345-5p[165]. These findings suggest that the lncRNA-mediated ceRNA network may provide new clues for overcoming radioresistance in HCC.

## PROBLEMS AND PERSPECTIVES

The lncRNA-mediated ceRNA regulatory network provides a new mode of posttranscriptional regulation and plays a critical role in the initiation and progression of HCC. Nevertheless, investigations into the detailed mechanism of the ceRNA network and its relationship with HCC are still in the preliminary stage. Although there are increasing reports about lncRNAs as ceRNAs in HCC, several fundamental problems facing these studies need to be addressed. First, information on the roles of lncRNAs that act as ceRNAs in current studies is derived from overexpression and/or knockout experiments, and only when the abundance of lncRNAs is remarkably high can lncRNAs act as ceRNAs. As a result, the abundance of artificially controlled lncRNAs often far exceeds the abundance range of any endogenous lncRNA. Therefore, it is urgent to verify whether the lncRNA-mediated ceRNA network has the same effects under normal cellular conditions. Second, most of the current research on ceRNAs is still in the prediction stage of bioinformatics, and most studies lack biological validation; the regulatory relationships in the ceRNA network need to be effectively verified. Third, methodologically, there are few predictive tools available; most miRNA-mRNA predictions focus only on the binding of a miRNA with its target in the 3′-UTR. However, this is not always the case; miRNAs can also target the 5'-untranslated region and coding sequences of mRNAs[166-170]. Thus, provided that the prediction of a ceRNA is not limited to the 3′-UTR of its mRNA, the range of predicted ceRNAs should be improved. Fourth, one miRNA generally interacts with one target mRNA. However, some miRNAs may modulate many target mRNAs, and *vice versa* [167,171]. Thus, it is necessary to model the effect of ceRNAs in real scenarios. Fifth, the ceRNA hypothesis maintains that a miRNA is stably expressed; in fact, intracellular miRNA expression is dynamic, which inevitably influences the effectiveness of ceRNAs. Sixth, although ceRNA prediction methods are constantly updated, the current prediction algorithms cannot fully encompass several factors affecting ceRNA susceptibility (quantity and characteristics of MREs, miRNA/mRNA abundance, and subcellular location of RNAs)[172,173]. Therefore, the prediction methods and experimental methods still need to be further updated and improved. Seventh, the initiation and progression of HCC are a complex event, and it is unclear whether other mechanisms interact with lncRNAs acting as ceRNAs in HCC cells. Addressing the above problems will enable a better understanding of the lncRNA-mediated ceRNA network that can be used to more effectively diagnose and treat HCC.

Given the critical roles and the complex interactions among lncRNA-mediated ceRNA regulatory networks in HCC, future investigations with validation in large sample sizes and the exploration of in-depth molecular mechanisms are needed to

probe the HCC-specific lncRNA-mediated ceRNA axis, which should identify new diagnostic and prognostic markers of HCC and provide promising targets for the treatment of patients with HCC.

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*MINIREVIEWS*

# Gadoxetic acid magnetic-enhanced resonance imaging in the diagnosis of cholangiocarcinoma

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## Abstract

The use of liver magnetic resonance imaging is increasing thanks to its multiparametric sequences that allow a better tissue characterization, and the use of hepatobiliary contrast agents. This review aims to evaluate gadoxetic acid enhanced magnetic resonance imaging in the diagnosis and staging of cholangiocarcinoma and its different clinical and radiological classifications proposed in the literature. We also analyze the epidemiology, risk factors in correlation with clinical findings and laboratory data.

**Key words:** Hepatobiliary contrast materials; Gadoxetic acid; Cholangiocarcinoma; Magnetic resonance imaging; Liver; Cirrhosis



**Core tip:** Cholangiocarcinoma is the second most common primary hepatic tumor. Magnetic resonance imaging, with its multiparametric study, with the use of cholangiographic sequences and with the hepatospecific contrast medium can allow a

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complete diagnostic evaluation, with a correct non-invasive staging, to choose the best therapeutic option.

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## INTRODUCTION

### *Epidemiology and risk factors*

Cholangiocarcinoma (CCA) is the most widespread primary biliary tract neoplasia[1] and the second most common primary hepatic tumor, considering that hepatocellular carcinoma (HCC) is the most frequent[2,3]. CCA represents 3% of all gastrointestinal tumors, typical of elderly adults, with a peak incidence in the 7th decade.

Recent updates showed a higher number of deaths due to CCA, in particular surpassing HCC[2], first of all, because the diagnosis of CCA occurs at an advanced stage.

The majority of cases is sporadic, however, all medical conditions that lead to chronic inflammation are considered risk factors, first of all, primary sclerosing cholangitis (PSC), viral hepatitis, biliary malformations, hepatolithiasis, bile ducts adenomas, biliary-pancreatic abnormal junction, parasite infections (*O. viverrini*, *C. sinensis*), chemical products (*e.g.* Thorotrast), and, finally, cirrhosis[4].

In a recent metanalysis, Bergquist *et al*[5] overviewed all possible risk factors for CCA: It was established a high risk for patients with choledochal cysts [Risk Ratio (RR): 36.9-47.1], cirrhosis (RR: 22.9), choledochal lithiasis (22.5-34.0) and hepatolithiasis (RR: 6.7-16.5), while the lower risk was evaluated in case of liver parasitosis (RR: 4.7), viral hepatitis (RR: 3.17-5.10), especially HCV, diabetes mellitus type II (RR: 1.89), obesity (RR: 1.56) and alcohol use (RR: 2.81). PSC represents a high-risk factor in developing CCA, with a range from 7% to 14%[6-8]. IBD is suggested to be a risk factor for CCA (RR: 1.72-3.95).

### *Clinical findings and laboratory data*

In the early onset, patients with CCA are asymptomatic or manifest no typical symptoms. The most common clinical symptom in patients with extrahepatic CCA is jaundice. Other non-specific symptoms can be present such as weight loss, abdominal pain, night sweats, dark urine, fatigue, vomiting, pruritus, increase of cholestasis-related lab parameters (Alanine transaminase, Aspartate transaminase, Gamma-glutamyltransferase, Bilirubin), increased prothrombin time, reduction in fat-soluble vitamins[9].

The protein CA 19-9 can be elevated in the majority of patients with CCA. In particular, the value of CA 19-9 higher than 100 U/mL in PSC patients has very good sensitivity and specificity for the diagnosis of CCA (89% and 86%, respectively). However, according to literature data and clinical experience, CA 19-9 values alone are not sufficient in the diagnosis of CCA. Besides, in patients without PSC, a CA 19-9 value higher than 1000 U/mL has been linked to metastatic and unresectable CCA[10]. On the other hand, Carcinoembryonic Antigen (Carcinoembryonic antigen) and CA 125 are non-specific markers.

### *Classification*

There are many classifications of CCA based on clinical, radiologic and pathological features.

CCA can be anatomically differentiated in intrahepatic and extrahepatic forms. Depending on anatomical location it's possible to recognize different types of CCA[11]: (1) Intrahepatic cholangiocarcinoma (iCCA), involving the periphery of the second-order bile ducts; (2) Extrahepatic cholangiocarcinoma: Perihilar cholangiocarcinoma (pCCA), involving the hepatic duct (left or right) or the junction; and (3) Distal cholangiocarcinoma (dCCA) involving the common bile duct.

The "Liver Cancer Study Group of Japan" classification is considered the most complete one because it's based on appearance, biological behaviour, growth

characteristics, clinical prognosis, and imaging characterization[12]. The "Liver Cancer Study Group of Japan" classification permits also to stage the disease, evaluating tumor size, vascular, and/or serosal invasion (Table 1).

Intrahepatic CCA presents three patterns of growth: Mass forming (MF-CCA), periductal infiltrating (PI-CCA), and intraductal growing (IG-CCA) (Figure 1)[11,13].

MF is the most common, accounting for 65% of iCCA, presenting as a mass, usually large and characterized by central necrosis or scarring. The typical presentation is a solitary mass lesion, more frequently located in the right lobe (35%), followed by the left lobe (22%). Finally, 12% is centrally located, and 31% is multifocal[11].

Accounting for 6% of iCAA, PI-CCA develops lengthways within the wall of the bile duct and spreads along the portal tracts. The affected ducts show wall thickening and progressive narrowing[14].

IG-CCA, accounting for 4% of iCAA, develops as a polypoid or papillary mass within the bile duct lumen. A partial biliary obstruction can be found out, due to a large amount of mucin yielded[12,13].

A combined form, composed by PI and MF types, represents a 25% of iCCA[11].

According to the site of involvement and histologic features, iCCA is classified into two types: Perihilar large duct, and peripheral small duct. This new classification, in particular taking into account location, clinical and genetic factors, has a potential role in predicting prognosis[14].

Extrahepatic CCA can be further subdivided into perihilar form and distal form, according to Bismuth-Corlette classification (Table 2), in particular, useful to assess longitudinal spread. The American Joint Committee on Cancer defined classification to evaluate radial spread because perihilar and distal forms differ in presentation, natural history, and management.

### *Pathological findings*

CCA has been classified into two wide groups: (1) The classical one, represented by adenocarcinoma, subdivided in by well, moderately, and poorly differentiated; and (2) The variant forms (*e.g.*, adenosquamous, squamous carcinomas). However, a newer histological classification has been introduced in clinical practice[15,16].

This most recent classification divides CCA into the conventional type (ductal), the ductular type, the intraductal type, and rare variants. Moreover, in the case of iCCA, the ductal type can be subdivided, according to bile duct involvement, into small and large type. While ductular type tumors are present exclusively in iCCA, the intraductal type can be seen in iCCA, pCCA, or dCCA. Additionally, ductular and small bile duct CCA may be grouped as the small duct type[16].

Many authors demonstrated that histologic data represent an important prognostic factor of iCCA. Shamis *et al*[17] classified iCCA into type 1 and type 2, according to on mucin production and immunophenotype, establishing a link between histopathology and prognosis[17].

### *Management*

To date, liver resection and liver transplantation are the only management options available for CCA. Imaging plays a fundamental role in the preoperative staging to establish the best treatment approach for each patient.

The pathologic staging system, developed for ductal CCA, has a limited value in the assessment of extra-hepatic CCA. Moreover, tumour node metastases classification does not correlate with resectability in patients with extrahepatic CCA while, conversely, the Memorial Sloan-Kettering staging system permits to determine which patients are suitable for resection and the prognosis (Table 3).

## MAGNETIC RESONANCE PROTOCOL

Nowadays magnetic resonance imaging (MRI), due to its high intrinsic contrast resolution, in particular in the evaluation of biliary tree, is considered the best imaging technique to diagnose and stage CCA. The diagnostic potential of magnetic resonance cholangiopancreatography (MRCP) is now almost as good as ERCP, eliminating the need for most invasive studies[18].

An optimal protocol for CCA evaluation should include conventional T1 in- and out-of-phase imaging, T2-weighted sequences in axial and coronal planes, diffusion-weighted imaging, completed with contrast-enhanced sequences. MRI should be performed on a high-field scanner (1.5T or 3T)[19]. According to clinical practice, the dynamic sequences should be performed by using standardized time points or with

**Table 1 Liver cancer study group of Japan tumour node metastases staging for intrahepatic cholangiocarcinoma**

| | Type | Result |
|---|---|---|
| T | Number of tumors | Solitary |
| | Size of tumor | 2 cm or less |
| | Negative invasion | Portal vein, hepatic vein, serous membrane |
| N | N0 | No metastasis to nodes |
| | N1 | Metastasis to nodes |
| M | M0 | No distant metastasis |
| | M1 | Positive distant metastasis |

T1: A tumor that meets all 3 requirement; T2: Meets 2/3 requirements; T3: Meets 1/3 requirements; T4: Meets none of the requirements.

**Table 2 Bismuth-Corlette classification system for perihilar cholangiocarcinoma**

| Type | Definition |
|---|---|
| I | Below the confluence of the left and right hepatic ducts |
| II | Reaching confluence but not involving left or right hepatics ducts |
| III | Occluding common hepatic duct and either right (A) or left (B) hepatic duct |
| IV | Multicentric or bilateral intrahepatic segmental involvement; or involving confluence and both right and left hepatics ducts |

**Table 3 Memorial Sloan Kettering T stage for hilar cholangiocarcinoma**

| Stage | Criteria |
|---|---|
| T1 | Tumor involving biliary confluence and/or unilateral extension to second-order biliary tracts |
| T2 | T1 and/or ipsilateral portal vein involvement and/or ipsilateral hepatic lobar atrophy |
| T3 | Tumor involving biliary confluence, the biliary extension to second-order biliary tracts; or unilateral extension to second-order biliary tracts with contralateral portal vein involvement; or unilateral extension to second-order radicals with contralateral hepatic lobar atrophy; or main or bilateral portal vein involvement |

bolus-triggering technique.

### *MRCP*

According to literature and clinical practice, MR study should be completed with MRCP sequences that nowadays are considered the non-invasive reference for biliary system assessment. MRCP is a non-contrast MR technique in which the contrast between bile and adjacent tissues, showing long and short T2 relaxation time respectively, is accentuated by using heavily T2-weighted sequences. MRCP sequences should be aligned to the common bile duct in the head of the pancreas, by using axial T2-weighted images. The patient is asked to breathe regularly during acquisition, which takes between 3-5 min to acquire.

Thin multi-slice MRCP allows to obtain a high-resolution visualization of the biliary tree by 3D-images in particular with multiplanar reconstructions (MPR) and maximum intensity projection reformation can be generated to better assess the hepatic biliary tree and pancreatic ducts.

As a general recommendation, patients should fast for at least 4 h to reduce gastrointestinal peristalsis and gastric secretions. Moreover, this approach allows for obtaining a gallbladder distention. Negative contrast agents permit to reduce fluid signal in the stomach and duodenum, such as blueberry juice or iron oxide[20].

### *MR imaging findings and contrast agents*

On T1-weighted images, CCA shows a hypo-to isointense signal, while on T2-weighted images the signal is slight to high hyperintense, thus reflecting tumor components, in particular fibrosis, mucin, and necrosis.

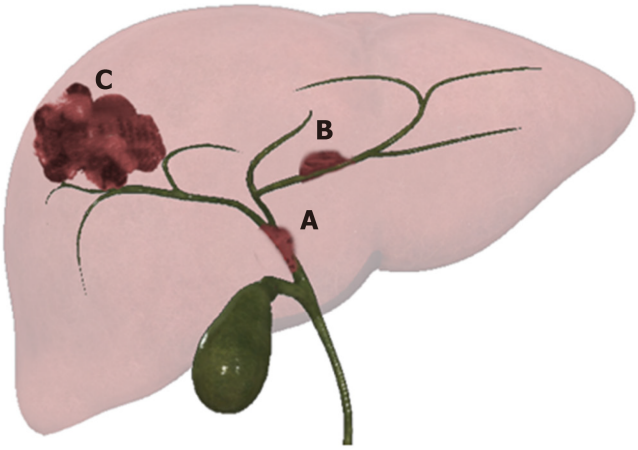


**Figure 1 Graphic representation of 3 different patterns of growth of intrahepatic cholangiocarcinoma.** A: Intraductal growing-cholangiocarcinoma; B: Periductal infiltrating-cholangiocarcinoma; and C: Mass forming-cholangiocarcinoma.

The dynamic enhancement pattern after gadolinium-based contrast administration may be variable. In the arterial phase, it shows a rim enhancement while a progressive, centripetal, enhancement can be appreciable in the delayed phases, due to its intrinsic fibrotic appearance. If the tumor is predominantly fibrotic, enhancement may only be visible in the delayed phase.

Ancillary features include vascular encasement, biliary obstruction, lobar atrophy, capsular retraction, and lymphadenopathy.

Dynamic imaging can be performed with both extracellular and hepatocyte-specific contrast media. Liver post-contrast signal intensity is greater with the use of hepatocyte-specific agents compared with traditional gadolinium-based extracellular contrast agents. In this setting, CCA appears as a hypointense lesion in the hepatobiliary phase (HBP), considering the absence of functioning hepatocytes[21].

## ROLE OF Gd-EOB-DTPA

Gd-EOB-DTPA (Bayer Schering Pharma, Berlin, Germany) is a gadolinium-based MRI hepatocyte-specific contrast agent. It shows a biphasic mechanism of action: First distribution in the extracellular space and then selective uptake by functioning hepatocytes and biliary excretion through the organic anionic transporting polypeptide (OATP8). In patients with preserved liver function, the hepatic uptake can be evident after 20 min and lasts for several hours after EOB-DTPA injection (*i.e.* the hepatobiliary phase).

This phenomenon allows evaluating both vascular features and the functional status of the nodules with a single examination. Indeed, the nodules with low or no OATP expression (*i.e.* CCA and most of the other hepatic lesions) show no hepatocyte-specific contrast agent s uptake and appear hypointense in the HBP.

Regarding the Gd-EOB-DTPA administration, the European society of gastrointestinal and abdominal radiology consensus statement provides recommendations[22]. The approved dose is 0.025 mmol/kg with a flow-rate of 1-2 mL/s followed by a saline flush. Dynamic sequences should be obtained by using a bolus triggering technique. Optimal multiphasic dynamic contrast-enhanced imaging, with fewer artifacts, is feasible using multi-arterial phase imaging[23] and in fact, its role in the evaluation of hepatic lesions particularly HCC has been well studied[24,25].

As mentioned in the first chapter, the imaging features of CCA have been classified into four different growth patterns: MF-CCA, PI-CCA, IG-CCA, and mixed type (MF-CCA and PI-CCA). There is a close correlation between growth pattern and anatomical location: PI-CCA and IG-CCA are uncommon in iCCA and are usually seen in pCCA and dCCA, while the majority of CCA arising in the large perihilar bile duct shows a mixed type growth pattern.

Several characteristics of EOB-DTPA MR cholangiography allow differential diagnosis between different subtypes of cholangiocarcinoma with high accuracy[26].

### MF-CCA

The vascular dynamic enhancement pattern of MF-CCA with EOB-DTPA is similar to CT contrast agents and the non-specific extracellular gadolinium-based agents (Figure 2): An arterial ring-like or band-like contrast enhancement in the early dynamic phase (arterial phase and late portal phase) with delayed progression (a progressive or concentric filling in the portal and delayed phase). This enhancement pattern is related to the fibro-cirrhotic nature of the disease with a fibrous central scar. A less frequent pattern is an HCC-like hypervascularity[27], which is more common in small lesions due to relatively less fibrous tissue.

Contrary to the extracellular contrast agents, sometimes the delayed EOB-DTPA enhancement is less evident in the transitional phase. The transitional phase is stated as the time frame between the portal venous phase and the HBP, representing the transition of the contrast media from the extracellular space into bile ducts[28]. Therefore, the lesions appear hypointense relative to the surrounding hyperintense liver parenchyma (pseudo-wash-out).

In the HBP phase, MF-CCA is usually heterogeneously hypointense due to the absence of OATP8 (*i.e.*, absence of Gd-EOB-DTPA uptake). An additional feature of MF-CCA in the HBP is the presence of target sign (*i.e.*, central hyperintensity less than surrounding parenchyma associated with a peripheral hypointense rim)[29] that reflects the presence of fibrosis. However, those with HCC-like arterial hyperenhancement seem to have less fibrosis in comparison to ones with typical rim-enhancement[30].

Other typical imaging features of MF-CCA are capsular retraction, bile duct dilatation, vascular involvement, and central scars[31]. All these features can be reliably evaluated using Gd-EOB-DTPA-enhanced MR cholangiography and in particular in a pre-surgical setting with 3D T1-weighted spoiled gradient-echo images with high flip angle (a flip angle more than 20° is recommended and 35-40° seemed to be the best) in the HBP. It revealed an improvement of diagnostic accuracy compared to the conventional magnetic resonance cholangiopancreatography with only T2 acquisition[32].

### PI-CCA and IG-CCA

PI-CCA presents an irregular wall thickening with stenosis or obliteration of the involved bile ducts and upstream dilation (Figure 3). Typically this tumor enhances slowly and gradually and at the delayed phase reveals an enhancement peak[31]. In the HBP, it is difficult to distinguish PI-CCA from enhanced liver parenchyma. Due to the impaired function of biliary cells, the HBP showed almost no EOB-DTPA in the common bile duct.

IG-CCA may present a variety of appearances depending on the ductal dilation (diffuse, cystic, or minimal dilation), the amount of mucin, and the extension (focal or diffuse) (Figure 4). This growth pattern has a typical heterogeneous arterial enhancement with delayed progression[33]. In HBP the polypoid nodules were homogeneously hypointense, and Gd-EOB-DTPA cannot be excreted into the dilated bile ducts, because these ducts are excluded from the biliary tree. Thus MRCP will show only bright and dilated excluded duct.

It should be underlined that the enhancement, especially in the PI-CCA and IG-CCA is better seen on MRI than on CT due to the higher contrast resolution[27]. In the HBP, the contrast between the healthy liver parenchyma and CCA may allow a more accurate assessment of tumor extent, helping to address patients to the correct management and, consequently, to evaluate prognosis[34,35].

### Advantages of EOB-DTPA

The use of EOB-DTPA is also important in terms of prognosis: The presence of daughter nodules[36] and intrahepatic metastasis[37] are poor prognostic factors. The high signal intensity of liver parenchyma in the HBP allows improving lesion conspicuity and detection of satellite nodule and intrahepatic metastasis[35]. Moreover, a recent study highlighted the role of capsule penetration, (*i.e.*, pathologically defined serosal perforation or invasion) and tumor size; the latter better define with low interobserver variability in the HBP, as prognostic factors for postoperative outcomes in MF-CCA[38].

Finally, HBP seemed to be effective in estimating regional liver functional reserve[39] and liver volume to guide surgical procedures. This technique has been reported to be also useful in the setting of surgical complications, in particular for bile leaks detection after hepatobiliary surgery[40].

The use of EOB-DTPA could be useful also for differential diagnoses[22]. A small nodule (< 3 cm) in a patient at risk for HCC without typical hallmark imaging features

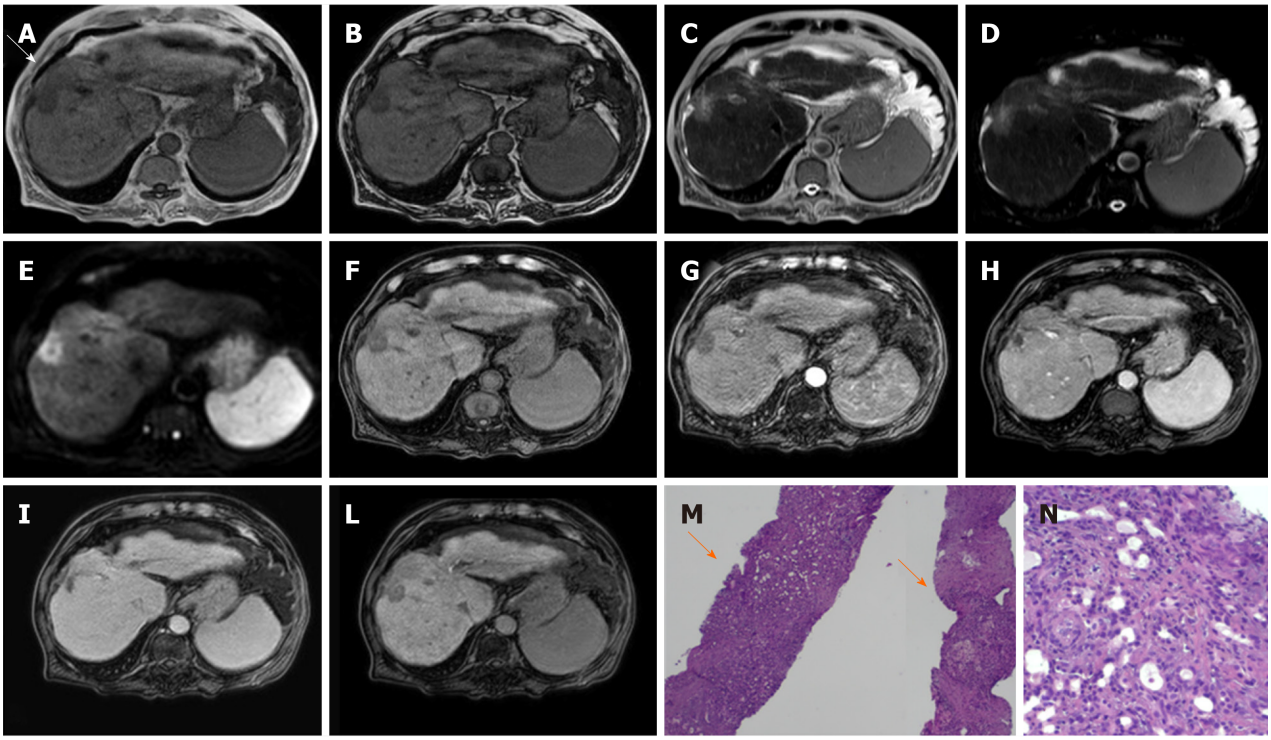


**Figure 2 Mass forming cholangiocarcinoma.** On a background of the alcohol-related cirrhotic liver, there is a 2 cm lesion in segment VIII with capsular retraction (white arrow). The lesion is hypointense on T1 IP and OP images. A and B: Slightly hyperintense on T2 and SPAIR; C and D: DWI restriction; E: The dynamic enhancement pattern after gadoxetic acid administration is a peripheral rim of enhancement in arterial and portal phase; G and H: Progressive centripetal enhancement on the delayed phase; I: The lesion demonstrates hypointensity in the hepatobiliary phase; L: The patient underwent percutaneous liver biopsy. Biopsy specimen stained with hematoxylin and eosin respectively at 4 × and 20 ×; M and N: Showed an adenocarcinoma (orange arrow) on a background of the cirrhotic liver (orange arrow); N: The magnification better depicts the appearance of the adenocarcinoma with the tubular aspect. The immunohistochemistry confirmed the positivity for PDX1 and CK7 and negativity for CDX2 and CK20, in keeping with cholangiocarcinoma.

cannot be certainly differentiated from CCA. However, a recent meta-analysis[41] identified eleven MRI features for differentiating HCC from MF-CCA such as capsular retraction, rim enhancement in the arterial phase, progressive enhancement in the delayed phases, target appearance in diffusion-weighted imaging, and, finally, bile duct dilation. These features are included in Liver Imaging Reporting and Data System (Liver imaging reporting and data system) as the LR-M category, indicating a probably or malignant lesion without specific HCC features[42].

Moreover, it has been recently shown[27] that the presence of thicker arterial ring enhancement, during the EOB-MRI study, associated with dot-/band-like internal enhancement could help differentiate MF-CCA carcinoma from IG-CCA and PI-CCA. Moreover, Gd-EOB-DTPA with MRCP can differentiate between the different growth patterns.

A recent meta-analysis[43] indicated that MRI with extracellular contrast agent had a sensitivity of 94% and specificity of 71% with an area under the curve of 0.92, comparable to CT in the evaluation of the resectability of hilar cholangiocarcinoma, but the use of EOB-DTPA seemed to increase both sensitivity and specificity. However, both CT and MRI show low sensitivity for nodal status while PET/CT appears to be the best technique, however, it has no clear role for evaluating surgical resectability.

Overall, MRI with EOB-DTPA provides an accurate assessment of tumor extent, biliary tree, vessels, and invasions of adjacent structures and is, therefore, a fundamental evaluation system before surgery which also allows differential diagnosis and providing prognostic information.

## CONCLUSION

Cholangiocarcinoma is the second most common hepatic tumor and is often asymptomatic and can be diagnosed late. Regardless of its intra or extrahepatic presentation, it requires an imaging technique that allows evaluating both anatomical

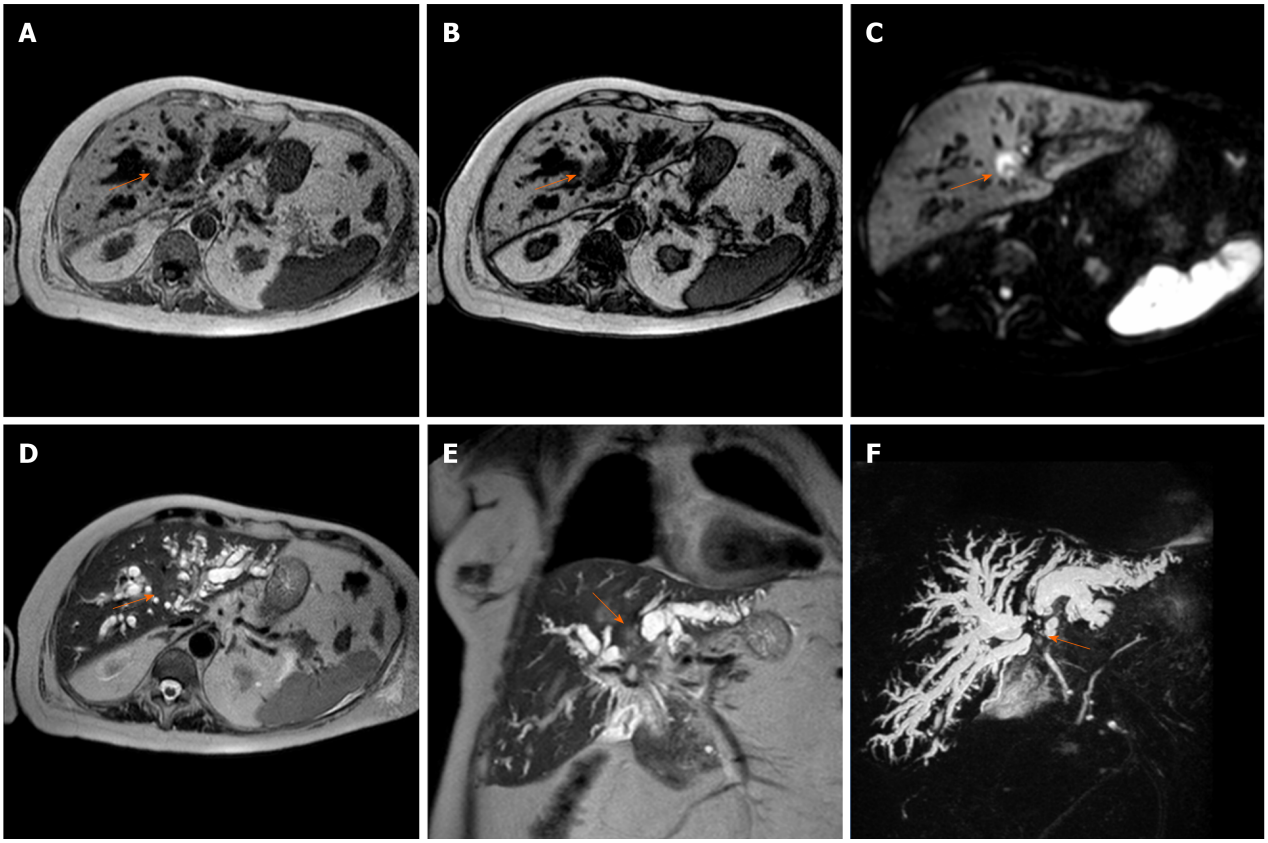


**Figure 3 Perihilar cholangiocarcinoma.** A case of pCCA (orange arrow), arising at the junction with the involvement also of in the right and left hepatic duct, in an 86-year-old female. The magnetic resonance cholangiopancreatography is reported. The lesion is a hypointense mass in T1-weighted images. A and B: Hyperintense in the higher b-value of diffusion-weighted images; C: Mild hyperintense on T2-weighted images; D and E: Finally the use of the 3D respiratory-triggered heavily T2-weighted FSE sequences; and F: Maximum intensity projection reconstruction is useful to detect the strictures at the junction of the biliary tree.

sites. MRI, strengthened by a multiparametric study, with the use of cholangiographic sequences and with the use of the hepatospecific contrast medium can allow a complete diagnostic evaluation to manage the best therapeutic option.

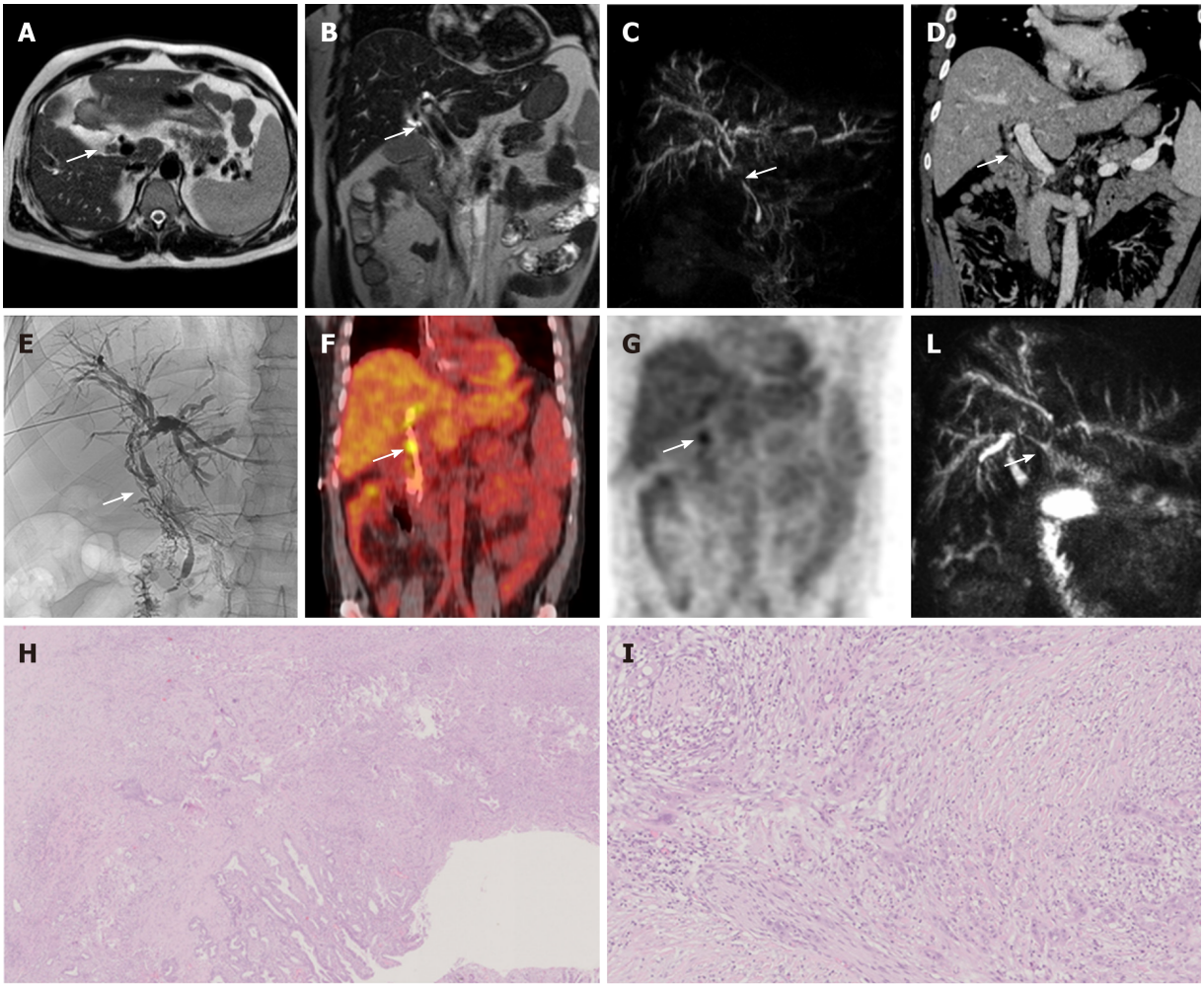


**Figure 4 Distal cholangiocarcinoma.** A case of distal cholangiocarcinoma (white arrow) involving the common bile duct in a 52-year-old male patients with primary sclerosing cholangitis and ulcerative colitis. The patients underwent magnetic resonance cholangiopancreatography with axial. A: Coronal; B: T2-weighted images and a maximum intensity projection reconstruction of the 3D respiratory-triggered heavily T2-weighted FSE sequences; C: Then contrast-enhanced Computed Tomography; D: For staging the disease. Moreover, a percutaneous transhepatic biliary drainage for diagnostic confirmation with tissue collection was performed; E: And finally, PET scan; F and G: It was used to resolve a diagnostic dilemma about a pulmonary nodule. The patient underwent surgery; H and I: It was reported the surgical resection specimen at histology stained with hematoxylin and eosin respectively at 2 × and 10 ×: The tumor was an adenocarcinoma, with an invasion of the wall of the bile duct for 6 mm with also perilesional papillary epithelial dysplasia, the final stage according to the VIII edition of the Union for International Cancer Control is T2, N0. L: The maximum intensity projection reconstruction of the 3D respiratory-triggered heavily T2-weighted FSE sequences, 6 mo after the surgery: The irregular dilatation of the intrahepatic biliary tract persists (in patients with primary sclerosing cholangitis) and the patency of the biliodigestive anastomosis is highlighted with the white arrow.

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ORIGINAL ARTICLE

Basic Study

# Rno_circ_0005139 regulates apoptosis by targeting *Wnt5a* in rat anorectal malformations

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## Abstract

### *BACKGROUND*

The molecular mechanisms underlying anorectal malformations (ARM) are not fully established. Circular RNAs (circRNAs) are new born non-coding RNAs, and their role in ARM is unclear. We assumed that rno_circ_0005139 influences apoptosis and proliferation by acting as a miR-324-3p sponge, and downregulating *Wnt5a* in ARM.

### *AIM*

To identify the differential expression of circRNAs and mRNAs in a rat ARM model.

### *METHODS*

Sixty-six pregnant Wistar rats were randomly divided into two groups: ARM group (2-imidazolidinethione-induced) and control groups. Embryos were harvested by cesarean delivery, and anorectal tissue was taken on embryonic days 16 (E16), 17 (E17), 19 (E19), and 21 (E21). RNA sequencing and gene microarray analysis was used to identify differentially expressed circRNAs and mRNAs in the ARM in a rat model. We selected 6 circRNAs and 3 mRNAs in the Wnt signal pathway from the result of the RNA sequencing and gene microarray analysis, and quantitative reverse transcription polymerase chain reaction was performed to evaluate their tissue expression. According to bioinformatics prediction, rno_circ_0005139 acted as a miR-324-3p sponge to regulate the expression of *Wnt5a*. We chose rno_circ_0005139 and Wnt5a as the final candidates. We tested the function of rno_circ_0005139 and the binding sites between rno_circ_0005139 and miR-324-3p, miR-324-3p and *Wnt5a* by luciferase assays. Co-transfection of rno_circ_0005139 and miR-324-3p was to verify their functional consistency.

### *RESULTS*

We identified 38 upregulated and 42 downregulated circRNAs on E17 ($P < 0.05$), and 301 mRNAs were upregulated and 256 downregulated in the ARM on E17 ($P$

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< 0.05, fold-change > 2.0). We found that rno_circ_0006880 and rno_circ_0011386 were upregulated, whereas rno_circ_0000436, rno_circ_0005139, rno_circ_0009285, rno_circ_0014367, *Wnt5a*, *Wnt10b*, and *Wnt2b* were downregulated in ARM tissues. According to bioinformatics prediction, rno_circ_0005139 acted as a miR-324-3p sponge to regulate the expression of *Wnt5a*. We chose rno_circ_0005139 and *Wnt5a* as the final candidates. Because the role and molecular mechanism of rno_circ_0005139 are poorly understood, its effect on apoptosis and proliferation was investigated by *in vitro* plasmid transfection. A luciferase experiment showed that rno_circ_0005139 could bind with miR-324-3p, which negatively regulated *Wnt5a* expression. The expression of miR-324-3p was significantly higher in ARM anorectal tissues than that in control group on E17 and E19; *Wnt5a* expression showed the opposite trend. In addition, a miR-324-3p inhibitor attenuated the effects of rno_circ_0005139 knockdown on ARM development.

***CONCLUSION***

Rno_circ_0005139 influences cell proliferation and apoptosis by acting as a miR-324-3p sponge, thereby downregulating *Wnt5a* in ARM. Accordingly, rno_circ_0005139, miR-324-3p, and *Wnt5a* could be targeted therapeutic factors for ARM.

**Key words:** Anorectal malformation; Circular RNA; MicroRNA; *Wnt5a*; Rno_circ_0005139



**Core tip:** Rno_circ_0005139, miR-324-3p, and *Wnt5a* play regulatory roles in the pathogenesis of anorectal malformations (ARM). Rno_circ_0005139 is a potential biomarker or therapeutic target in the ARM. In general, this study provides an important basis for further studies on the diagnosis, treatment, and prevention of ARM.

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## INTRODUCTION

Anorectal malformations (ARM) are the most common gastrointestinal malformations in pediatric surgery, with an incidence of 1/5000-1/1500 live births[1,2]. More than half of patients have genitourinary, cardiovascular, skeletal, and gastrointestinal malformations[3,4]. Wnt signaling pathway, SHH signaling pathway, BMP pathway, FGF signaling pathway, and Hox family are all associated with ARM[5-10]. *Wnt5a* is located on chromosome 3p14–p21, and *Wnt5a* plays a pivotal role in the development of anorectum, deformity, terminal rectal intestinal neuromuscular, and pelvic floor neuromuscular development in both human and rats[11]. In addition, ARM in *Wnt5a*$^{-/-}$ mice shows anal atresia with rectal urethral fistula[12]. However, the regulatory factors upstream of Wnt5a remain unknown.

Circular RNAs (circRNAs) were originally thought to be transcriptional mismatches or transcriptional byproducts; the first evidence for the existence of circRNAs was reported in 1976[13]. Unlike linear RNA molecules, circRNAs represent a class of single-stranded, unusually conserved, and stable RNAs with a covalently closed loop structure lacking 5′-3′ polarity or a polyadenylated tail[14]. The functions of circRNAs have been gradually revealed, and they mainly act as microRNA (miRNA) sponges to regulate gene expression in neurological diseases, cardiovascular diseases, and various types of cancers[15,16]. However, the role of circRNAs in ARM has not been widely examined. RNA sequencing was used to identify differentially expressed circRNAs in congenital ARM, and validated a novel circRNA, rno_circ_0005139. Bioinformatics analysis revealed that miR-324-3p was a potential target of rno_circ_0005139, and *Wnt5a* was the target gene of miR-324-3p. There have been no reports on the function of rno_circ_0005139 and its relationship with miR-324-3p.

In the present study, downregulation of rno_circ_0005139 and upregulation of miR-324-3p resulted in decreased *Wnt5a* expression in the anorectal tissues of ARM and intestinal epithelial cells (IECs) and promoted apoptosis. Overexpression of rno_circ_0005139 and miR-324-3p mimic inhibited apoptosis by blocking the interaction of miR-324-3p and the 3'-untranslated region (UTR) of *Wnt5a*. Rno_circ_0005139/miR-324-3p/Wnt5a pathway may have the crucial effect on cell apoptosis and proliferation in ARM, and circ_0005139 may thus be a targeted therapeutic factor for ARM.

## MATERIALS AND METHODS

### Preparation of tissues

Prior to the study, approval was obtained from the Medical Research and New Technology Ethics Committee of Shengjing Hospital, affiliated with China Medical University (2016PS045K). Female (250-280 g) and male (280-300 g) Wistar rats were provided by the Chang Sheng Biotechnology Co., Ltd. (Changchun, China). The Wistar rats were in the specific pathogen free grade (12 h light-dark cycle, 37°C, get water and feed anytime) animal litter. Wistar rats were mated overnight in a cage (a ratio of female: male, 4:1). Pregnant rats were identified by sperm in vaginal smears in the morning, and the day was defined E0. A total of 66 pregnant Wistar rats were randomly divided into 2-imidazolidinethione (ETU)-induced ARM group [a single dose of ETU (Aldrich Chemical, Penzberg, Germany)] and control group (an equal dose of saline without ETU) on E10. The concentration of ETU was 0.01 g/L and the dose was 0.125 g/kg given *via* oral gavage. Embryos were harvested by cesarean delivery on embryonic days 16 (E16), 17 (E17), 19 (E19), and 21 (E21). The embryos in ARM group were short or no tail, and in control group were long tail. The hindgut tissues were removed under a microscope and immediately frozen in liquid nitrogen.

### Library preparation, RNA sequencing, and data analysis

Six cDNA libraries were constructed, *i.e.*, three for E17 rats with ARM and three for E17 normal control rats. Three micrograms of RNA per sample was used as the input material for sample preparation with TRIzol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions, followed by DNase I treatment to remove DNA contamination. The total RNA level was assessed on a denaturing agarose gel and quantified by the NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, United States). First, total RNA was treated with an Epicentre Ribo-Zero kit (Epicentre Technologies, Madison, WI, United States) to remove all rRNAs. The remaining RNAs were processed using a TruSeq RNA Sample Prep Kit according to the Illumina protocol (San Diego, CA, United States). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with Phusion High-Fidelity DNA polymerase (Agilent Technologies, United States), Index (X) Primer (Agilent Technologies, United States), and universal qRT-PCR primers (Agilent Technologies, United States). Finally, the products were purified using the AMPure XP system (Beckman, Brea, CA, United States), and library quality was evaluated on an Agilent Bioanalyzer 2100 system (Santa Clara, CA, United States). The RNA library was sequenced on an Illumina Hiseq 2500 platform and find_circ[17] was used to identify circRNA. The expression of known and predicted circRNAs were normalized by transcripts per million, and differential expression analysis was performed using DEGseq. CircRNAs with $Q < 0.01$ and $|\log2 \text{ fold-change}| > 1$ were considered as differentially expressed.

Another six samples (three for E17 rats with ARMs and three for E17 normal rats) were used for gene microarray analysis. Total RNA was quantified using Nano Drop ND-1000, and RNA integrity was assessed by standard denaturing agarose gel electrophoresis. According to manufacturer's protocols, the following steps were taken: Preparing samples, hybridizing microarray, amplifying and transcribing into fluorescent complementary (c) RNA. The labeled cRNAs were hybridized onto a Whole Genome Oligo Array (444K, Agilent Technologies). The Whole Rat Genome Oligo Microarray Kit is a tool for modeling human biology in the rat model organism. The arrays were scanned to analyze the acquired array images by Agilent Scanner G2505C and Agilent Feature Extraction software (version 11.0.1.1). A Gene Spring GX v11.5.1 software package (Agilent Technologies) was used to perform the quantile normalization and subsequent data procession.

### IECs culture and processing

Rat IECs were acquired from the BeNa Culture Collection (Beijing, China) and were cultured in α-minimum Eagle,s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY, United States) at 37°C and 5% $CO_2$ (Gibco).

### RNA extraction and qRT–PCR analysis

Total RNA was isolated from IECs and anorectal tissues using TRIzol reagent (Invitrogen), the synthesis of complementary (c)DNA was completed using RNA PCR Kit from TaKaRa (Dalian, China) and gene amplification was carried out using a SYBR Premix Ex Taq Kit from Takara the according to the manufacturer's instructions. qRT–PCR was performed with the 7500 Fast PCR System (Applied Biosystems, Foster City, CA, United States). Primers for *β-actin* (for circRNA and mRNA) and *U6* (for miRNA) were used as endogenous controls. Each experiment was performed in triplicate. The Ct values were recorded and melting curve was analyzed. Comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to calculate the expression of the genes[18].

Primers for rno_circ_0005139, rno_circ_0006880, rno_circ_0011386, rno_circ_0000436, rno_circ_0005139, rno_circ_0009285, and rno_circ_0014367 were synthesized by Geneseed (Guangzhou, China). Primers for miR-324-3p and *U6* were designed and synthesized by RiboBio (Guangzhou, China). *U6* was the normalization control for miRNAs. Primers for *Wnt5a*, *Wnt2b*, *Wnt10b* and *β-actin* were designed by Takara (Table 1).

### Western blotting

Chopped tissues and cells were placed in radioimmunoprecipitation assay lysis buffer containing 1% phenylmethylsulfonyl fluoride (Beyotime, Jiangsu, China). Supernatant was collected after 12000 *r*/min for 15 min. Protein lysates were separated by gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were exposed to anti-Wnt5a (Invitrogen) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies at 4°C overnight. The membrane was incubated in the anti-rabbit IgG secondary antibody (1:5000; Proteintech, Rosemont, IL, United States) for 2 h at room temperature. A ProtoBlot II AP System (Promega, Madison, WI, United States) was used to test the signals. The protein expression was compared with the expression of GAPDH. Densitometry quantified the protein relative expression with ImageJ software (National Institutes of Health, Bethesda, MD, United States).

### Luciferase assays

Rno_circ_0005139 wild-type, mutant sequences, the 3'-UTR of *Wnt5a* wild-type and the 3'-UTR of *Wnt5a* mutant-type were inserted into a pMIR-REPORT Vector (RiboBio). pMIR-REPORT with rno_circ_0005139 wild-type and mutant sequences were co-transfected with miR-324-3p and pMIR-REPORT with Wnt5a wild-type and mutant-type were co-transfected with miR-324-3p into HEK293T cells using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol. After 48 h, the luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega). Relative firefly luciferase activity (firefly luciferase activity/Renilla luciferase activity) was the final criterion.

### Apoptosis of IECs

IECs in the logarithmic phase were collected and seeded into 6-well plates at a density of $4 \times 10^5$ cells per well. After 48 h transfection, 10 μL annexin V-fluorescein isothiocyanate and 5 μL propidium iodide were added to each sample well. After incubation for 15 min at room temperature in the dark, the samples were diluted with another 300 μL binding buffer, followed by filtration through a 300-μmol/L mesh cell strainer. The apoptotic rate for each sample was measured by flow cytometry (BD Biosciences, San Jose, CA, United States).

### Proliferation assays

Cell Counting Kit (CCK)-8 assay (KeyGEN, Jiangsu, China) was used to assess the cell proliferation. IECs ($1 \times 10^4$) were added to each well of a 96-well plate. Next, 10 μL CCK-8 solution was added to each well at four time points. After 2 h incubation at 37°C in 5% $CO_2$, absorbance at 450 nmol/L was measured using an automatic microplate reader (Synergy4, BioTek, Winooski, VT, United States). Each experiment was repeated three times.

**Table 1 Circular RNA and mRNA primer sequences**

| | Forward | Reverse |
|---|---|---|
| Rno_circ_0000436 | 5′-CCACGGAGAACAAGGTAAAA-3′ | 5′-ATTGACCTTGTCTTCCTCAG-3′ |
| Rno_circ_0005139 | 5′-CATCCTGTTGTGAAGATCTTG-3′ | 5′-TGTGGTAAGCCAAGTGATGAA-3′ |
| Rno_circ_0006880 | 5′-ACTGTTTACTTCTCCCAGGAAG-3′ | 5′-GCTTCATACCGATAAACCAGTG-3′ |
| Rno_circ_0009285 | 5′-CTTATAACCTGAGTGATAATGTC-3′ | 5′-TGGCATTCTTGCTGTTGGCT-3′ |
| Rno_circ_0011386 | 5′-CCACTGGTCAAGCAGCCTGT-3′ | 5′-GATGATCCTTCTCGGTCAGAG-3′ |
| Rno_circ_0014367 | 5′-GCGCTTGTTTCCTCATAGATTTC-3′ | 5′-CTTTGATTCCTTGATCTCCTC-3′ |
| *Wnt5a* | 5′-AGACGGGCATCAAAGAGT-3′ | 5′-AAGCGGTAGCCATAGTC-3′ |
| *Wnt*2b | 5′-CGGGCCCTCATGAACTTACATAAC-3′ | 5′-CAGGGTACAGGAGCCACTCACA-3′ |
| *Wnt10b* | 5′-AGTCACAGAGTGGGTCAACG-3′ | 5′-CGAAATCCGAGCAAAGAGC-3′ |
| *β-actin* | 5′-GGAGATTACTGCCCTGGCTCCTA-3′ | 5′-GACTCATCGTACTCCTGCTTGCTG-3′ |

### *Statistical analysis*

SPSS 21.0 (SPSS, Inc., Chicago, IL, United States) and Prism 7.0 (GraphPad Software, San Diego, CA, United States) were used for statistical analysis using the Student's *t* test and one-way analysis of variance. Data were obtained from at least three biological replicates and the results are presented as the means ± SD. $P < 0.05$ or $P < 0.01$ indicated that the difference between the contrast groups was statistically significant.

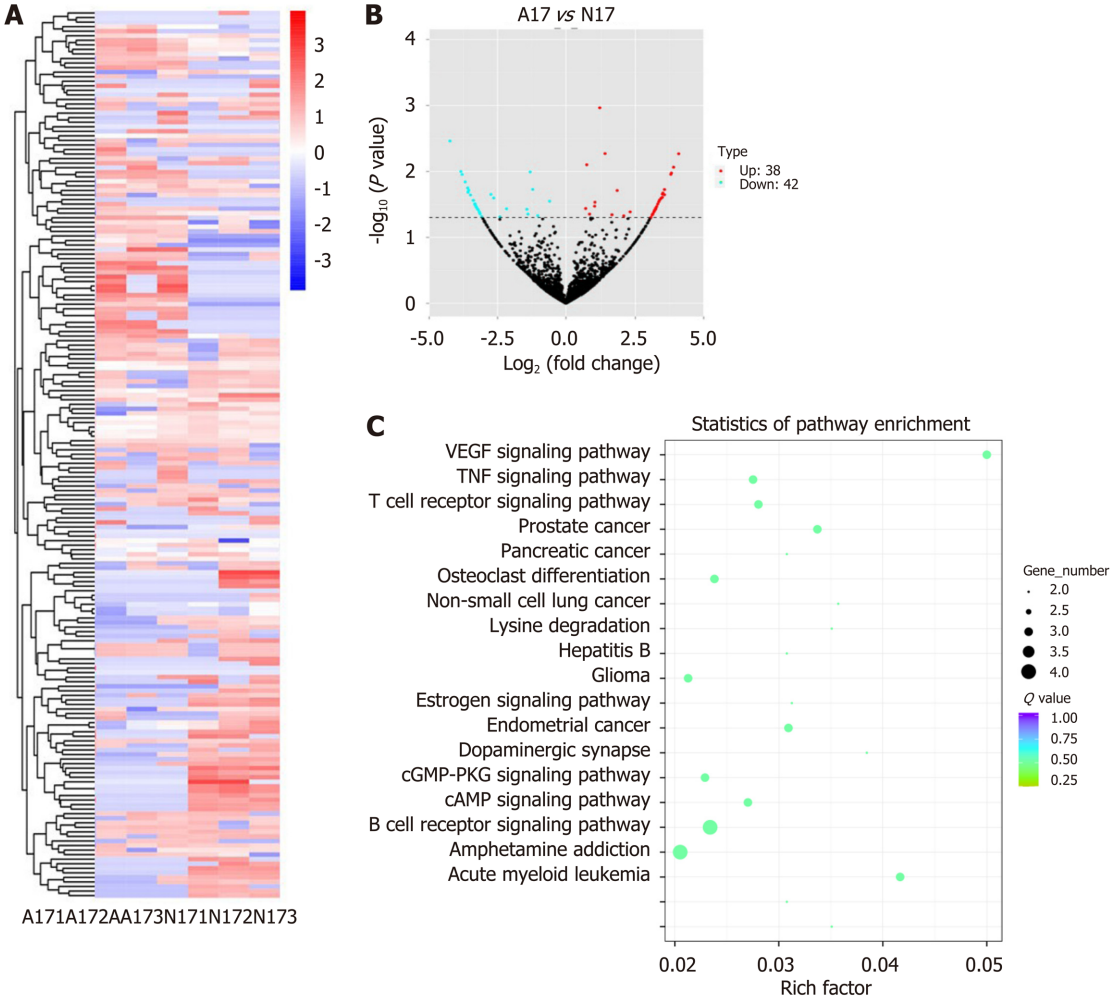
## RESULTS

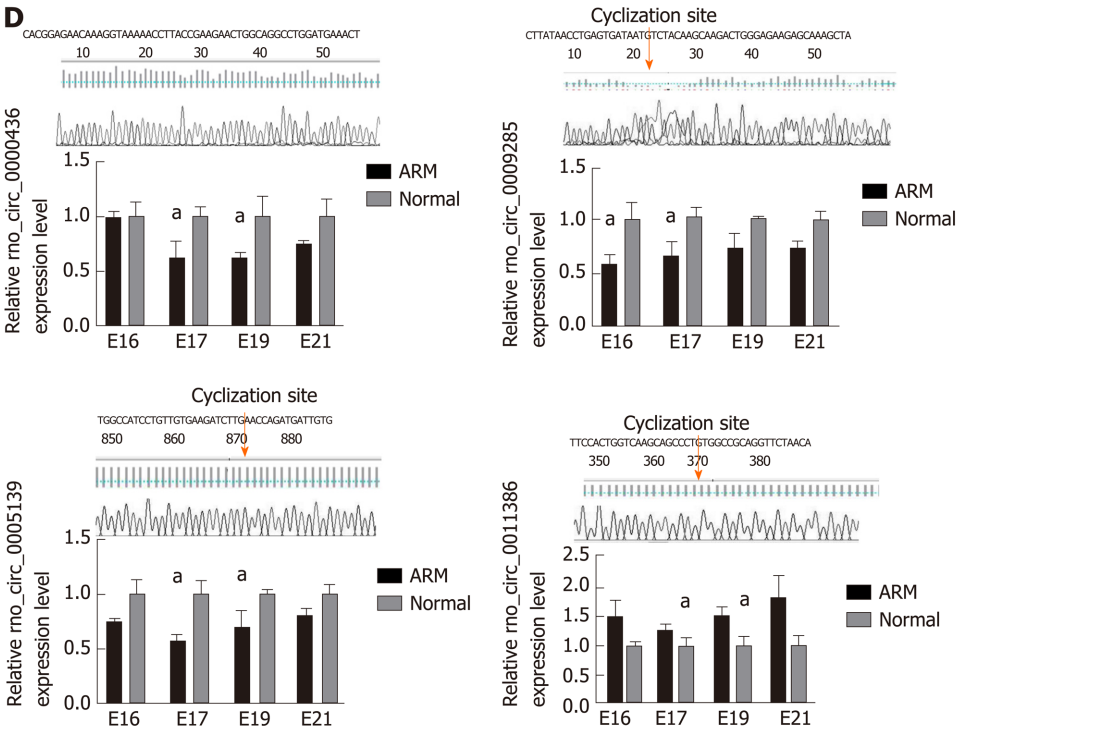
### *Analysis of circRNAs in ARM tissues and control tissues on E17*

We obtained a total of 652 fetal rats, including 310 in the ARM group and 342 in the control group. And 218 embryos had ARM (218/310, 70.32%) in the ARM group. And we randomly selected the same number of embryos at the same day. We sequenced RNA in rat ARM tissues and control fetuses on E17. The results of a cluster analysis of differentially expressed circRNAs on E17 are summarized in Figure 1A. We identified 38 circRNAs that were up-regulated in anorectal tissues compared to the control tissues ($P < 0.05$) and 42 circRNAs that were down-regulated ($P < 0.05$) on E17 (Figure 1B). To further explore the function of circRNAs which were differentially expressed on E17. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that the top 5 pathways of dysregulated genes were "VEGF signaling pathway", "B cell receptor signaling pathway", "Prostate cancer", "Estrogen signaling pathway", and "cGMP-PKG signaling pathway", which are known to be closely associated with proliferation, apoptosis, migration, and survival (Figure 1C). We randomly selected rno_circ_0006880, rno_circ_0011386, rno_circ_0000436, rno_circ_0005139, rno_circ_0009285, and rno_circ_0014367 as targets (Figure 1D). Based on the qRT–PCR results, rno_circ_0005139 was the final candidate. Additionally, we identified the cyclization site of rno_circ_0005139. We found that from E16 to E21, rno_circ_0005139 levels were decreased in the anorectal tissues of the ARM group compared to control tissues. Rno_circ_0005139 was significantly dysregulated on E16 and E17.

### *Rno_circ_0005139 modulates apoptosis and proliferation in IECs*

It was reported that IECs were used in cell proliferation and apoptosis during rat anorectal development experiments and were therefore used for subsequent functional experiments. . The relative rno_circ_0005139 expression levels after silencing and over-expression in IECs are shown in Figure 2A. Based on the results of the CCK-8 assay, rno_circ_0005139 silencing markedly inhibited cell proliferation and overexpression promoted cell proliferation (Figure 2B). Flow cytometry analysis showed that rno_circ_0005139 silencing increased the rate of apoptosis, whereas rno_circ_0005139 overexpression decreased the rate of apoptosis (Figure 2C and D).


A
A171A172AA173N171N172N173
B
A17 vs N17
-log10 (P value)
Log2 (fold change)
Type
Up: 38
Down: 42
C
Statistics of pathway enrichment
VEGF signaling pathway
TNF signaling pathway
T cell receptor signaling pathway
Prostate cancer
Pancreatic cancer
Osteoclast differentiation
Non-small cell lung cancer
Lysine degradation
Hepatitis B
Glioma
Estrogen signaling pathway
Endometrial cancer
Dopaminergic synapse
cGMP-PKG signaling pathway
cAMP signaling pathway
B cell receptor signaling pathway
Amphetamine addiction
Acute myeloid leukemia
Rich factor
Gene_number
Q value


D
Cyclization site
Relative rno_circ_0000436 expression level
Relative rno_circ_0009285 expression level
Relative rno_circ_0005139 expression level
Relative rno_circ_0011386 expression level
ARM
Normal
E16
E17
E19
E21

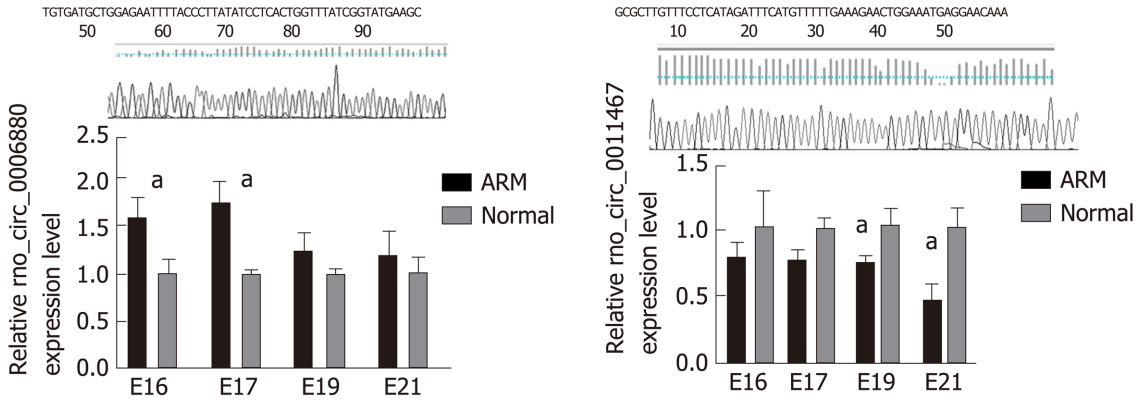



**Figure 1 Identification of differentially expressed circular RNAs between anorectal malformations anorectal tissues and control anorectal tissues.** A: Cluster analysis of differentially expressed circular RNAs (circRNAs) presented as a heatmap. Red indicates high expression and blue indicates low expression [red to blue represent high to low log10 (transcripts per million + 1) values]; B: Volcano plot of 38 up-regulated (red) and 42 down-regulated (green) circRNAs on embryonic day 17 (E17) in anorectal malformations (ARM) anorectal tissues compared with control tissues showing a two-fold change; C: Vertical axis represents the pathway name, and horizontal axis represents the enrichment factor. The size of the dots indicates the number of parent genes in this pathway, and the color of the dots indicates different q-value ranges; D: circRNA rno_circ_0000436, rno_circ_0005139, rno_circ_0006880, rno_circ_0009285, rno_circ_0011386, and rno_circ_0014367 were evaluated using quantitative reverse transcription polymerase chain reaction in ARM and control anorectal tissues. $^{a}P < 0.05$ *vs* normal. A: Anorectal malformations; ARM: Anorectal malformations; E: Embryonic day; N: Normal control.

### *Sequencing analysis of mRNAs in ARM and control tissues on E17*

To examine the expression of mRNAs, we used tissues from a rat ARM model and normal anorectal tissues collected on E17. Using an Agilent RNA Microarray Scanner, cluster analysis of differentially expressed mRNAs is shown in a heatmap scatter plot (Figure 3A). A total of 301 mRNAs were up-regulated and 256 mRNAs down-regulated in the ARM on E17 ($P < 0.05$, fold-change > 2.0) (Figure 3B). According to the KEGG analysis, 38 signaling pathways were enriched, with the top 10 listed in Figure 3C. Of these pathways, "Pathways in cancer" showed the greatest enrichment, followed by "Neuroactive ligand-receptor interaction". The enriched pathways included "MAPK signal pathway" and "Ras signal pathway", which are known to be closely associated with the process of embryonic development and ARM. RNA microarray scanner and the KEGG pathway analyses identified 3 mRNAs in ARM tissues that consistently responded to treatment: *Wnt5a*, *Wnt2b*, and *Wnt10b*. qRT–PCR analysis further showed that from E16 to E21, the expression levels of *Wnt5a*, *Wnt2b*, and *Wnt10b* were down-regulated in the anorectal tissues of the ARM group relative to normal tissues (Figure 3D). *Wnt5a* was selected as the final candidate.

### *Rno_circ_0005139 acts as a sponge for miR-324-3p, which interacts with Wnt5a*

We used TargetScan prediction software to predict the binding sites of six pairs of circRNAs (rno_circ_0000436, rno_circ_0005139, rno_circ_0006880, rno_circ_0009285, rno_circ_0011386, and rno_circ_0014367) and their target miRNAs, and miRNAs pairs and their three target *Wnt* genes (*Wnt5a*, *Wnt2b*, and *Wnt10b*). The results showed that rno_circ_0005139 shared a potential binding site with miR-324-3p, and the seed region of miR-324-3p was predicted to recognize the 3'-UTR of *Wnt5a*. qRT–PCR analysis showed that from E16 to E21, miR-324-3p was up-regulated in the anorectal tissues of the ARM group relative to normal control tissues (Figure 4A), in contrast with the expression changes in rno_circ_0005139 and *Wnt5a*. Furthermore, a luciferase assay revealed that co-transfection of cells with miR-324-3p mimic decreased the luciferase activity of wild-type rno_circ_0005139, but not of the mutant rno_circ_0005139 (Figure 4B). The results indicated that rno_circ_0005139 acted as a sponge for miR-324-3p. Moreover, we found that miR-324-3p was up-regulated after silencing of rno_circ_0005139 (Figure 4C). The prediction results showed that the miR-324-3p could bind with *Wnt5a* 3′-UTR; this prediction was confirmed by a luciferase reporter assay (Figure 4D). The ratios of miR-324-3p inhibitor and mimic in IECs are shown in Figure 4E. The expression of *Wnt5a* was negatively regulated by miR-324-3p, as shown by qRT–PCR and western blotting results (Figure 4F and G). These findings indicate that *Wnt5a* is a direct downstream target of miR-324-3p.

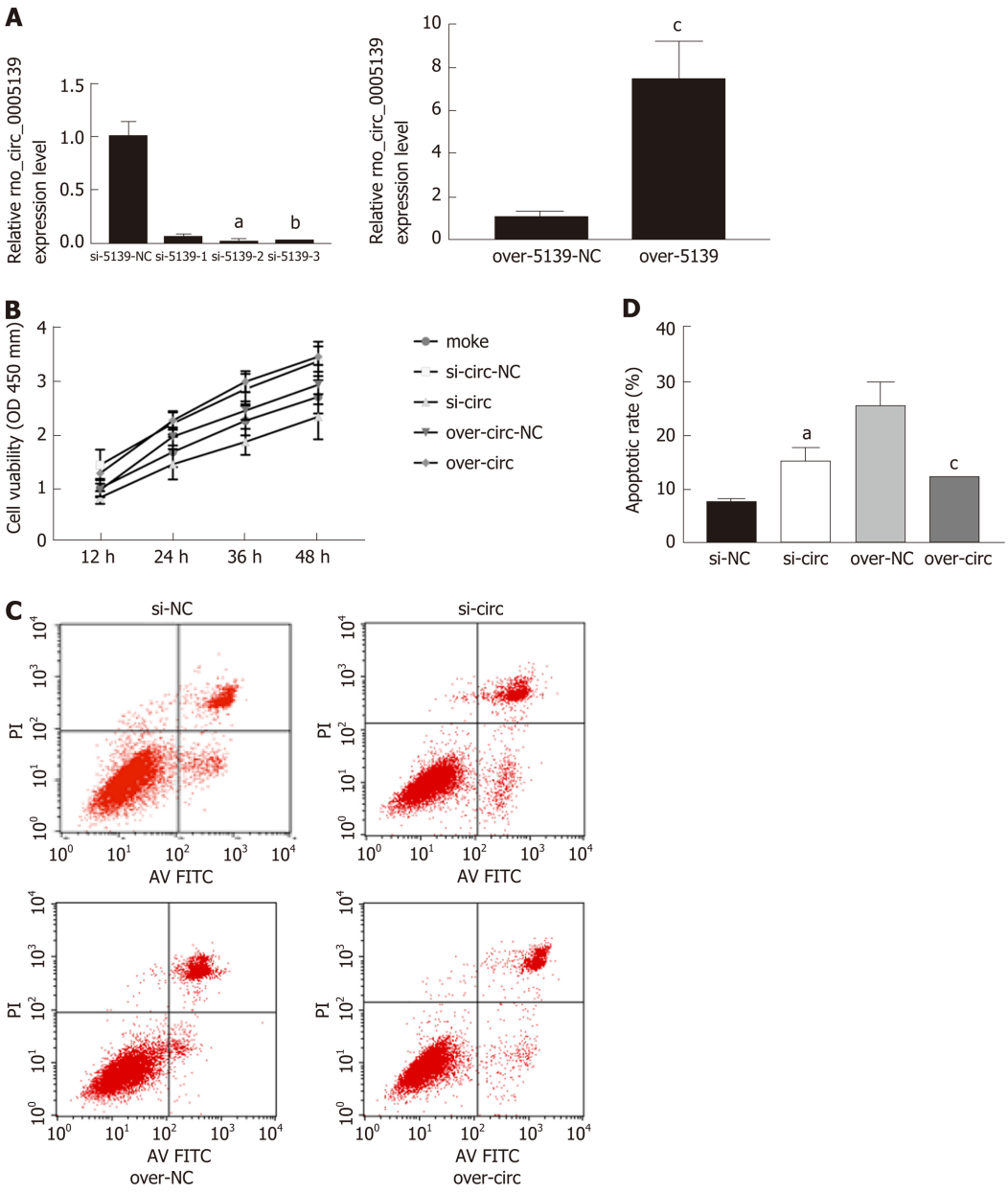



**Figure 2 Knockdown and overexpression of rno_circ_0005139 affected proliferation and apoptosis of intestinal epithelial cells.** A: Expression of rno_circ_0005139 was detected after transfection with si-rno_circ_0005139 (si-circ), pLO5-rno_circ_0005139 (over-circ), or a negative control (si-NC/vector) in intestinal epithelial cells (IECs), as determined by quantitative reverse transcription polymerase chain reaction; B: Cell Counting Kit-8 assays were used to measure the viability of IECs after transfection (rno_circ_0005139); C and D: Apoptosis detection using flow cytometry was conducted to measure apoptosis in IECs after transfection (rno_circ_0005139). $^{a}P < 0.01$ *vs* si-5139-NC, $^{b}P < 0.05$ *vs* si-5139-NC, $^{c}P < 0.05$ *vs* over-5139-NC.

### *Rno_circ_0005139 regulates apoptosis and proliferation via the miR-324-3p/Wnt5a pathway*

*Wnt5a* expression was lower in ARM anorectal tissues than in control tissues. Previous studies demonstrated negative regulatory relationships between rno_circ_0005139 and miR-324-3p, miR-324-3p, and *Wnt5a*. Therefore, the rno_circ_0005139/miR-324-3p/Wnt5a pathway may be a key regulator during the development of ARM. Silencing of rno_circ_0005139 decreased *Wnt5a* expression, whereas overexpression of rno_circ_0005139 increased the expression of *Wnt5a* (Figure 5A). These results suggest that rno_circ_0005139 influences the expression of *Wnt5a*. We further found that overexpression of rno_circ_0005139 partially up-regulated *Wnt5a* expression after co-transfection with a miR-324-3p mimic (Figure 5B). rno_circ_0005139 overexpression partially rescued the suppressive effects of an miR-324-3p mimic on cell proliferation (Figure 5C and D). rno_circ_0005139 overexpression also partially reversed the promotion of apoptosis by an miR-324-3p mimic (Figure 5E and F). These findings indicate that rno_circ_0005139 exerts its effects by targeting the miR-324-3p/Wnt5a

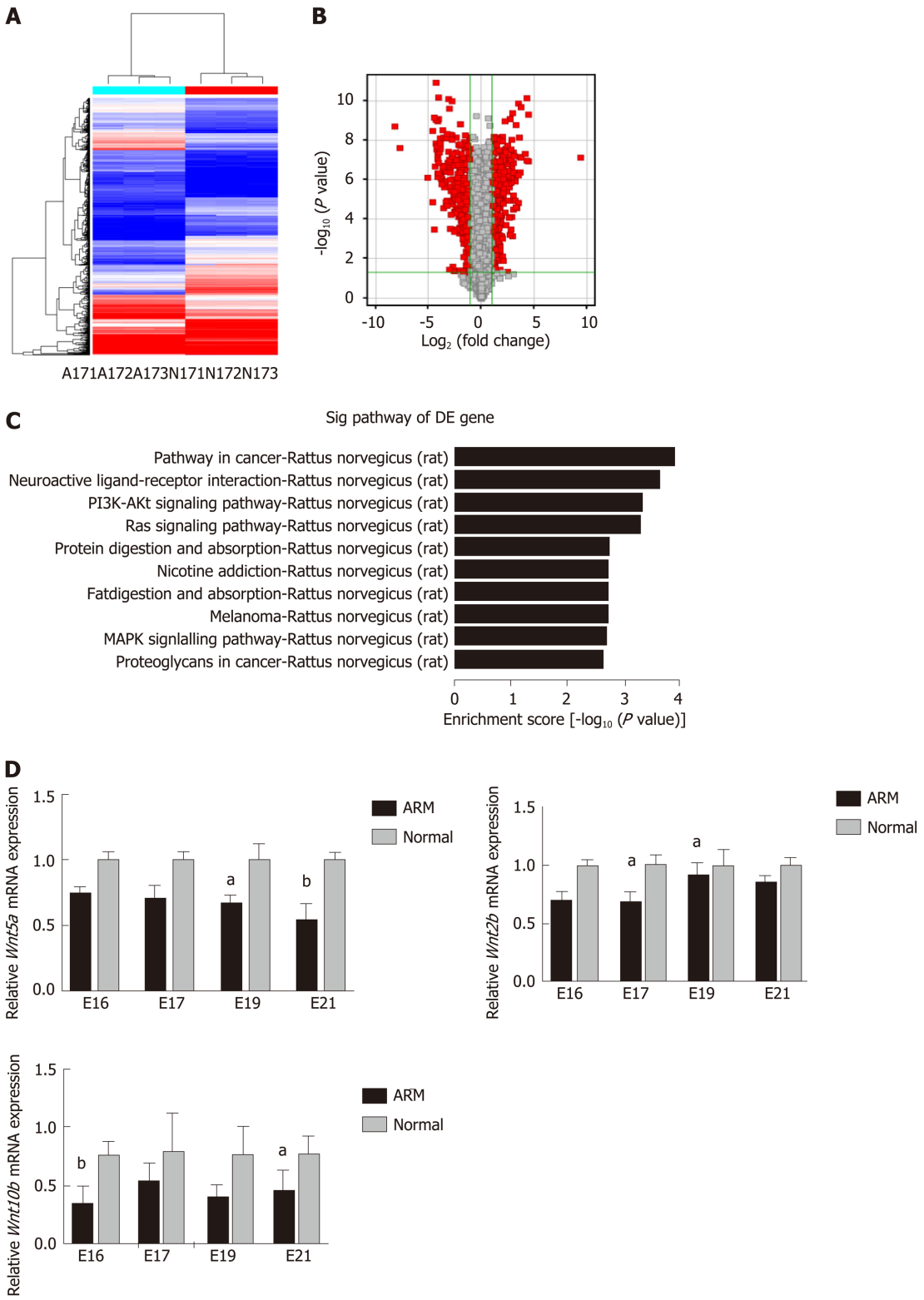
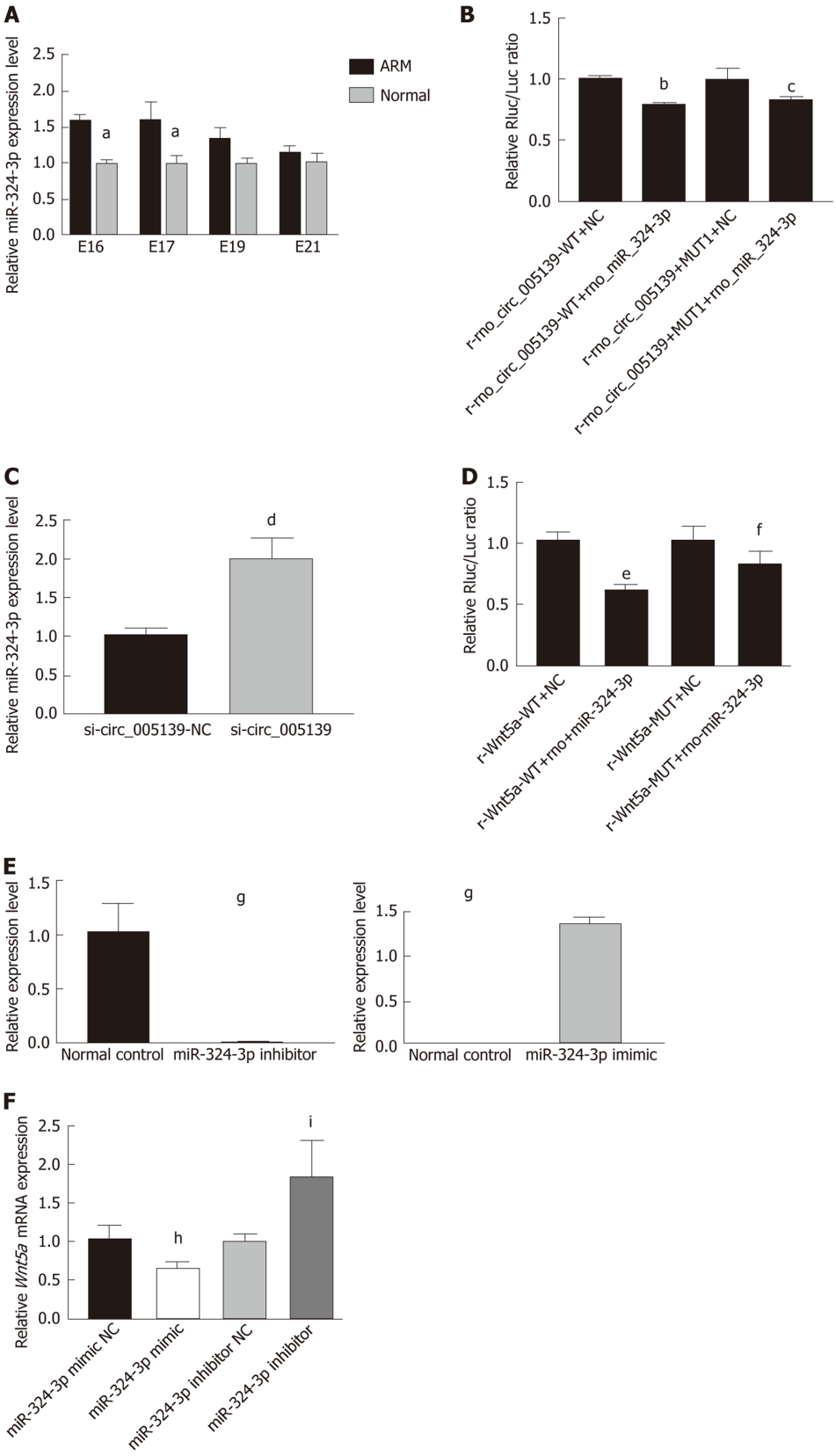



**Figure 3 Identification of mRNAs in ARM and normal anorectal tissues.** A: Cluster analysis of differentially expressed mRNAs was conducted with a heatmap on E17. In the heatmap, red and blue indicate high and low expression, respectively. The color ranges from red to blue, indicating that log10 (transcripts per million + 1) expression ranges from large to small; B: Volcano plot showing 301 up-regulated (red) and 256 down-regulated (green) circular RNAs profiled on E17 in anorectal malformations (ARM) anorectal tissues compared to normal tissues showing a two-fold change ($P < 0.05$); C: Pathway analysis was performed using Kyoto Encyclopedia of Genes and Genomes pathways. The top 10 pathways in mRNA analysis are shown; D: Three mRNAs were evaluated by quantitative reverse transcription polymerase chain reaction in ARM and normal anorectal tissues. $^{a}P < 0.05$ *vs* normal, $^{b}P < 0.01$ *vs* normal. A: Anorectal malformations; ARM: Anorectal malformations; E: Embryonic day; N: Normal; DE: Differentially expression.

pathway.


A
Relative miR-324-3p expression level
2.5
2.0
1.5
1.0
0.5
0.0
a
a
E16
E17
E19
E21
ARM
Normal
B
Relative Rluc/Luc ratio
1.5
1.0
0.5
0.0
b
c
r-rno_circ_005139-WT+NC
r-rno_circ_005139-WT+rno_miR_324-3p
r-rno_circ_005139+MUT1+NC
r-rno_circ_005139+MUT1+rno_miR_324-3p
C
Relative miR-324-3p expression level
2.5
2.0
1.5
1.0
0.5
0.0
d
si-circ_005139-NC
si-circ_005139
D
Relative Rluc/Luc ratio
1.5
1.0
0.5
0.0
e
f
r-Wnt5a-WT+NC
r-Wnt5a-WT+rno+miR-324-3p
r-Wnt5a-MUT+NC
r-Wnt5a-MUT+rno-miR-324-3p
E
Relative expression level
1.5
1.0
0.5
0.0
g
Normal control
miR-324-3p inhibitor
Relative expression level
1.5
1.0
0.5
0.0
g
Normal control
miR-324-3p imimic
F
Relative *Wnt5a* mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
h
i
miR-324-3p mimic NC
miR-324-3p mimic
miR-324-3p inhibitor NC
miR-324-3p inhibitor

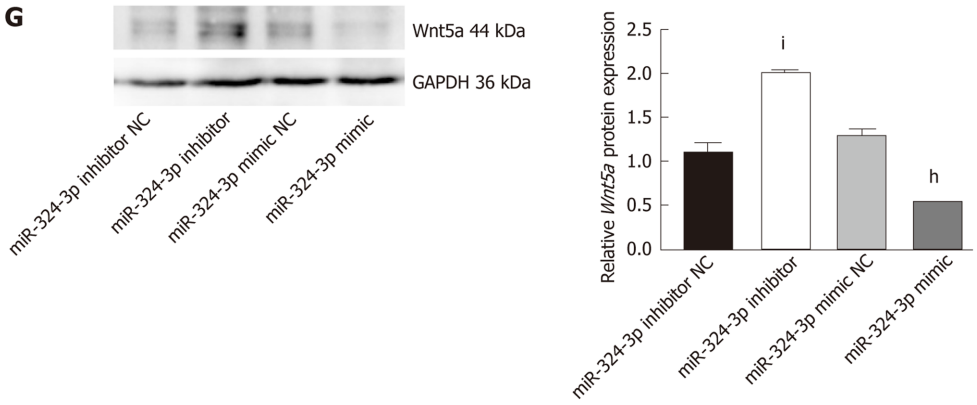



**Figure 4 Rno_circ_0005139 directly binds to miR-324-3p, a direct upstream target of *Wnt5a*.** A: Relative expression of microRNA (miR)-324-3p in anorectal malformations and normal control anorectal tissues was detected by quantitative reverse transcription polymerase chain reaction (qRT–PCR); B: Luciferase reporter assays were conducted to explore the correlation between rno_circ_0005139 and miR-324-3p; C: Expression of miR-324-3p was detected after transfection with si-rno_circ_0005139 (si-circ) and a negative control (si-NC) in IECs by qRT–PCR; D: Luciferase reporter assays were conducted to explore the correlation between miR-324-3p and *Wnt5a*; E: Expression of miR-324-3p was detected after transfection with miR-324-3p mimic, miR-324-3p inhibitor, or a negative control (mimic-NC/inhibitor-NC) by qRT–PCR. Expression of *Wnt5a* was detected after transfection with miR-324-3p mimic, miR-324-3p inhibitor, or negative control (mimic-NC/inhibitor-NC) by qRT–PCR; F and G: mRNA and protein expression levels of *Wnt5a* were detected after transfection with miR-324-3p mimic, miR-324-3p inhibitor, or negative control (mimic-NC/inhibitor-NC) by qRT–PCR and western blotting. $^{a}P < 0.05$ *vs* normal, $^{b}P < 0.01$ *vs* r-rno_circ_0005139-WT+NC, $^{c}P < 0.05$ *vs* r-rno_circ_0005139-MUT1+NC, $^{d}P < 0.05$ *vs* si-circ_0005139-NC; $^{e}P < 0.05$ *vs* r-*Wnt5a*-WT+NC, $^{f}P < 0.05$ *vs* r-*Wnt5a*-MUT+NC, $^{g}P < 0.05$ *vs* Normal control, $^{h}P < 0.05$ *vs* miR-324-3p mimic NC and $^{i}P < 0.05$ *vs* miR-324-3p inhibitor NC. ARM: Anorectal malformations; miR: microRNA.

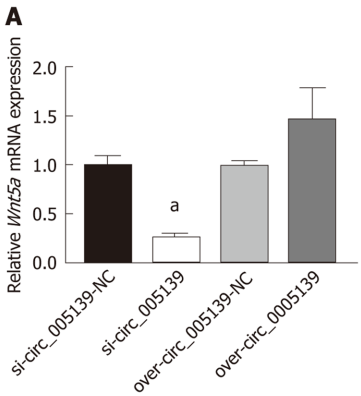
## DISCUSSION

Previous studies have indicated that the Wnt signaling pathway is involved in the formation of ARM. However, the roles of circRNAs associated competing endogenous RNAs in Wnt signaling are not well understood. In this study, we validated rno_circ_0005139 and predicted miR-324-3p as its target miRNA. We found that miR-324-3p was up-regulated, whereas rno_circ_0005139 and *Wnt5a* were down-regulated in ARM. The rno_circ_0005139-miR-324-3p-Wnt5a axis was validated by luciferase assays in rat ARM, and modulated cell proliferation and apoptosis in IECs. Our findings revealed a novel regulatory mechanism by which rno_circ_0005139 acts as a sponge for miR-324-3p to regulate the expression of *Wnt5a*. This provides a new therapeutic target for ARM diseases.

As a member of non-coding RNAs, circRNAs can bind with miRNAs to regulate the target genes expression, indicating that circRNAs regulate the occurrence and the function of diseases[19]. We identified differentially expressed circRNAs on E17, and KEGG pathway analysis showed that both the vascular endothelial growth factor and cyclic guanosine monophosphate-dependent protein kinase signaling pathways, which are involved in the process of apoptosis and migration, are related to ARM development. In our study, transfection of IECs with si-rno_circ_0005139 increased apoptosis and decreased proliferation, and our results are consistent with ARM being associated with high rates of apoptosis during anorectal development[20]. The results indicate that rno_circ_0005139 had a significant influence on IECs. We used the interference of rno_circ_0005139 in IECs to simulate decreased rno_circ_0005139 expression in ARM, which showed a crucial role of rno_circ_0005139 in ARM.

The circRNAs may act as miRNAs "sponges", which combine miRNAs to regulate the expression and function of targeting functional genes[21]. At the same time, circRNAs have been shown to regulate many diseases by interacting with mRNAs[22]. Luciferase reporter assays confirmed that down-regulated rno_circ_0005139 targets miR-324-3p, which is upregulated in ARM. And *Wnt5a* is a predicted target of up-regulated miR-324-3p. This suggests that rno_circ_0005139 acts via miR-324-3p to regulate *Wnt5a* function in ARM.

The Wnt signaling pathway is highly conserved, regulates numerous processes in tissue development, cellular metabolism, and apoptosis, and is associated with processes of urogenital and anorectal embryogenesis[23]. As an important member in Wnt family, Wnt5a has demonstrated its decreased expression in ARM group compared with the control group[24]. And the ARM is closely related to high levels of apoptosis in anorectal tissue development[25]. Thus, it is reasonable that the rno_circ_0005139/miR-324-3p/Wnt axis contributes to ARM. Transfected with si-


A
Relative Wnt5a mRNA expression
2.0
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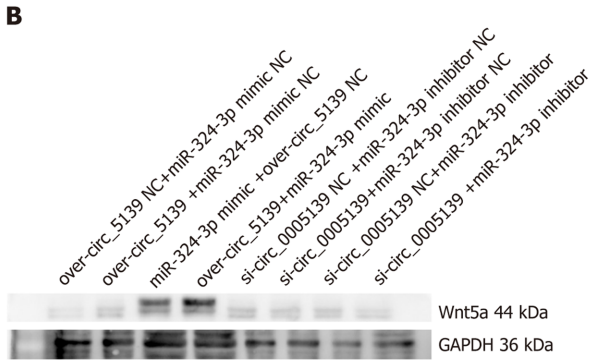
over-circ_0005139


B
over-circ_5139 NC+miR-324-3p mimic NC
over-circ_5139 +miR-324-3p mimic NC
miR-324-3p mimic +over-circ_5139 NC
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si-circ_0005139 NC +miR-324-3p inhibitor NC
si-circ_0005139+miR-324-3p inhibitor NC
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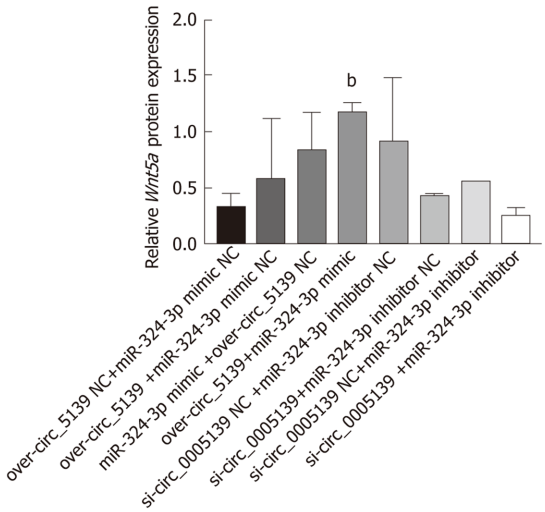
si-circ_0005139 +miR-324-3p inhibitor
Wnt5a 44 kDa
GAPDH 36 kDa


Relative Wnt5a protein expression
2.0
1.5
1.0
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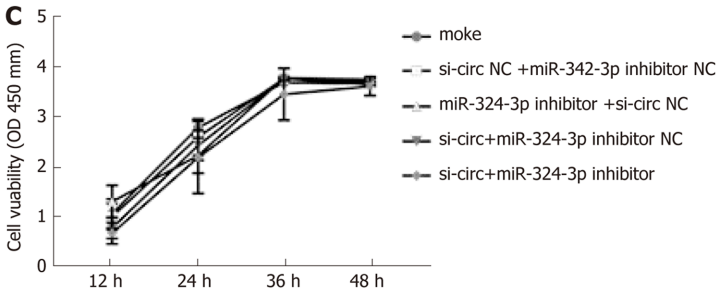
b
over-circ_5139 NC+miR-324-3p mimic NC
over-circ_5139 +miR-324-3p mimic NC
miR-324-3p mimic +over-circ_5139 NC
over-circ_5139+miR-324-3p mimic
si-circ_0005139 NC +miR-324-3p inhibitor NC
si-circ_0005139+miR-324-3p inhibitor NC
si-circ_0005139 NC+miR-324-3p inhibitor
si-circ_0005139 +miR-324-3p inhibitor


C
Cell vuability (OD 450 mm)
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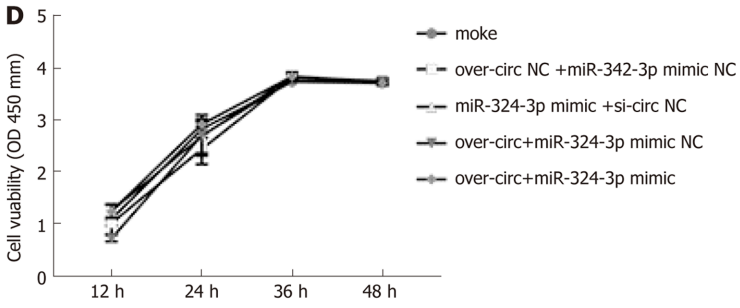
si-circ NC +miR-342-3p inhibitor NC
miR-324-3p inhibitor +si-circ NC
si-circ+miR-324-3p inhibitor NC
si-circ+miR-324-3p inhibitor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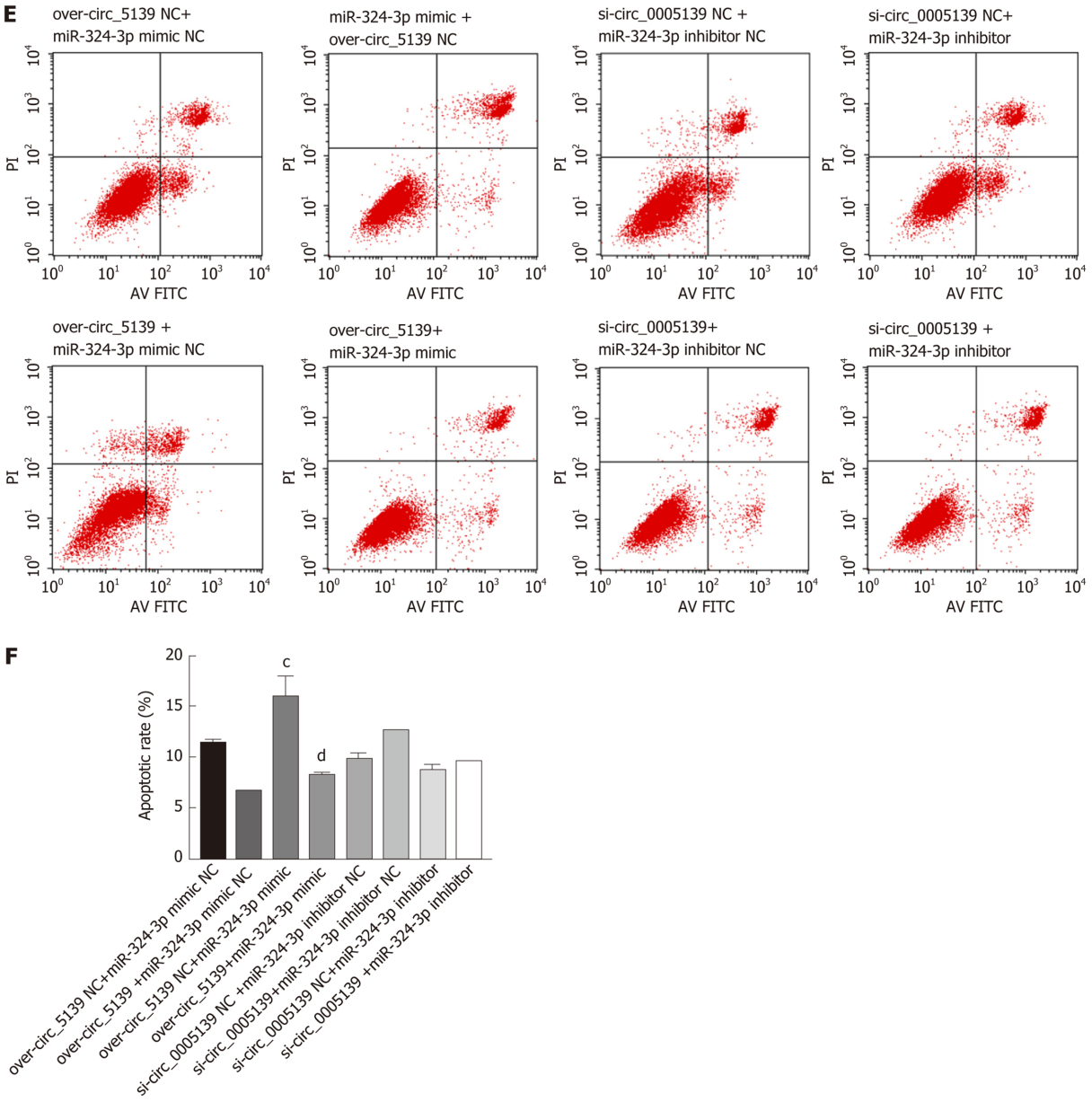


**Figure 5 Rno_circ_0005139 regulated cell proliferation and apoptosis *via* miR-324-3p/Wnt5a.** A: Relative expression of *Wnt5a* was measured by quantitative reverse transcription polymerase chain reaction with si-rno_circ_0005139 (si-circ), pLO5-rno_circ_0005139 (over-circ), or a negative control (si-NC/vector); B: *Wnt5a* expression was measured by western blotting after co-transfection with si-rno_circ_0005139 (si-circ), miR-324-3p inhibitor, or a negative control (si-NC/inhibitor-NC), and co-transfection with pLO5-rno_circ_0005139 (over-circ), miR-324-3p mimic, or a negative control (over-NC/mimic-NC); C and D: A Cell Counting Kit-8 assay was used to measure viability in intestinal epithelial cells (IECs) after co-transfection (rno_circ_0005139 and miR-324-3p); E and F: An apoptosis assay with detection by flow cytometry was used to measure apoptosis in IECs after co-transfection (rno_circ_0005139 and miR-324-3p). $^{a}P < 0.05$ *vs* si-circ_0005139-NC, $^{b}P < 0.05$ *vs* over-circ_0005139-NC+miR-324-3p mimic NC, $^{c}P < 0.01$ *vs* over-circ_0005139-NC+miR-324-3p mimic NC and $^{d}P < 0.01$ *vs* over-circ_0005139-NC+miR-324-3p mimic.

rno_circ_0005139 in IECs, higher levels of apoptosis and lower proliferation rates similar to those in ARM abnormalities were observed. However, co-transfection with the rno_circ_0005139 over-expression vector and miR-324-3p mimic attenuated these effects. Therefore, rno_circ_0005139 positively regulated the expression and function of *Wnt5a* by acting as a sponge for miR-324-3p. Additionally, E16 and E17 appear to be a critical period for the development of ARM, and rno_circ_0005139 may regulate miR-324-3p/Wnt5a mainly in the middle and late stages of anorectal development.

We further found that rno_circ_0005139 positively regulated the expression and function of *Wnt5a* by acting as a sponge for miR-324-3p. More broadly, clarifying the crosstalk between circRNA associated competing endogenous RNAs networks in Wnt signaling will provide insight into ARM development as well as new therapeutic targets.

However, our study had several potential limitations. Particularly, the

corresponding time of the rat IECs transfection moment in ARM model was unclear. Therefore, gene expression and function deviations were possible.

In conclusion, our findings improve our understanding of the regulatory roles of rno_circ_0005139, miR-324-3p, and *Wnt5a* in the pathogenesis of ARM. Rno_circ_0005139 is a potential biomarker or therapeutic target in ARM. In general, this study provides an important basis for further studies of the diagnosis, treatment, and prevention of ARM.

## ARTICLE HIGHLIGHTS

### Research background

Anorectal malformations (ARM) are common in pediatric surgery. During normal embryonic development of the anus and rectum, the Wnt signaling pathway is one of the most mature and important pathways in ARM, but its upstream regulatory mechanism is not clear. Due to its strong stability and high conservation, circular RNA (circRNA) is considered to be the most competitive microRNA (miRNA) "sponge", which competitively binds to the 3'-non-coding region of the common target gene and regulates the expression process of the target gene. circRNA is rarely reported in the research of ARM, and circRNA acts on miRNA, and then regulates the expression of Wnt signaling pathway in ARM, and affects cell proliferation and apoptosis, which are of research significance.

### Research motivation

ARM is often combined with multiple system malformations. Although they can be treated surgically, children with multiple system malformations often require long-term multidisciplinary treatment, and some children even need psychological treatment. The quality of life is poor, and the prognosis is not ideal. The mechanism of ARM is still unclear. Therefore, an in-depth study of circRNA and the upstream of the *Wnt* genes in ARM will provide a new research direction for ARM and a new approach to prenatal intervention therapy to improve the children' quality of life.

### Research objectives

To investigate differentially expressed circRNAs and mRNAs in a rat ARM model and identify the mechanism of rno_circ_0005139 targeting *Wnt5a* in proliferation and apoptosis.

### Research methods

We chose 66 pregnant Wistar rats and randomly divided them into two groups: Ethylenethiourea-induced ARM group and control group. A total of 652 embryos was harvested by cesarean delivery and anorectal tissue was taken on embryonic day 16 (E16), 17 (E17), 19 (E19) and 21 (E21). RNA sequencing and gene microarray analysis were used to identify differentially expressed circRNAs and mRNAs in ARM in a rat model. We selected 6 circRNAs and 3 mRNAs in the Wnt signal pathway from the result of the RNA sequencing and gene microarray analysis, and quantitative reverse transcription polymerase chain reaction was performed to evaluate their tissue expression. We tested the function of rno_circ_0005139 and the binding sites between rno_circ_0005139 and miR-324-3p, miR-324-3p and *Wnt5a* by luciferase assays. Co-transfection of rno_circ_0005139 and miR-324-3p was performed to verify their functional consistency.

### Research results

We found 38 upregulated and 42 downregulated circRNAs on E17, and 301 mRNAs were upregulated and 256 downregulated in the ARM on E17. We also observed that rno_circ_0006880 and rno_circ_0011386 were upregulated, whereas rno_circ_0000436, rno_circ_0005139, rno_circ_ 0009285, rno_circ_0014367, *Wnt5a*, *Wnt10b*, and *Wnt2b* were downregulated in ARM tissues. A luciferase experiment showed that rno_circ_0005139 was a sponge for miR-324-3p, which negatively regulated *Wnt5a* expression. MiR-324-3p expression was significantly higher in ARM group anorectal tissues than in normal control tissues on E17 and E19; *Wnt5a* expression showed the opposite trend. The knockdown of rno_circ_0005139 promoted cell apoptosis. In addition, a miR-324-3p inhibitor attenuated the effects of rno_circ_0005139 knockdown on ARM development and cell apoptosis.

### Research conclusions

Rno_circ_0005139 influences cell proliferation and apoptosis by acting as a miR-324-3p sponge, thereby downregulating *Wnt5a* in ARM. Accordingly, rno_ circ_0005139, miR-324-3p, and *Wnt5a* are potential therapeutic targets for ARM.

### Research perspectives

There are a large number of circRNAs in ARM, and in terms of the mechanism of rno_circ_0005139/miR-324-3p/*Wnt5a*, the specific regulatory mechanism of circRNAs is the focus of future research. In line with this, future directions should include studies exploring the diagnostic and therapeutic potential of circRNAs.

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ORIGINAL ARTICLE

Clinical and Translational Research

# Role of nutritional status and nutritional support in outcome of hepatitis B virus-associated acute-on-chronic liver failure

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**Author contributions:** Chang Y and Li H provided the ideas; Chang Y and Liu QY drafted the report; Chang Y, Zhang Q, Rong YM, Lu CZ, and Li H delivered experimental data; all authors have read and contributed to editing this manuscript and presentation of the paper.

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**Clinical trial registration statement:** This study was registered with ClinicalTrials.gov (NCT03108794 and NCT01938820).

**Informed consent statement:** All participants provided informed written consent.

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## Abstract

### *BACKGROUND*

Hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) is an important type of liver failure in Asia. There is a direct relationship between HBV-ACLF and gastrointestinal barrier function. However, the nutritional status of HBV-ACLF patients has been poorly studied.

### *AIM*

To investigate the nutritional risk and nutritional status of HBV-ACLF patients and evaluated the impact of nutritional support on the gastrointestinal barrier and 28-d mortality.

### *METHODS*

Nutritional risk screening assessment and gastrointestinal barrier biomarkers of patients with HBV-ACLF ($n$ = 234) and patients in the compensatory period of liver cirrhosis (the control group) ($n$ = 234) were compared during the period between 2016 and 2018. Changes were analyzed after nutritional support in HBV-ACLF patients. Valuable biomarkers have been explored to predict 28-d death. The 28-d survival between HBV-ACLF patients with nutritional support ($n$ = 234) or no nutritional support (2014-2016) ($n$ = 207) was compared.

**Conflict-of-interest statement:** No conflicts of interest, financial or otherwise, are declared by the authors.

**Data sharing statement:** The dataset is available from the corresponding author *via* email (haili_tj@sina.com).

**CONSORT 2010 statement:** The manuscript was prepared and revised according to the CONSORT 2010 Statement.



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*RESULTS*

The nutritional risk of the HBV-ACLF patients was significantly higher than that of the control group. The nutritional intake of the patients with HBV-ACLF was lower than that of the control group. The decrease in skeletal muscle and fat content and the deficiency of fat intake were more obvious ($P < 0.001$). The coccus-bacillus ratio, secretory immunoglobulin A, and serum D-lactate were significantly increased in HBV-ACLF patients. The survival group had a lower nutritional risk, lower D-lactate, and cytokine levels (endotoxin, tumor necrosis factor alpha, interleukin-10, and interleukin-1). Interleukin-10 may be a potential predictor of death in HBV-ACLF patients. The 28-d survival of the nutritional support group was better than that of the non-nutritional support group ($P = 0.016$).

*CONCLUSION*

Patients with HBV-ACLF have insufficient nutritional intake and high nutritional risk, and their intestinal barrier function is impaired. Individualized and dynamic nutritional support is associated with a better prognosis of 28-d mortality in HBV-ACLF patients.

**Key words:** Liver failure; Hepatitis B; Nutrition therapy; Intestinal host defense; Cytokine; Prognosis



**Core tip:** In this study, we investigated the nutritional risk, nutritional status, and the biomarkers of the gastrointestinal barrier in patients with hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF). We found poor nutritional intake and high nutritional risk, with some markers of impaired gastrointestinal barrier function, in HBV-ACLF patients. Blood cytokines, endotoxin, and D-lactate seemed potential predictors of death. Individualized and dynamic nutritional support was associated with a better prognosis of 28-d mortality. Moreover, nutritional support can also improve the gastrointestinal barrier function in survivors. Thus, nutritional status is not only the prognostic factor but also the therapeutic target in HBV-ACLF.

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## INTRODUCTION

Liver failure, in which liver cell function cannot meet the physiological needs of the human body, is a clinically common serious liver disease[1]. Globally, hepatitis B virus (HBV)-related liver failure is an important issue. Due to the high level of HBV infection in China, HBV-associated acute-on-chronic liver failure (HBV-ACLF) is a major burden as well[2]. The condition of patients with HBV-ACLF results in impaired nutrient synthesis and absorption, due to inadequate nutritional intake, abnormal liver structure, and functional failure[3]. At the same time, malnutrition will further aggravate the liver injury, affecting the quality of life and survival time[4].

At present, malnutrition in most patients with liver disease progresses slowly, so it is particularly important to screen for nutritional risk in the early stage of the disease[5,6]. Therefore, more attention should be paid to nutritional risk screening and corresponding nutritional support[7]. However, in the past, the treatment of acute-on-chronic liver failure relied too much on drugs and artificial liver support[8], ignoring basic nutritional support. In addition, the data on nutritional risk screening and nutritional status assessment in patients with liver failure is still insufficient.

The gastrointestinal tract is not only involved in digestion and absorption but also has an important barrier function[9]. In the gastrointestinal tract, there is the largest pool of bacteria in the human body. Intestinal flora plays an important role in

immunity and biological antagonism to the host[10]. The gastrointestinal tract is the central organ of the body to produce a stress response, and for the liver, it is the first organ to be impacted after the intestinal barrier is damaged[11]. The destruction of the intestinal barrier can be seen in the animal model of liver failure, indicating that liver injury will further aggravate the damage to the intestinal function[12]. In addition, intestinal endotoxin and cytokines are important causes of liver failure, that is, these findings indirectly proved that the damage of the intestinal barrier has a serious effect on the liver[13,14]. Thus far, the effect of improving nutritional status on gastrointestinal barrier function in patients with acute-on-chronic liver failure has not been determined.

As the nutritional status of HBV-ACLF patients has been poorly studied, we investigated the nutritional risk and nutritional status of HBV-ACLF patients and evaluated the impact of nutritional support on the gastrointestinal barrier and 28-d mortality in patients with HBV-ACLF.

## MATERIALS AND METHODS

### Patients

This study consisted of two parts. For Study 1, the changes in serum markers and cytokines related to nutritional risk, nutritional status, and intestinal barrier function were compared between HBV-ACLF patients and patients with compensated cirrhosis (control group). A total of 234 HBV-ACLF patients were recruited and admitted to Characteristic Medical Center of People's Armed Police Force, Tianjin Xiqing Hospital, and Tianjin Second People's Hospital from January 2016 to December 2018. All participants provided written informed consent in line with the Declaration of Helsinki. This study was registered with ClinicalTrials.gov (NCT03108794 and NCT01938820).

The inclusion criteria were: (1) HBV-ACLF diagnosis and staging according to the consensus recommendations of the Asian-Pacific Association for the Study of the Liver (APASL): Jaundice (serum bilirubin ≥ 5 mg/dL) and coagulopathy [international normalized ratio (INR) ≥ 1.5 or prothrombin activity (PTA) < 40%] complicated within four week by ascites and/or encephalopathy[15]; and (2) Hospitalization for more than 48 h. Patients with hepatocellular carcinoma or other tumors; who had undergone hepatectomy; combined with other liver diseases and unconscious; and who could not cooperate with the investigation were excluded from this study.

Propensity score matching was used to screen patients with HBV-related compensated liver cirrhosis at a ratio of 1:1. The matched patients had the same sex and age as the HBV-ACLF group. None of them were complicated with other hepatitis virus infections.

For Study 2, HBV-ACLF patients treated in the same hospital from January 2014 to December 2016 were collected retrospectively as a control group. None of these patients had undergone nutritional risk screening or nutritional status assessment. The 28-d survival of the control group was compared with HBV-ACLF patients in Study 1. The flowchart of this study is shown in Figure 1.

### Demographic and clinical characteristics

The venous blood of the subjects included in Study 1 was collected at admission for laboratory tests. Platelet (PLT) was tested using an automatic blood analyzer (Mindray BC-6800, China). The biochemical indexes alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), Total bilirubin level (TBIL), gamma-glutamyl transferase, and creatinine (Cr) were detected by biochemical analysis (Mindray BS-200, China). The coagulation function indicators prothrombin time (PT), INR, and PTA were detected with an automatic coagulation analyzer (SysmexCS5100, Japan). HBV-DNA was detected by fluorescence quantitative polymerase chain reaction (ABI 7500FAST, Life Technologies, United states). The model for end-stage liver disease (MELD) score was calculated as follows: $R = 9.6 \times loge\ (Cr, mg/dL) + 3.8 \times loge\ (TBIL, mg/dL) + 11.2 \times loge\ (INR) + 6.4$[16]. All these data were also collected or calculated for the non-nutritional support group in Study 2.

### Nutritional risk screening and nutritional status assessment

All nutritional evaluation and marker tests were performed at the time of diagnosis and 2 wk and 4 wk after treatment.

Nutritional risk screening (NRS) 2002 was used to assess the nutritional status, severity, and age of the patients. If the three scores added up to more than or equal to

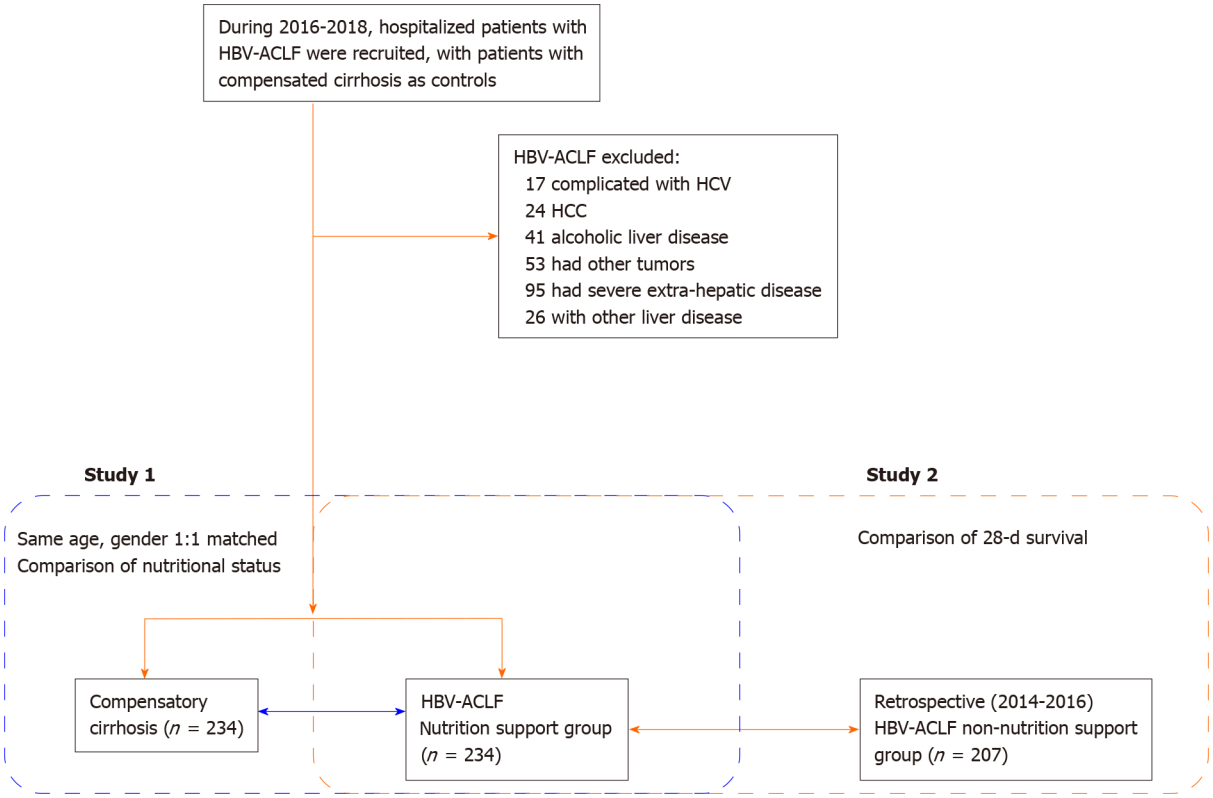


**Figure 1 Overview of this study.** HBV-ACLF: Hepatitis B virus-associated acute-on-chronic liver failure; HCV: Hepatitis C virus infection; HCC: Hepatocellular carcinoma.

3 points, the patient was considered to have a nutritional risk[17].

According to the improved subjective global assessment (SGA) score sheet, the nutritional status of the patients was evaluated by specialized medical personnel. Muscle consumption was defined when fatigue, muscle soreness, and elevated blood creatine kinase were present, and the thickness of the triceps skinfold (TSF) was evaluated. Each indicator consisted of A, B, and C corresponding to 1, 2, and 3 points, respectively. Finally, the patient's total SGA score was calculated, which consisted of 7 to 21 points. TSF was measured by two professional trainers using a fixed soft ruler and fold meter. The right hand was measured with a sebum thickness meter three times.

Human body composition was observed in the HBV-ACLF patients and the control group. According to the instructions, the body composition analyzer (Inbody 3.0, BiospaceCo., Ltd., South Korea) was used to determine the protein, fat, and skeletal muscle contents using the multifrequency bioelectrical impedance method.

### *Biomarkers of the gastrointestinal barrier*

Enzyme linked immunosorbent assay (ELISA) was used to detect secretory immunoglobulin A (sIgA) in feces (Abcam, Cambridge, UK). The coccus-bacillus ratio was measured by direct smear in the laboratory department.

### *Biomarkers of inflammation*

ELISA was used to detect D-lactate (Becton, Dickinson and Company, United states) and endotoxin (Abcam, Cambridge, UK) in serum at the time of diagnosis and 2 wk and 4 wk after treatment.

Centralized testing for cytokines was performed by Jingmei Beijing Central Laboratory. Serum interleukin-1 (IL-1), interleukin-10 (IL-10), and tumor necrosis factor alpha (TNF-α) were detected with a Luminex-100 flow fluorescence microsphere detector (Luminex, Austin, TX, United states) according to the detection procedure. The levels of IL-1, IL-10, and TNF-α were detected using a human cytokine commercial kit (Becton, Dickinson and Company, United states).

### *Nutritional intervention*

All HBV-ACLF patients had a light diet, licorice preparation, glutathione, albumin,

fresh plasma, and other drugs to protect liver cells. Artificial extracorporeal liver support was considered in critical patients[15].

In Study 1, HBV-ACLF patients were given individualized nutritional support by the nutrition support team. Nutritional support was initiated promptly for malnourished HBV-ACLF patients. Patients without malnutrition were provided with enteral nutrition (preferentially) and/or parenteral nutrition if they were viewed as unlikely to resume normal nutrition within the next 5-7 d. The nutritional composition and calories in the intestine of the patients were calculated by using the principle of small multi-meals and referring to the nutritional composition table. HBV-ACLF patients were encouraged to receive late evening oral nutritional supplementation in dietary habit. During hospitalization, total energy intake for HBV-ACLF patients was not lower than 35 kcal/(kg·d), while protein intake was not lower than 1.2-1.5 g/(kg·d)[18]. Total nutrition, carbohydrate, protein, and lipid were collected and calculated daily by 24-h dietary recalls[19]. Nutrition was supplemented by fresh plasma, albumin, glucose, and medium/long-chain fat emulsion on the second day of deficiency, while water-soluble and fat-soluble vitamins were supplemented to maintain the water-electrolyte balance. Branched-chain amino acids were supplied when adequate nitrogen intake was not achieved. If necessary, parenteral nutrition and calories were combined. The nutritional intake was re-assessed every day. According to the EASL guidelines, sodium intake was restricted to 80 mmol·d in patients with ascites[7]. Vitamin D was supplemented in patients with a vitamin D level < 20 ng/mL until serum 25 (OH) D > 30 ng/mL[18].

In Study 2, the HBV-ACLF non-nutritional support group was also treated with licorice preparation, glutathione, albumin, and fresh plasma to protect liver cells, but no individualized total intake calculation and nutritional support were performed. Only albumin and fresh plasma were given intermittently, and no long-chain fat emulsion was used.

### Statistical analysis

Statistical analyses were performed using Student's *t*-test, Welch's *t*-test, Mann-Whitney U test, chi-square test, or ANOVA, as appropriate. A receiver operating characteristic (ROC) curve was constructed using different markers to predict death in patients with HBV-ACLF. Youden's index was used to determine the best cut-off value. The prognosis of patients with HBV-ACLF in the nutritional support group and the non-nutritional support group was analyzed using the Kaplan-Meier method. All statistical analyses were performed using Statistical Product and Service Solutions version 22.0 (IBM Corp., New York State, United states) and GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United states). $P < 0.05$ was accepted as statistically significant.

## RESULTS

### Nutritional status of the two groups

According to the NRS 2002 assessment, the nutritional risk of the HBV-ACLF group at admission was significantly higher than that of the control group (214/234 *vs* 158/234, $P < 0.001$) (Figure 2A). The average energy intake [18.1 ± 5.0 kcal / (kg·d)] was significantly lower in the HBV-ACLF group than in the control group [36.6 ± 4.8 kcal/(kg·d)] ($P < 0.001$; Figure 2B). According to the results of the body composition analysis, the skeletal muscle (25.3 ± 2.4 kg *vs* 26.8 ± 2.6 kg), protein (5.9 ± 0.9 kg *vs* 7.8 ± 1.12 kg), and body fat (16.1 ± 1.3 kg *vs* 21.4 ± 3.1 kg) levels in the HBV-ACLF group were significantly lower than those of the control group, while the decrease in skeletal muscle and fat content was more pronounced (Figure 2C). At hospital admission, the intake of calories in patients with HBV-ACLF (13.9 ± 1.5 kg *vs* 26.7 ± 3.2 kg), protein (1.7 ± 0.3 kg *vs* 3.0 ± 0.3 kg), and fat (2.8 ± 0.5 kg *vs* 7.4 ± 1.2 kg) were lower than those of the control group, and the deficiency of fat intake was more obvious (Figure 2D). The baseline characteristics of the HBV-ACLF group and the control group are shown in Table 1.

The SGA scores in the HBV-ACLF group were significantly higher than those in the control group ($P < 0.001$), while the TSF in the HBV-ACLF group was lower ($P < 0.001$), indicating that there was excessive fat consumption in patients with HBV-ACLF (Figure 3).

**Table 1 Baseline characteristics of compensated cirrhosis and hepatitis B virus-associated acute-on-chronic liver failure patients enrolled in Study 1, *n* (%)**

| Characteristic | HBV-ACLF | Control | *P* value |
|---|---|---|---|
| *n* | 234 | 234 | |
| Age, yr | 46.7 ± 10.8 | 46.7 ± 10.8 | 1.000 |
| Male | 202 (86.3) | 202 (86.3) | 1.000 |
| HBeAg positive | 98 (41.9) | 118 (50.4) | 0.064 |
| HBV-DNA (log10 IU/mL) | 6.14 (1.85, 8.13) | 5.95 (1.74, 7.14) | 0.112 |
| PLT ($10^9$/L) | 120.2 ± 49.1 | 109.6 ± 43.7 | 0.014 |
| ALT (IU/L) | 680 (421, 1191) | 53 (37,114) | < 0.001 |
| AST (IU/L) | 592 (313, 980) | 41 (23,60) | < 0.001 |
| ALB (g/L) | 33.6 ± 4.9 | 39.9 ± 6.8 | < 0.001 |
| TBIL (μmol/L) | 274.1 (166.1, 588.7) | 17.3 (12.8, 22.5) | < 0.001 |
| PTA (%) | 34 (14, 40) | 59 (43, 119) | < 0.001 |
| INR | 2.46 ± 0.18 | 1.13 ± 0.09 | < 0.001 |
| BUN (mmol/L) | 6.1 (3.1, 8.7) | 4.8 (3.4, 7.2) | 0.001 |
| Cr (μmol/L) | 73 (66, 104) | 68 (51, 89) | 0.012 |
| SBP | 124 (53.0) | 0 | < 0.001 |
| Hepatic encephalopathy | 97 (41.5) | 0 | < 0.001 |
| Hepatorenal syndrome | 24 (10.3) | 0 | < 0.001 |
| MELD score | 25.4 ± 4.8 | 8.2 ± 3.2 | < 0.001 |

Data are presented as the mean ± SD, median (IQR), or *n* (%). ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatinine; HBeAg: Hepatitis B e antigen; HBV-ACLF: hepatitis B virus-associated acute-on-chronic liver failure; INR: International normalized ratio; SBP: Spontaneous bacterial peritonitis; MELD: Model for end-stage liver disease; PLT: Platelet; PTA: Prothrombin activity; TBIL: Total bilirubin level.

### *Difference in biomarkers of the gastrointestinal barrier and inflammation*

Compared with the control group, the coccus-bacillus ratio in feces, sIgA in stool, and serum D-lactate were significantly higher in the HBV-ACLF group (Figure 4A-C), which indicated that the intestinal barrier was impaired in HBV-ACLF patients.

For biomarkers of inflammation, the levels of serum endotoxin, IL-1, IL-10, and TNF-α in the HBV-ACLF group were significantly higher than those of the control group (Figure 4D).

### *Nutritional support and prognosis of HBV associated acute-on-chronic liver failure*

According to the survival conditions of the patients at 2 d, the HBV-ACLF patients were divided into a survival group and a death group. The survival group had a lower nutritional risk (SAG scores) ($P < 0.001$), lower serum D-lactate ($P = 0.007$), and lower levels of cytokines ($P < 0.05$) at admission (Table 2). The differences in biomarkers were used to establish an ROC for predicting 28 d mortality. IL-10 had the best result in predicting the death of HBV-ACLF patients (Figure 5A). The cutoff value was 80.5 μg/mL. Other detailed results of ROC are shown in Supplemental Table 1.

After individualized nutritional support for HBV-ACLF patients, the TSF of survivors did not continue to decrease ($P = 0.673$). By week 4, the SGA score was significantly improved ($P < 0.001$). There was no significant change in the coccus-bacillus ratio, but the levels of serum D-lactate ($P < 0.001$) and fecal sIgA ($P < 0.001$) decreased gradually. Serum endotoxin ($P = 0.021$), TNF-α ($P < 0.001$), and IL-1 ($P < 0.001$) decreased gradually as well, but IL-10 had no significant change ($P = 0.402$) (Figure 5B).

In Study 2, the non-nutritional support group had similar age, liver function, renal function, complications, and other clinical characteristics to the nutritional support group ($P < 0.05$; Table 3). A total of 91 patients (38.9 %) patients died within 28 d in the HBV-ACLF nutritional support group and 102 (49.3%) died in the non-nutritional

**Table 2 Characteristics of nutritional and gastrointestinal barrier between survival group and death group in hepatitis B virus-associated acute-on-chronic liver failure patients at admission, *n* (%)**

| Characteristic | Survival group | Death group | *P* value |
|---|---|---|---|
| *n* | 143 | 91 | |
| Male | 98 (68.53) | 63 (69.23) | 0.910 |
| TSF (mm) | 17.1 ± 5.5 | 16.5 ± 5.7 | 0.391 |
| SGA score | 18.1 ± 2.7 | 19.8 ± 2.7 | < 0.001 |
| Fecal cocci-bacillus ratio | 1.2 ± 0.2 | 1.3 ± 0.3 | 0.342 |
| D-lactate (μg/mL) | 13.2 ± 4.0 | 14.6 ± 3.8 | 0.007 |
| sIgA (mg/L) | 341.7 ± 95.7 | 371.0 ± 97.9 | 0.024 |
| Endotoxin (U/mL) | 518.0 ± 162.6 | 627.4 ± 179.1 | < 0.001 |
| IL-1 (μg/mL) | 86.1 ± 48.5 | 126.8 ± 50.9 | < 0.001 |
| IL-10 (μg/mL) | 39 (24, 87) | 147 (59, 246) | < 0.001 |
| TNF-α (pg/mL) | 53 (21, 104) | 72 (46, 147) | < 0.001 |

Data presented as mean ± SD or median (IQR). IL-1: Interleukin-1; IL-10: Interleukin-10; SGA: Subjective global assessment; SIgA: Secretory immunoglobulin A; TSF: Triceps skin fold; TNF-α: Tumor necrosis factor alpha.

support group. There was a difference in 28-d survival between the two groups ($\chi^2$ = 5.812, $P$ = 0.016; Figure 6).

## DISCUSSION

This study comprehensively assessed the nutritional status, nutritional risk, and gastrointestinal barrier function in patients with HBV-ACLF. We found that improving the nutritional status of HBV-ACLF patients can improve gastrointestinal barrier function and associate with a better prognosis.

We found that most patients with HBV-ACLF had inadequate nutrient intake and malnutrition at admission or diagnosis. Clinical nutritionists believe that patients with liver cirrhosis should be given nutritional treatment when there is a nutritional risk[20,21]. Thus, the determination of the nutritional status and various nutritional components of the patients at the time of diagnosis or admission is necessary. On this basis, an individualized nutritional support treatment plan has been developed for each patient to meet the needs of the human body and promote the recovery from the disease[7,18].

We further verified malnutrition in patients with HBV-ACLF by comparing TSF and SGA score. Although anthropometric indexes such as TSF have limitations, they are not affected by ascitic or other factors. Due to their ease of use, anthropometric indexes are used as the evaluation criteria for nutritional status in many studies of malnutrition in liver diseases[22]. However, the time lag is certain regardless of what type of physiologic parameters. In contrast, SGA is a comprehensive index to reflect the nutritional status, which is widely used in the field of liver disease and recommended by the European Association of Parenteral Nutrition for comprehensive nutritional assessment[18]. In addition, Kyle *et al*[23] screened 995 patients and found that NRS 2002 had higher sensitivity and specificity. NRS 2002 and SGA scores were both used in our study.

The presence of gastrointestinal barrier impairment in patients with HBV-ACLF is also one of our main findings. Gastrointestinal function and liver function are closely related. Gastrointestinal dysfunction can occur in patients with liver failure in the early stage. At the same time, portal hypertensive gastropathy, portal hypertensive enteropathy, increased abdominal pressure caused by ascites, and intestinal flora disorders, which are the results of liver failure, are also the causes of malnutrition in patients[24]. Finally, a vicious circle is formed, which further affects the function of the gastrointestinal barrier.

Moreover, this study confirmed that the level of serum D-lactate in patients with chronic liver failure was significantly increased. When the barrier function of the

**Table 3 Baseline characteristics of hepatitis B virus-associated acute-on-chronic liver failure patients enrolled in Study 2, *n* (%)**

| Characteristic | Nutritional support | None-nutritional support | *P* value |
|---|---|---|---|
| *n* | 234 | 207 | |
| Age (yr) | 46.7 ± 10.8 | 47.5 ± 11.6 | 0.454 |
| Male | 202 (86.3) | 172 (83.9) | 0.345 |
| HBeAg positive | 98 (41.9) | 96 (46.4) | 0.342 |
| HBV-DNA ($log_{10}$ IU/mL) | 6.14 (1.85, 8.13) | 6.54 (1.53, 8.71) | 0.097 |
| PLT ($10^9$/L) | 120.2 ± 49.1 | 112.6 ± 52.6 | 0.117 |
| ALT (IU/L) | 680 (421, 1191) | 652 (447, 1352) | 0.243 |
| AST (IU/L) | 592 (313, 980) | 613 (320, 1014) | 0.381 |
| ALB (g/L) | 33.6 ± 4.9 | 34.1 ± 5.2 | 0.299 |
| TBIL (μmol/L) | 274.1 (166.1, 588.7) | 291.5 (162.5, 604.3) | 0.227 |
| PTA (%) | 34 (14, 40) | 32 (11, 40) | 0.135 |
| INR | 2.46 ± 0.18 | 2.49 ± 0.21 | 0.107 |
| BUN (mmol/L) | 6.1 (3.1, 8.7) | 6.4 (3.2, 9.2) | 0.295 |
| Cr (μmol/L) | 73 (66, 104) | 76 (59, 98) | 0.530 |
| SBP | 124 (53.0) | 106 (51.2) | 0.708 |
| Hepatic encephalopathy | 97 (41.5) | 82 (39.6) | 0.695 |
| Hepatorenal syndrome | 24 (10.3) | 19 (9.2) | 0.703 |
| MELD scores | 25.4 ± 4.8 | 26.1 ± 5.2 | 0.142 |
| Artificial extracorporeal liver support | 141 (60.3) | 124 (59.9) | 0.940 |

Data presented as mean ± SD, median (IQR) or *n* (%). ALB: Albumin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatinine; HBeAg: Hepatitis B e antigen; INR: International normalized ratio; SBP: Spontaneous bacterial peritonitis; MELD: Model for end-stage liver disease; PLT: Platelet; PTA: Prothrombin activity; TBIL: Total bilirubin level.

gastrointestinal tract is impaired, intestinal bacteria show excess propagation or intestinal flora translocation occurs, and the production and absorption of endotoxin increase, which further leads to damage to the liver and other organs[25]. Endotoxin has a variety of physiological and pathological effects, such as causing a febrile reaction, activating the complement system, and causing local or systemic Shwartzman reactions and disseminated intravascular coagulation[26]. In addition to the direct effect of endotoxin, the toxic mechanism can also induce the cascade release of cytokines, strongly increase tumor necrosis factor, result in an Th1/Th2 imbalance, and further aggravate the disease and cause death[27]. D-lactate is the product of bacterial metabolism and lysis, which can be produced by a variety of intestinal bacteria[28]. When intestinal permeability increases abnormally, high levels of D-lactate produced by intestinal bacteria enter the circulation through the intestine. The liver cannot metabolize D-lactate, so the level of serum D-lactate can reflect the extent of the impaired gastrointestinal barrier[29].

In addition to the intestinal mechanical barrier and biological barrier, there are many plasma cells in the lamina propria of the intestinal mucosa, which mainly secrete sIgA. Intestinal sIgA plays an important role in maintaining intestinal immune surveillance, clearing bacteria, and preventing bacteria from adhering to the mucosa[30]. The detection of intestinal mucosa sIgA content requires intestinal mucosa biopsies for radioimmunity, which is not applicable for patients with HBV-ACLF[31]. Thus, sIgA was measured in the feces. In Study 1, the TSF of the surviving patients did not continue to decrease, indicating that the nutritional status of the patients did not deteriorate further. The further decreases of biomarkers of impaired gastrointestinal function and inflammation in survival were also found after nutritional support. Furthermore, we found that IL-10 is a potential predictor of 28-d death.

In Study 2, a retrospective cohort, we demonstrated that individualized and dynamic nutritional support reduced 28-d mortality. In practice, we found that intravenous infusion of albumin and medium/long-chain fat emulsion can be well

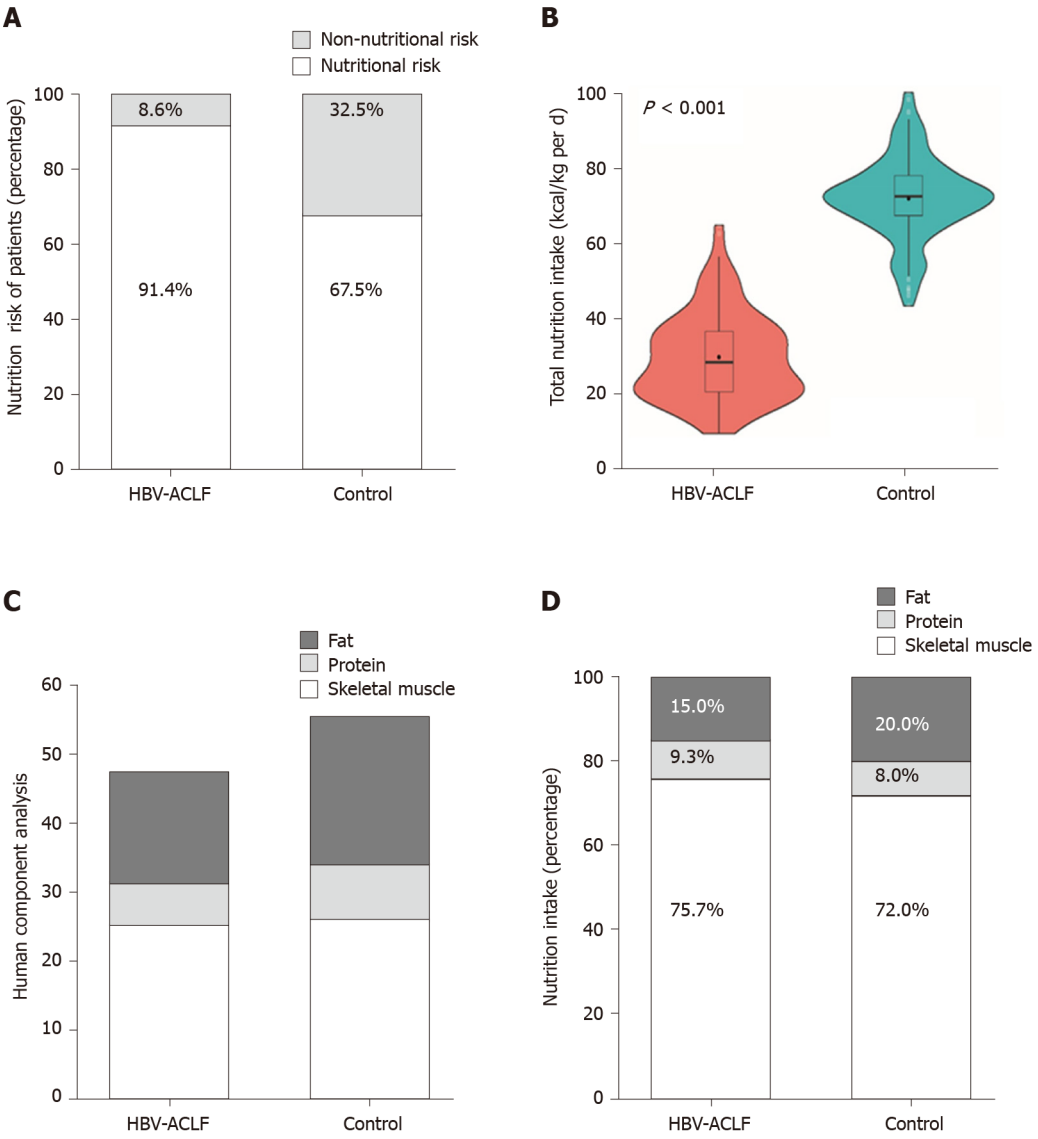


**Figure 2 Comparison of nutritional status between the hepatitis B virus-associated acute-on-chronic liver failure group and the control group.** A: According to the Nutritional risk screening 2002 assessment, 91.4% of hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) patients and 67.5% of compensated cirrhosis patients had a nutritional risk; B: The energy intake in HBV-ACLF patients was lower than that in compensated cirrhosis patients ($P < 0.001$); C: The body composition analysis in HBV-ACLF and control group. Significant decreases in skeletal muscle and fat in HBV-ACLF patients were observed; D: The intake of calories, protein, and fat in HBV-ACLF were lower than those in the control group at hospital admission ($P < 0.001$). HBV-ACLF: Hepatitis B virus-associated acute-on-chronic liver failure.

tolerated in HBV-ACLF patients. Protein and fat are the dominant source of energy for liver regeneration and hepatocyte turnover. So protein and fat should not be overly restricted in the diet. The administration of parenteral nutrition, including fat emulsion, did not worsen liver function. However, Pierce *et al*[32] noted that too much fat increases the burden on the liver in mice. This change leads to or aggravates jaundice, increases transaminase and blood glucose, and decreases immune function. Therefore, the supplemental dose of fat should be limited to < 1 g/(kg·d)[33].

There are some limitations and shortcomings in this study: (1) There are some differences in the definition of acute-on-chronic liver failure among the American Association for the Study of the Liver Diseases, the European Association for the Study of the Liver, and APASL[15]. The subjects were selected according to the consensus recommendations of the APASL; (2) The non-nutritional support group with liver failure was from a retrospective cohort, which may not reflect the situation of all hospitalized patients at the same time; and (3) Some biomarker tests for predicting death in HBV-ACLF patients were based on ELISA, and this result may be affected by the manufacturers of test reagents and measurement errors.

Overall, we performed the nutritional assessment of HBV-ACLF patients for the first time and found that their intestinal peristaltic ability decreased and the intestinal

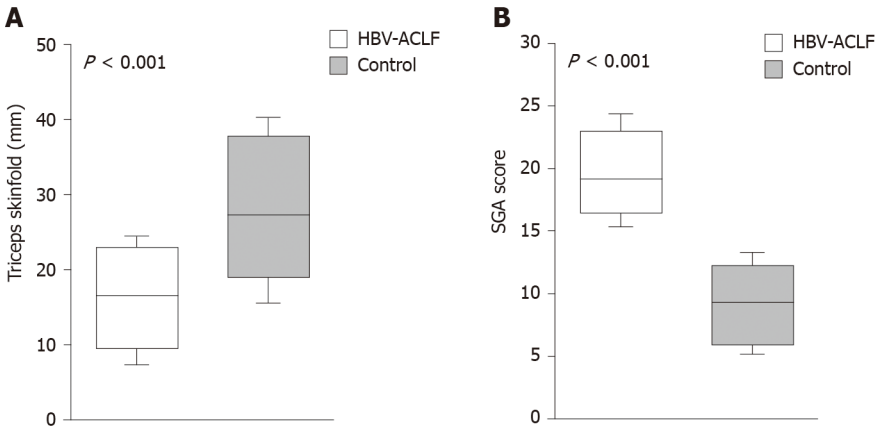


**Figure 3 Difference in triceps skinfold thickness and subjective global assessment score.** A: Patients with hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) had a higher subjective global assessment score; B: Patients with HBV-ACLF had a lower triceps skinfold thickness. SGA: Subjective global assessment; HBV-ACLF: Hepatitis B virus-associated acute-on-chronic liver failure.

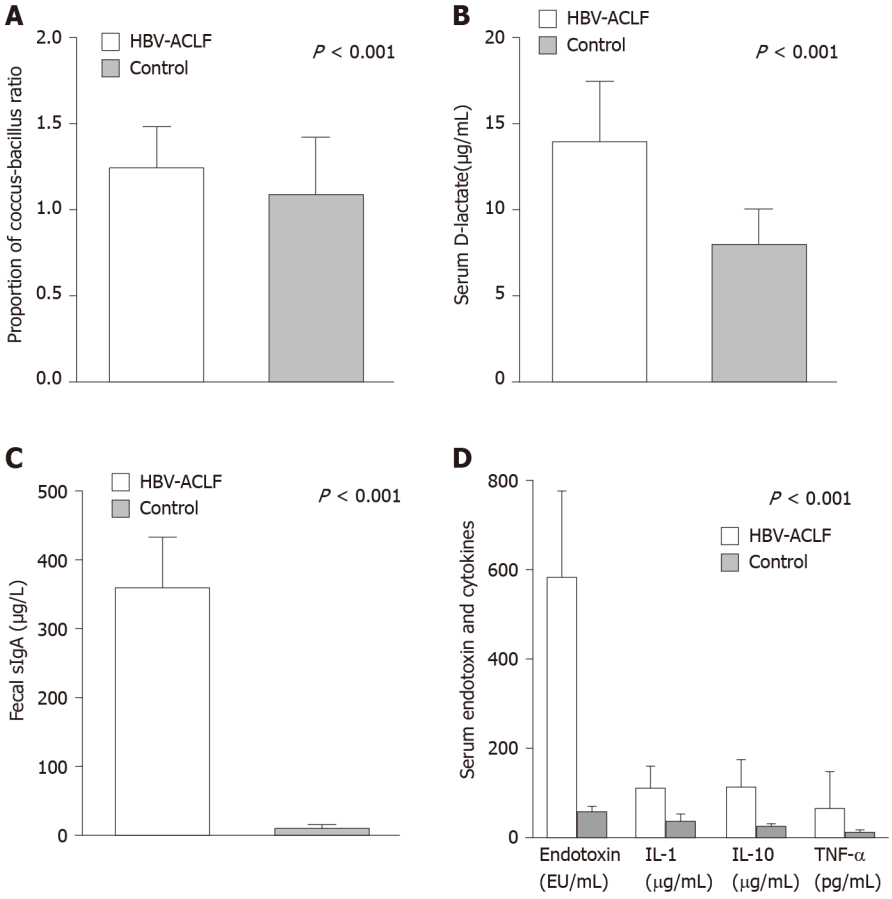


**Figure 4 Comparison of biomarkers of the gastrointestinal barrier and inflammation.** A: The coccus-bacillus ratio in feces in the hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) group (1.2 ± 0.2) was higher than that of the control group (1.1 ± 0.3; $P < 0.001$); B: The level of serum D-lactate in the HBV-ACLF group (358.1 ± 74.6 μg/mL) was higher than that of the control group (8.0 ± 2.1 μg/mL; $P < 0.001$); C: The level of sIgA in the HBV-ACLF group (358.1 ± 74.6 mg/L) was higher than that of the control group (12.5 ± 4.2) mg/L; $P < 0.001$); D: The levels of endotoxin, IL-1, IL-10, and TNF-α in HBV-ACLF patients were higher than those in the control group ($P < 0.001$). HBV-ACLF: Hepatitis B virus-associated acute-on-chronic liver failure; IL-1: Interleukin-1; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor alpha.

barrier function was damaged. In addition, individualized and dynamic nutritional support was associated with a better prognosis of 28-d mortality in HBV-ACLF patients and can improve the nutritional status of survivors.

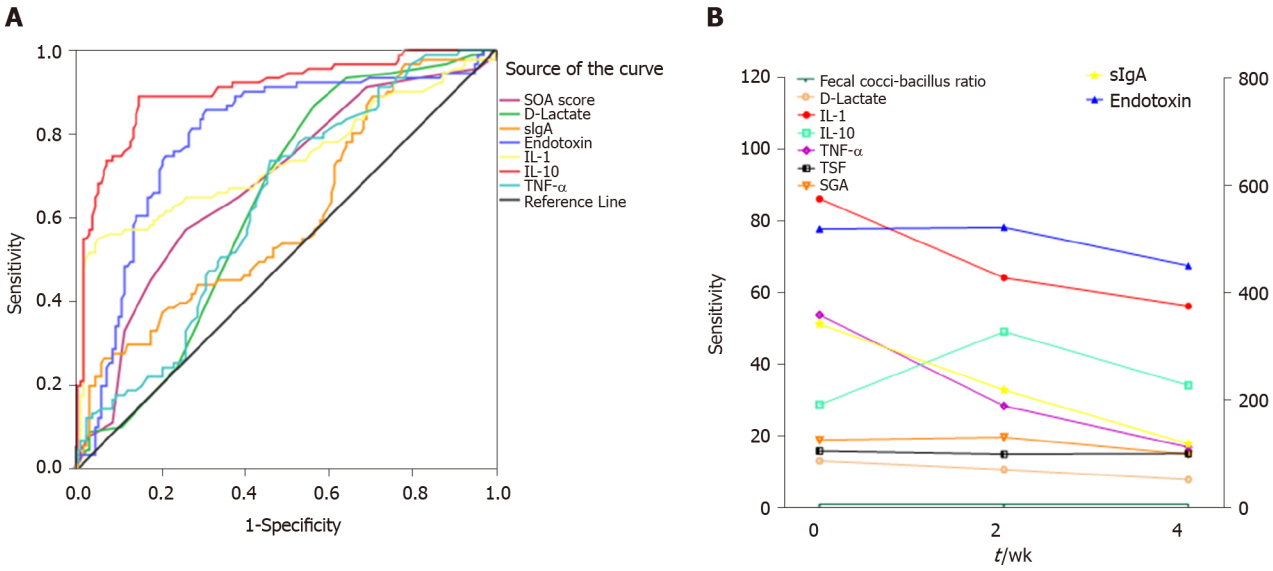


**Figure 5 Role of biomarkers in hepatitis B virus-associated acute-on-chronic liver failure patients.** A: Value of biomarkers in predicting death in HBV-ACLF patients. The area under the receiver operating characteristic curve of IL-10 was the largest (area under the curve = 0.907, $P < 0.001$); B: Trends of biomarkers in survivors. HBV-ACLF: Hepatitis B virus-associated acute-on-chronic liver failure; SGA: Subjective global assessment; SIgA: Secretory immunoglobulin A; IL-1: Interleukin-1; IL-10: Interleukin-10; TNF-α: Tumor necrosis factor alpha.

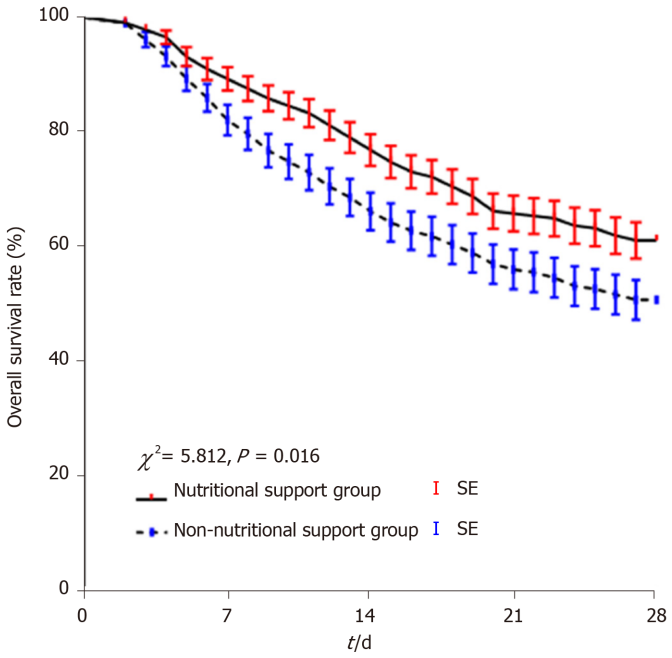


**Figure 6 Comparison of 28-d mortality between the nutritional support group and the non-nutritional support group (Kaplan-Meier method).** The hepatitis B virus-associated acute-on-chronic liver failure nutrition support group had a better prognosis than the non-nutrition support group ($P$ = 0.016).

## ARTICLE HIGHLIGHTS

### *Research background*

Patients with hepatitis B virus-associated acute-on-chronic liver failure (HBV-ACLF) have inadequate nutrient intake and malnutrition at admission or diagnosis. However, the nutritional status of HBV-ACLF patients has been poorly studied.

### *Research motivation*

We have found that HBV-ACLF patients had inadequate nutrient intake and malnutrition. Little is known about the impact of nutritional support on the outcome

and function of the gastrointestinal barrier.

### Research objectives

To investigate the nutritional risk and nutritional status of HBV-ACLF patients and evaluated the impact of nutritional support on the gastrointestinal barrier and 28-d mortality.

### Research methods

The nutritional risk screening assessment, baseline characteristics, and biomarkers of the gastrointestinal barrier of patients with HBV-ACLF ($n$ = 234) and patients with compensatory cirrhosis ($n$ = 234) were analyzed. The 28-d survival of the nutritional support group ($n$ = 234) and non-nutritional support group ($n$ = 207) was compared.

### Research results

The nutritional risk of the HBV-ACLF patients was significantly higher than that of the control group. The coccus-bacillus ratio, secretory immunoglobulin A, and serum D-lactate were significantly increased in HBV-ACLF patients. Interleukin-10 may be a potential predictor of death in HBV-ACLF patients. The 28-d survival of the nutritional support group was better than that of the non-nutritional support group ($P$ = 0.016).

### Research conclusions

This is the first study to investigate the nutritional risk and status of HBV-ACLF patients and evaluated the impact of nutritional support. We found that nutritional support initiated promptly for malnourished HBV-ACLF patients was associated with a better prognosis of 28-d mortality and improved the status of the gastrointestinal barrier.

### Research perspectives

More attention should be paid to nutritional risk screening and corresponding nutritional support for HBV-ACLF patients. Nutritional support initiated promptly for malnourished HBV-ACLF patients is associated with a better prognosis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Evaluation of intrahepatic manifestation and distant extrahepatic disease in alveolar echinococcosis

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**Author contributions:** The plan for this manuscript was developed during a personal XUUB meeting of the authors; the data were evaluated by the authors at a meeting in Besançon; all authors were involved in the interpretation of the results; Graeter T and Bao HH contributed equally to this work; Graeter T, Bao HH, Kratzer W, Delabrousse E and Liu WY prepared the first draft; the statistical analysis was performed by Schmidberger J, Brumpt E, Shi R, Li WX and Jiang Y; all partners read, amended, and approved the final version of the manuscript.

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## Abstract

### *BACKGROUND*

The main endemic areas of alveolar echinococcosis (AE) are in Central Europe and Western China. Both the infiltration of intrahepatic vascular and bile duct structures as well as extrahepatic disease can lead to further complications and may increase morbidity in patients with AE.

### *AIM*

To evaluate vascular/biliary involvement in hepatic AE and its distant extrahepatic disease manifestations in an international collective was the aim.

### *METHODS*

Consecutively, five experienced examiners evaluated contrast-enhanced abdominal computed tomography (CT) scans for 200 patients with hepatic AE of each of four locations ($n$ = 50) in Germany, France and China. Therefore, we retrospectively included the 50 most recent abdominal contrast-enhanced CT

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**Institutional review board statement:** The study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki (ref. No. 409/15). Because of its retrospective design and pseudonymized evaluation of imaging, no ethics approval was necessary for France and China. All data were analyzed anonymously.

**Informed consent statement:** Because of the retrospective and anonymous character of this study the need for informed consent was waived by the institutional review board.

**Conflict-of-interest statement:** The authors declare that they have no competing interests.

**Data sharing statement:** The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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examinations at each center, performed because of hepatic AE from September 21, 2007 to March 21, 2018. AE liver lesions were classified according to the echinococcosis multilocularis Ulm classification for CT (EMUC-CT). Distant extrahepatic manifestations were documented either by whole body positron emission tomography–CT or with the addition of thoracic CT and cranial magnetic resonance imaging. Vascular/biliary involvement of the hepatic disease as well as the presence of distant extrahepatic manifestations were correlated with the EMUC-CT types of liver lesion. Statistical analysis was performed using SAS Version 9.4 (SAS Institute Inc., Cary, NC, United States).

*RESULTS*

Distant extrahepatic AE manifestations were significantly more frequent in China than in Europe ($P$ = 0.0091). A significant relationship was found between the presence of distant extrahepatic disease and AE liver lesion size ($P$ = 0.0075). Vascular/biliary structures were involved by the liver lesions significantly more frequently in China than in Europe ($P$ < 0.0001), and vascular/biliary involvement depended on lesion size. Different morphological types of AE liver lesions led to varying frequencies of vascular/biliary involvement and were associated with different frequencies of distant extrahepatic manifestations: Vascular/biliary involvement as a function of lesions primary morphology ranged from 5.88% of type IV liver lesions to 100% among type III lesions. Type IV differed significantly in these associations from types I, II, and III ($P$ < 0.0001). With respect to extrahepatic disease, the primary morphology types IV and V of liver lesions were not associated with any case of distant extrahepatic disease. In contrast, distant extrahepatic manifestations in types I–III were found to varying degrees, with a maximum of 22% for type III.

*CONCLUSION*

Different CT morphological patterns of hepatic AE lesions influence vascular/biliary involvement and the occurrence of distant extrahepatic manifestations. There are intercontinental differences regarding the characteristics of AE manifestation.

**Key Words:** Alveolar echinococcosis; Echinococcus multilocularis; Echinococcus multilocularis Ulm classification for computed tomography; Vascular/biliary involvement; Extrahepatic manifestation; XUUB project



**Core tip:** This study demonstrates for the first time how different computed tomography morphological types of liver lesions in alveolar echinococcosis (AE) affect the intrahepatic involvement pattern as well as distant extrahepatic disease manifestations. The disease shows different characteristics in an intercontinental comparison between Europe and China. These results may provide information about the behavior of this disease during its initial manifestation and its progression. A morphological classification of AE liver lesions seems therefore not only useful in order to facilitate the initial differential diagnosis but also indicates a direct clinical impact.

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## INTRODUCTION

Human alveolar echinococcosis (AE) is a rare malignant parasitic disease that results

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from infection with the larval stage of echinococcus multilocularis[1]. Because of the frequently exogenous tumor-like proliferation and destructive growth, AE resembles a malignant tumor in its behavior and appearance and can lead to infiltration of the affected organs and even to severe disease and death[2]. AE has become a serious global problem, occurring in moderate to cold climate zones in the northern hemispheres and being prevalent particularly across central Europe, a large part of north and central Asia, and some parts of North America[3].

Imaging tools such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and 18F fluorodeoxyglucose (FDG)-positron emission tomography (PET) are used to diagnose AE lesions, combined with results of immunodiagnosis (specific serology) and epidemiological findings[4-8]. CT scans can reveal the shape, number, size, and location of lesions more accurately than can ultrasound and also demonstrate the typical calcifications most clearly[9]. MRI best captures the structural alveolar characteristics in AE. Combined with CT or MRI, FDG-PET can be used to evaluate the local inflammatory activity induced by the lesion. Absence of metabolic activity, however, does not necessarily mean that the parasite is nonviable and may indicate suppressed immune defenses[10]. Despite abundant clinical resources and technical advances, the diagnosis of AE in infected individuals remains challenging, especially among inexperienced clinicians. With delayed diagnosis, therapy often begins in a late stage of the disease, which significantly limits treatment options[11].

The liver usually is the first organ affected by larval infestation. A manifestation outside the liver without liver involvement is rare[12]. Hepatic AE (HAE) can affect intrahepatic blood vessels and bile ducts. Especially with involvement of such structures in the hilum, a radical resection is difficult or impossible. In the literature (cases and small series), hepatobiliary complications in AE are reported with an incidence of 10%–30%[13-17]. Vascular complications include Budd–Chiari and vena cava syndromes, which are caused by occlusions of the hepatic veins or inferior vena cava, respectively[18-21]. Hepatobiliary complications represent a turning point in HAE and are crucial for the further clinical course of the disease[11,13].

This multicenter study was based in two Chinese and two European university clinics that are international leaders in research and in treatment of AE. These centers are located in AE-endemic areas and established a research cooperation in 2016 to carry out the Xining-Urumqi-Ulm-Besançon (XUUB) imaging project. Xining is the regional capital of Qinghai province in central China, and Urumqi is the capital of the Uyghur autonomous region of Xinjiang in northwest China. Ulm is situated in the state of Baden-Württemberg in southwest Germany. Besançon is a city in the Franche-Comté region of eastern France (Figure 1).

The aim of the study was to assess the vascular/biliary involvement and the distant extrahepatic disease manifestations of the different cases in a collective of 200 German, French, and Chinese patients with HAE.

## MATERIAL AND METHODS

### *Ethics statement*

For German patients, the study was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki (ref. No. 409/15). Because of its retrospective design and pseudonymized evaluation of imaging, no ethics approval was necessary for France and China. All data were analyzed anonymously.

### *Inclusion and exclusion criteria*

The following inclusion criteria were defined. Retrospectively, we included the 50 most recent abdominal contrast-enhanced CT examinations at each center ($n$ = 200), performed because of hepatic AE from 09/21/07 to 03/21/18. The number of cases was estimated after consultation with the respective countries involved and the number of CT examinations in recent months. The previous clinical and imaging morphological findings had to have been classified as confirmed AE according to Brunetti *et al*.'s case definition[2]. Antibody status, possible subsequent therapeutic strategies, and socioeconomic factors were not considered in the inclusion criteria.

### *Examination and classification*

The echinococcus multilocularis Ulm classification for CT (EMUC-CT) provides a scheme for classifying the very different morphological appearances of HAE lesions (Table 1)[5]. The classification of all HAE cases according to the EMUC-CT was carried out by the first reader from April 09, 2018 to April 14, 2018. Only venous-phase CT

**Table 1 Overview of the echinococcosis multilocularis Ulm classification for computed tomography**

| Primary morphology *(Subcriteria)* | Pattern of calcification |
|---|---|
| Type I, Diffuse infiltrating (with cystoid portion / without cystoid portion) | Without calcifications |
| Type II, Primarily circumscribed tumor-like (with cystoid portion / without cystoid portion) | With feathery calcifications |
| Type III, Primarily cystoid - intermediate (IIIa), widespread (IIIb) - (with more solid portions at the edge / without more solid portions at the edge) | With focal calcifications |
| | With diffuse calcifications |
| Type IV, Small-cystoid/metastasis-like | With calcifications primarily at the edge |
| Type V, Mainly calcified | With a central calcification |

The two pillars of the classification (Primary morphology and Pattern of calcification) are considered separately in the first instance and then, in principle, can be freely combined. There are two exceptions: the pattern "with a central calcification" can occur only with type IV primary morphology, while type V is not further characterized by a specific pattern of calcification. The focus for further evaluations in this study was on the determination of the main pillar of the classification, the primary morphology.

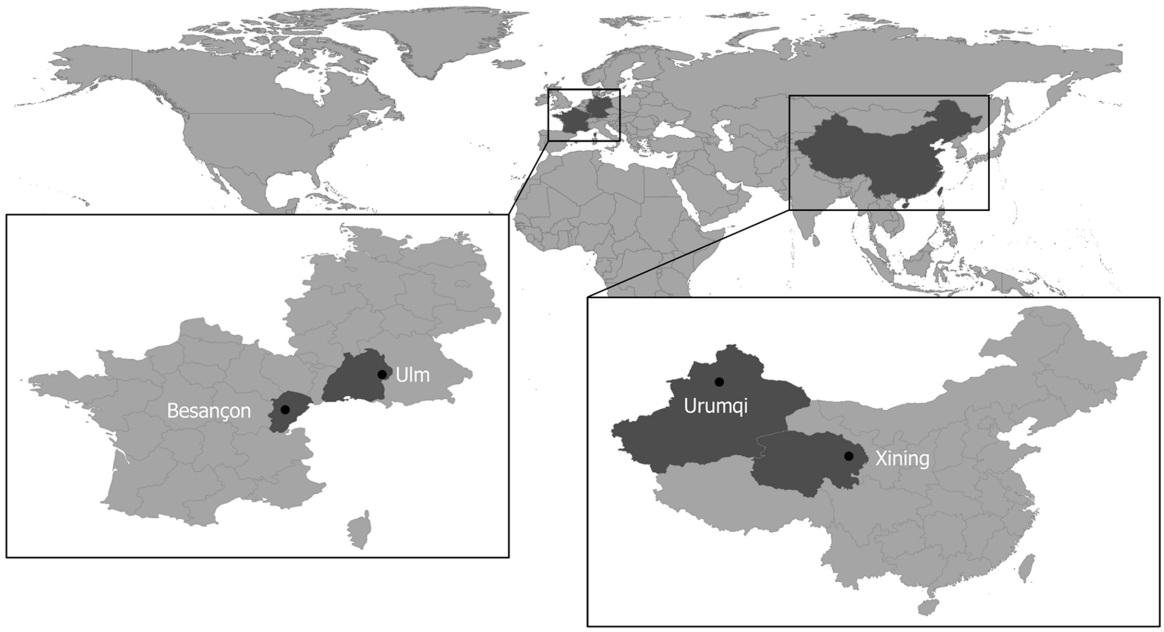


**Figure 1 Geographical locations of the centres.** The map shows the four centers in China and Europe: Xining in central China and Urumqi in the northwest of the country; Besançon in eastern France, and Ulm in southwestern Germany.

scans were used to evaluate the lesions. The largest lesion within a liver was used to determine the primary morphological type, and all further evaluations in this study reference these. A local experienced radiologist at each of the four centers became the second reader for their own 50 cases and independently re-classified the local cases (Figure 2). Criteria concerning the classification of the lesions, as well as further technical and disease-related information, were collected on a detailed report form. In addition to the essential patient data (sex and age), technical information included the basic technical modality of the CT scan. The following CT scanners were used in the different centers: Philips ICT, United UCT (Xining); CT-Discovery CT 750 HD, GE Healthcare (Urumqi); Biograph mCT-S (40), Siemens Healthcare (Ulm) and Biograph; Siemens; CTI; Knoxville, TN (Besançon).

Disease-related information included the affected hepatic lobes and a detailed listing of the liver segments involved, as well as the number of lesions, any vascular/biliary involvement and the overall dimension of the biggest lesion. The evaluation regarding vascular/biliary involvement of the liver lesions was based on a detailed joint case discussion and the consensus of all five readers, all experienced radiologists. The involvement of large central or medium-sized peripheral portal venous, venous, or arterial vessel sections and a central or peripheral cholestasis

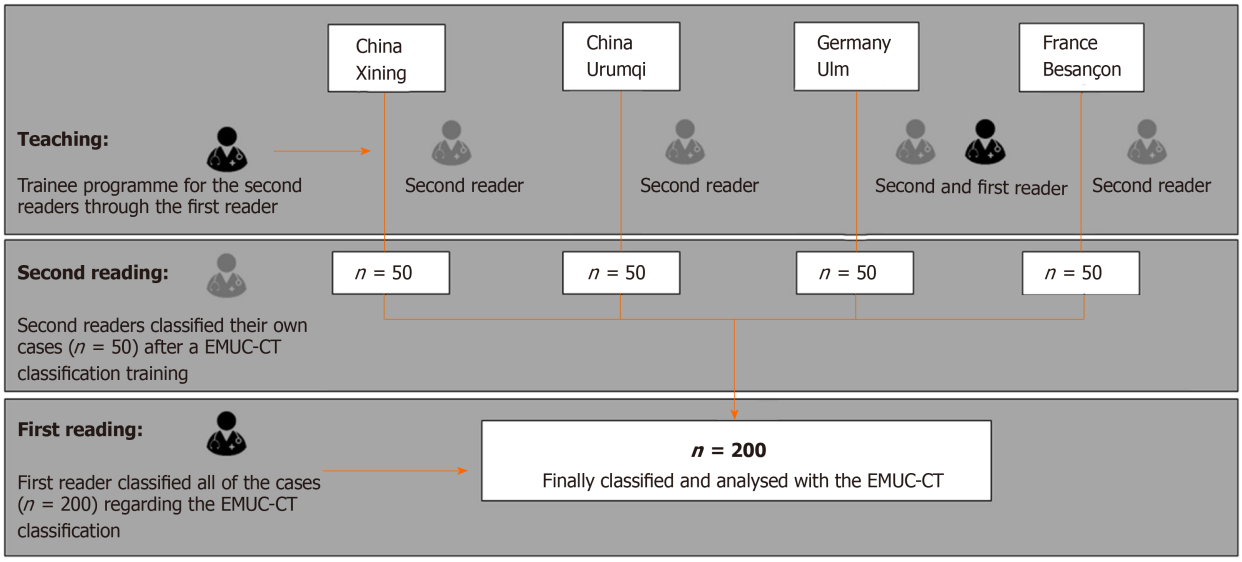


**Figure 2 Flowchart and study design.** EMUC-CT: Echinococcus multilocularis Ulm classification-computed tomography.

(peripheral bile ducts more than 2 mm) caused by lesions was evaluated using CT. From an anatomical point of view, this association points to an involvement of the jointly running portal biliary and vascular structures. The criteria for vascular and biliary involvement were therefore considered to be common criteria.

Whether a distant extrahepatic disease manifestation was present was determined retrospectively based on respective whole-body staging examinations. The different centers occasionally handled these differently, depending on local conditions and practices. In Ulm and Besançon, full body imaging was performed during the present PET-CT examination. In the two Chinese centers, where no PET-CT examinations were performed, the chest was also examined using CT, and the cranium was examined by using complementary MRI, assessing the corresponding clinical symptoms. All distant extrahepatic manifestations were histologically confirmed as AE.

Solely accentuated but well-circumscribed lymph nodes without infiltrating aspect were not evaluated as extrahepatic manifestations. Furthermore, the direct infiltration of organs adjacent to the liver or an infiltration of parahepatic connective tissue or diaphragm, respectively, through the liver lesion was also not evaluated as a separate (metastasis-like) extrahepatic manifestation. For further calculations concerning extrahepatic disease manifestations, these cases were not included unless distant extrahepatic manifestations were simultaneously recorded, but they were documented separately. The presence of vascular/biliary involvement by the AE liver lesion as well as of distant extrahepatic disease manifestations was finally associated with the presented EMUC-CT type of liver lesion.

### *Statistical analysis*

We performed statistical analyses using SAS Version 9.4 (SAS Institute Inc., Cary, NC, United States). Descriptive analysis of the data was performed to obtain absolute and relative frequencies, as well as measures of central tendency and dispersion. Pearson's $\chi^2$ and exact fisher tests were used to determine possible relationships and differences in the frequency distribution between dichotomous variables. Differences in metric variables from the four study centers were evaluated by post hoc Tukey and Kruskal–Wallis tests based on an analysis of variance. Inter-rater reliability between the reader was determined by kappa coefficients. The level of significance was set at $\alpha = 0.05$, and a $P$ value < 0.05 was considered to be statistically significant with a five percent probability of error.

### *Biostatistics*

The statistical methods of this study were reviewed by Dr. Julian Schmidberger, MPH, PhD, from the Department of Internal Medicine I, University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany.

## RESULTS

In the overall collective ($n$ = 200), 55% were women. The complete demographic data can be found in Table 2. The fleiss kappa (k) inter-rater reliability for reporting the findings using the EMUC-CT was 76% (95%CI: 69%-83%; $P$ < 0.0001). The distribution of the primary morphologies of the different centers is shown in Table 3.

Involvement of the right hepatic lobe was present in 80%, which is also reflected in similar values in the intercontinental comparison of 78% for Europe and 82% for China. For each of the four centers, segment VIII, which is centrally located on the right hepatic side, was most frequently involved, occurring in 57%.

Distant extrahepatic manifestations were rather rare in the total collective, occurring in 16 of 200 (8%). Table 4 provides an overview of the localization of the respective distant extrahepatic manifestations. However, Europe and China differed significantly in rates of these features, with only three cases in Europe 3/100 (3%) compared to thirteen in China 13/100 (13%) ($\chi^2$ = 6.7935; $P$ = 0.0091). In the Chinese group, the presence of a distant extrahepatic manifestation was approximately balanced, with six cases in Urumqi and seven for Xining. The three European cases were all from the French data, with no cases recorded in the German cases.

With respect to the primary morphology types of liver lesions, types IV and V were not associated with any case of distant extrahepatic disease. In contrast, distant extrahepatic manifestations in types I–III were found to varying degrees, with a maximum of 22% for type III.

We identified a significant differences between the presence and non-presence of distant extrahepatic disease and the size of the liver lesion, for the total dataset (130.13 ± 49.81 *vs* 92.65 ± 51.05; $P$ = 0.0075) and for the Chinese cases (145.15 ± 42.25 *vs* 116.69 ± 51.16; $P$ = 0.0251). Overall, distant extrahepatic manifestations were significantly more common in larger lesions of the liver (Figures 3 and 4).

We did not adjudicate the following as distant extrahepatic manifestations: solely accentuated, well-circumscribed lymph nodes, and a continuous infiltration of neighboring organs or liver-adjacent connective tissue (including diaphragm) through the liver lesion. These features had the following distribution in this group of 200 cases: lymph nodes, $n$ = 6; diaphragm, $n$ = 3; retroperitoneum close to the liver, $n$ = 3; right adrenal gland, $n$ = 3; mediastinum/pericardium/right atrium, $n$ = 1; and gallbladder, $n$ = 2. Within those cases, simultaneous distant extrahepatic manifestations were recorded for two of six with accentuated lymph nodes, one of three with infiltration of the diaphragm, and one of three with local retroperitoneal infiltration.

Vascular/biliary involvement of the largest liver lesion was found in 153 of the total 200 cases (76.50%). In the Chinese data, 92/100 (92%) showed this involvement, which was significantly higher than in the European data, with 61/100 (61%) ($\chi^2$ = 26.7279, $P$ < 0.0001, Figure 5).

The presence of vascular/biliary involvement highly significantly correlated with liver lesion size for the total group (110.45 ± 46.99 *vs* 47.44 ± 35.03; $P$ < 0.0001) as well as for the European (88.04 ± 32.54 *vs* 44.07 ± 35.20; $P$ < 0.0001) and Chinese datasets separately (125.30 ± 49.30 *vs* 63.87 ± 31.02; $P$ = 0.0009).

Among the 200 cases, vascular/biliary involvement as a function of primary lesion morphology ranged from 5.88% of type IV liver lesions to 100% among type III lesions. Other primary morphological types occasionally were associated with very high rates of vascular/biliary involvement. Type IV differed significantly in these associations from types I, II, and III (fisher exact test; $P$ < 0.0001) (Figure 6). In Europe, type I was most commonly associated with involvement of the corresponding structures, whereas in China it was type II, ($P$ = 0.0056 for comparison between the two geographic regions). Figure 7 shows the distribution of primary morphology types depending on vascular and biliary involvement and non-involvement considering the lesion size.

## DISCUSSION

In this study, a large international collective ($n$ = 200) of German, French, and Chinese patients with HAE was evaluated after prior classification of the liver lesions based on EMUC-CT[5] concerning findings of vascular/biliary involvement and distant extrahepatic disease. The aim was to obtain information about the different manifestations of this parasitosis at the respective sites and yield useful information about the development and dynamics of this disease. Information on criteria such as extrahepatic manifestations and vascular/biliary involvement have never been

**Table 2 Patient characteristics**

| | XUUB overall (*n* = 200) | Xining (*n* = 50) | Urumqi (*n* = 50) | Ulm (*n* = 50) | Besançon (*n* = 50) |
|---|---|---|---|---|---|
| **Sex, *n* (%)** | | | | | |
| Male | 90 (45.0) | 22 (44.0) | 23 (46.0) | 21 (42.0) | 24 (48.0) |
| Female | 110 (55.0) | 28 (56.0) | 27 (54.0) | 29 (58.0) | 26 (52.0) |
| **Age, yr, *n* (%)** | | | | | |
| < 18 | 8 (4.0) | 6 (12.0) | 2 (4.0) | 0 (0.0) | 0 (0.0) |
| 18–40 | 59 (29.5) | 20 (40.0) | 26 (52.0) | 8 (16.0) | 5 (10.0) |
| 41–60 | 66 (33.0) | 24 (48.0) | 18 (36.0) | 12 (24.0) | 12 (24.0) |
| 61–80 | 50 (25.0) | 0 (0.0) | 4 (8.0) | 26 (52.0) | 20 (40.0) |
| > 81 | 17 (8.5) | 0 (0.0) | 0 (0.0) | 4 (8.0) | 13 (26.0) |
| **Lesion size (mm)** | 95.6 ± 51.8 | 108.0 ± 53.0 | 132.7 ± 46.0 | 71.4 ± 46.39 | 70.4 ± 32.3 |
| | (11–261) | (21–261) | (36–253) | (11–202) | (13–173) |
| **Number of lesions** | 3.2 ± 4.5 | 3.3 ± 5.3 | 1.8 ± 1.2 | 5.0 ± 6.5 | 2.7 ± 2.4 |
| | (1–29) | (1–27) | (1–6) | (1–29) | (1–12) |
| **Age (yr)** | 50.1 ± 20.5 | 35.5 ± 12.6 | 38.0 ± 13.9 | 61.3 ± 16.8 | 65.7 ± 18.3 |
| | (11–91) | (11–55) | (16–77) | (18–85) | (18–91) |

**Table 3 Primary morphological types classified according to the echinococcosis multilocularis Ulm classification for computed tomography, *n* (%)**

| | XUUB total (*n* = 200) | Xining (*n* = 50) | Urumqi (*n* = 50) | Ulm (*n* = 50) | Besançon (*n* = 50) |
|---|---|---|---|---|---|
| Type I | 85 (42.5) | 18 (36.0) | 17 (34.0) | 22 (44.0) | 28 (56.0) |
| With cystoid portion | 55 (64.7) | 13 (72.2) | 13 (76.5) | 11 (50.0) | 18 (64.3) |
| Without cystoid portion | 30 (35.3) | 5 (27.8) | 4 (23.5) | 11 (50.0) | 10 (35.7) |
| Type II | 67 (33.5) | 17 (34.0) | 26 (52.0) | 12 (24.0) | 12 (24.0) |
| With cystoid portion | 55 (82.09) | 13 (76.5) | 22 (84.6) | 10 (83.3) | 10 (83.3) |
| Without cystoid portion | 12 (17.9) | 4 (23.5) | 4 (15.4) | 2 (16.7) | 2 (16.7) |
| Type III | 27 (13.5) | 13 (26.0) | 7 (14.0) | 4 (8.0) | 3 (6.0) |
| With more solid portions at the edge | 23 (85.19) | 11 (84.6) | 6 (85.7) | 4 (100.0) | 2 (66.7) |
| Without more solid portions at the edge | 4 (14.8) | 2 (15.4) | 1 (14.3) | 0 (0.0) | 1 (33.3) |
| Type iiia | 8 (4.0) | 2 (4.0) | 1 (2.0) | 2 (4.0) | 3 (6.0) |
| With more solid portions at the edge | 7 (87.5) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (66.7) |
| Without more solid portions at the edge | 1 (12.5) | 2 (100.0) | 1 (100.0) | 2 (100.0) | 1 (33.3) |
| Type iiib | 19 (9.5) | 11 (22.0) | 6 (12.0) | 2 (4.0) | 0 (0.0) |
| With more solid portions at the edge | 16 (84.21) | 9 (81.8) | 5 (83.3) | 0 (0.0) | 0 (0.0) |
| Without more solid portions at the edge | 3 (15.8) | 2 (18.2) | 1 (16.7) | 2 (100.0) | 0 (0.0) |
| Type IV | 17 (8.5) | 2 (4.0) | 0 (0.0) | 9 (18.0) | 6 (12.0) |
| Type V | 4 (2.0) | 0 (0.0) | 0 (0.0) | 3 (6.0) | 1 (2.0) |

studied in relation to different morphological types of AE liver lesions. It may be possible that such information can provide an approximation to a suspected stadium-like development of AE.

AE lesions appear almost exclusively in the liver. In addition to lesion size, this infiltration into vascular and biliary structures is clinically important[11,13-17]. Extrahepatic localizations of primary AE lesions are rare, but there may be invasion

**Table 4 Patients with extrahepatic disease manifestation from alveolar echinococcosis**

| Patient | Center | Age | Sex | Involved organs |
|---|---|---|---|---|
| No. 1 | Besançon | 91 | Female | Spleen |
| No. 2 | Besançon | 86 | Male | Cranial calotte |
| No. 3 | Besançon | 89 | Female | Lung |
| No. 4 | Urumqi | 56 | Male | Lung |
| No. 5 | Urumqi | 49 | Male | Lung |
| No. 6 | Urumqi | 53 | Male | Retroperitoneal (distant from the liver) |
| No. 7 | Urumqi | 37 | Female | Retroperitoneal (distant from the liver) |
| No. 8 | Urumqi | 30 | Male | Lung |
| No. 9 | Urumqi | 45 | Female | Lung |
| No. 10 | Xining | 32 | Female | Lung |
| No. 11 | Xining | 51 | Female | Lung |
| No. 12 | Xining | 30 | Female | Brain, lung |
| No. 13 | Xining | 17 | Male | Lung |
| No. 14 | Xining | 29 | Male | Brain, lung |
| No. 15 | Xining | 50 | Male | Brain, lung |
| No. 16 | Xining | 49 | Male | Lung |

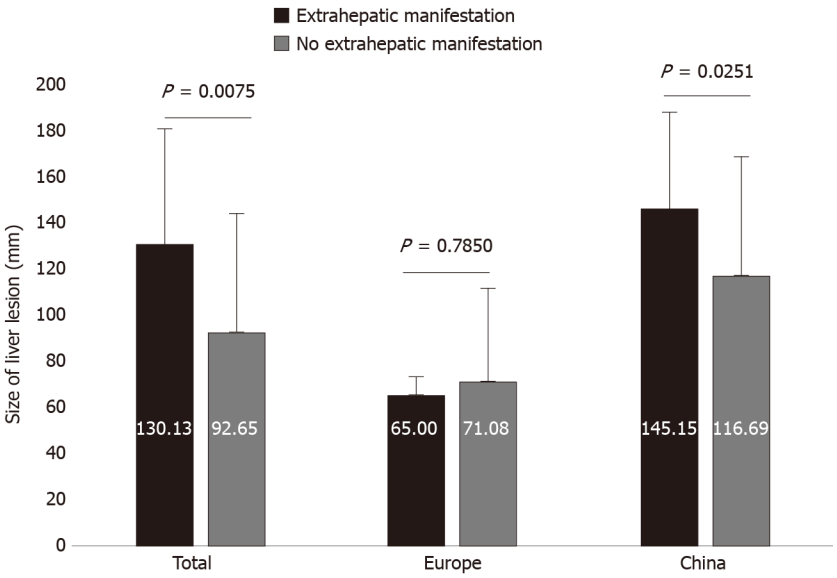


**Figure 3 Measure of dispersion for liver lesion sizes stratified according to extrahepatic manifestation in China *vs* Europe.** $P < 0.05$ was evaluated as statistically significant.

into neighboring organs or distant disease manifestations[1,12]. The exact prognostic relevance of distant extrahepatic manifestations in AE as well as the mechanism driving these so-called "distant metastases" of AE are debated[22-26]. Both the infiltration of intrahepatic vascular and bile duct structures as well as extrahepatic disease manifestations can lead to further complications and may increase morbidity in patients with AE.

Here, with regard to intrahepatic disease, the involvement of the right hepatic lobe was most common, occurring in 80% of the total cases. The same held for comparisons among regions (Europe *vs* China). The fact that segment VIII was most frequently involved for all four centers is certainly because of its central location and size, taking up a larger volume compared to the left lobe. Azizi *et al*[7] and Becce *et al*[27] also

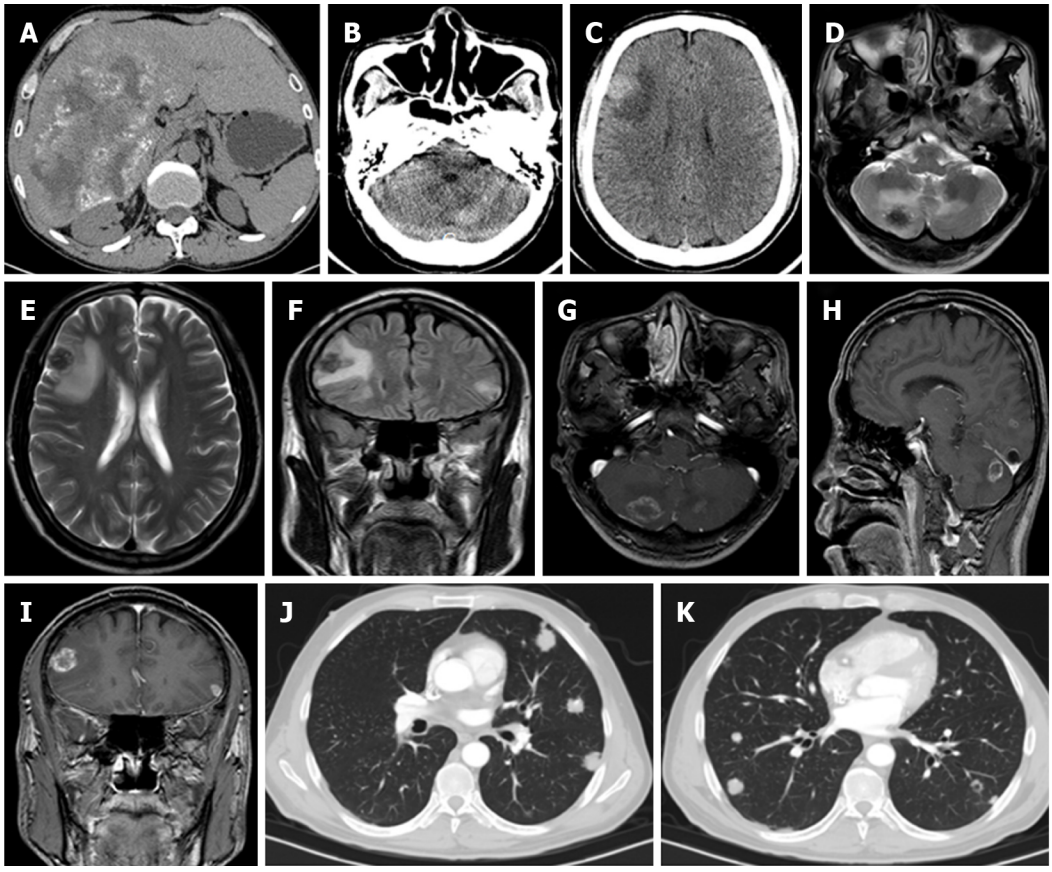


**Figure 4 Male, age 52 years, Han Chinese.** A: Abdominal computed tomography (CT) showing the hepatic alveolar echinococcosis lesion in the right liver lobe. echinococcus multilocularis Ulm classification-CT Type IIIb with more solid portions at the edge; B, C: Cranial CT scan showing multiple bilateral cerebellar hemisphere and frontal lobe calcified masses with surrounding edema; D, E: Coronal T2-weighted magnetic resonance imaging (MRI) showing multiple lesions with associated edema; F: Fluid-attenuated inversion recovery MRI showing two lesions with low signal intensity and surrounding edema; G-I: T1-weighted contrast-enhanced MRI showing nodular enhancement of the lesion; J, K: Chest CT scan showing multiple irregular solid nodules in both lungs and some lesions with "empty bubble sign" .

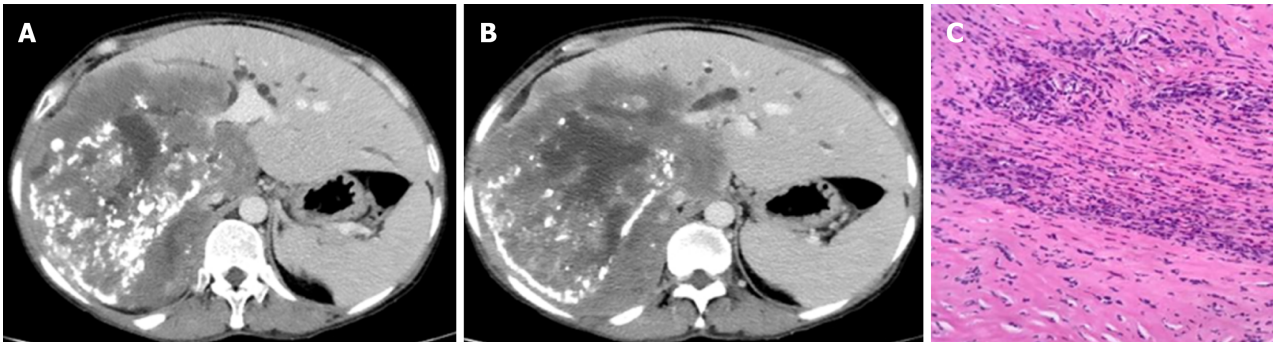


**Figure 5 Male, age 53 years, Tibetan.** A, B: Echinococcus multilocularis Ulm classification-computed tomography type IIIb with more solid portions at the edge. The images indicate invasion of the right hepatic vein and inferior vena cava. middle hepatic vein and bile duct at the primary and secondary hilum porta hepatis; C: Multiple cysts surrounded by necrosis and an intense granulomatous reaction characterized by epithelioid cells, multinucleated giant cells, and infiltration by lymphocytes (hematoxylin and eosin, original magnification 40 x).

described a predominately right hepatic distribution of AE lesions.

In this study, if only a continuous infiltration of the liver lesion was present locoregionally with some extrahepatic tendency, these cases were not adjudicated as extrahepatic disease. Similarly, solely accentuated, well-circumscribed lymph nodes were not evaluated as extrahepatic manifestations. These nodes can exhibit so-called "small particles of echinococcus multilocularis," which can lead to corresponding inflammatory reactivity without representing a confirmed parasitic disease manifestation[28].

True distant extrahepatic manifestations, which were quite rare overall, showed a

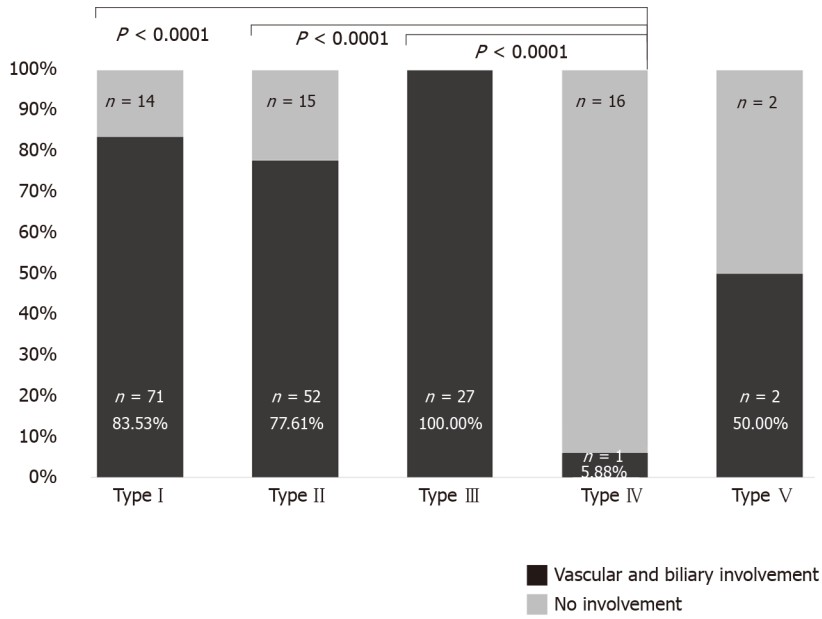


**Figure 6 Distribution of primary morphology types depending on vascular and biliary involvement and non-involvement.**

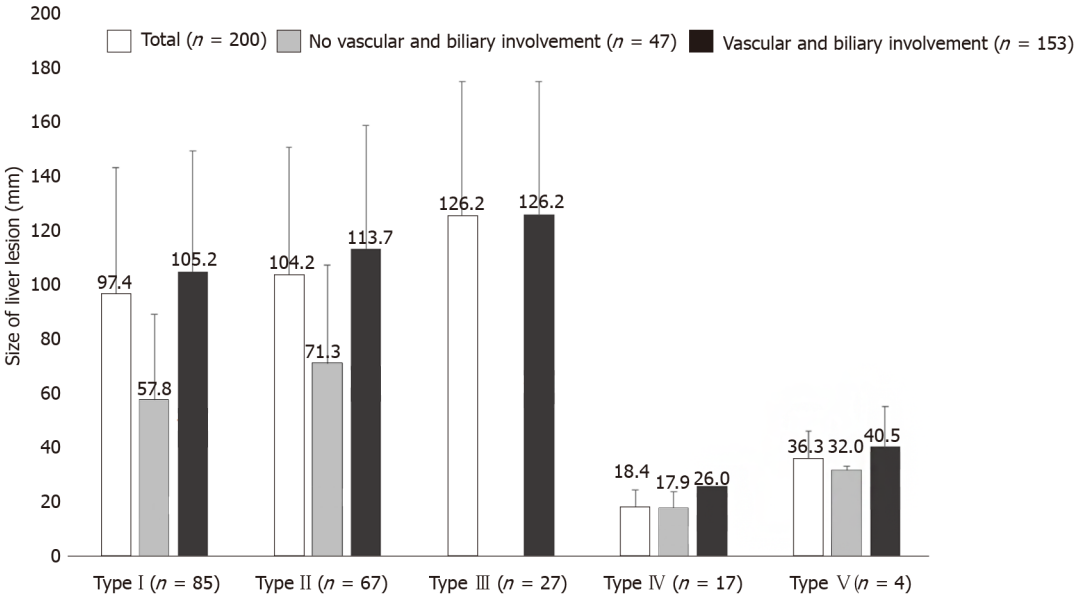


**Figure 7 Distribution of primary morphology types depending on vascular and biliary involvement and non-involvement considering the lesion size.**

significant difference in comparison between the European and Chinese centers, with more identified in the Chinese data. This finding could be viewed as an indication of more advanced cases in China at the time of diagnosis. Distant extrahepatic manifestations in the lung were most common in the overall group, followed by lesions in the brain. Distant extrahepatic AE manifestations are also described more frequently in these two organs in other studies[29-31].

With regard to the five primary morphology types of liver lesions after EMUC-CT, type IV and V were not associated with any cases of distant extrahepatic disease, and type III was most frequently associated with it (22.22%). This result may indicate different stages of development through the lesion types, with incrementally different degrees of progression of the overall disease. In agreement, in a recent comparative study of CT morphology and histology, type IV was described as the initial lesion and type III as the most advanced[32].

We found a significant relationship between the presence of distant extrahepatic disease and the size of the liver lesion in the overall group and in the Chinese data,

with significantly more frequent extrahepatic involvement and simultaneously larger lesions of the liver in China. These findings imply that detection of distant extrahepatic manifestations is related to disease progression.

The vast majority of cases in the overall dataset (76.50%) showed vascular/biliary involvement of the largest liver lesion, and the patient population in China had significantly more frequent involvement compared to the European patients. Involvement of vascular and biliary structures also significantly correlated with lesion size. These observations add further support to the earlier evidence that Chinese cases are more advanced at detection, based on lesion size and type distribution[33]. In addition, type IV had the least vascular/biliary involvement at 5.88%, differing significantly from types I–III, and type III had the most, at 100%. These results also support the idea of disease progression through type IV to types I, II, and III successively[33-35].

A limitation of this retrospective study is the assessment of vascular/biliary involvement of HAE lesions, which was based purely on image morphological criteria, although all experienced reviewers agreed on the conclusions. Since no standardized examination protocol was used in the preparation of the routine CT scans, this may in principle have led to method-related limitations in the assessment of individual criteria. The study design precluded a histopathological evaluation of these criteria. Furthermore, the presence of distant extrahepatic disease was determined retrospectively based on whole-body staging examinations. As described above, these exams were occasionally handled differently for the different centers, depending on local conditions and practices.

In summary, the current findings show differences between Chinese and European AE cases in terms of vascular/biliary involvement, distant extrahepatic disease manifestations, and EMUC-CT types of liver lesions associated with these features.

The results may give indications about the behavior of this disease in the context of initial manifestation and progression. A morphological classification of AE liver lesions seems therefore not only useful in order to facilitate the initial differential diagnosis but also indicates a direct clinical impact.

The designation and evaluation of distant extrahepatic AE manifestations as "metastases," as in the PNM (P = parasitic mass in the liver, N = involvement of neighbouring organs, and M = metastasis) classification[36], may be re-evaluated in future studies. Such a re-evaluation is warranted as it remains unclear whether these distant manifestations are “metastases” originating from the liver lesion or if they also represent possibly more slowly growing primary manifestations, which we think is more likely.

Many important scientific questions concerning AE could so far only be answered through networks and cooperation at international, European and national level[1,37-39]. There is a need for future prospective studies combining standardized international radiological expertise and terminology in an Echino network[40].

In future internationally standardized examination protocols will be necessary to generate valid imaging data in order to improve their comparability.

## ARTICLE HIGHLIGHTS

### *Research background*

Human alveolar echinococcosis (AE) is a zoonosis caused by the larval stage of the fox tapeworm echinococcus multilocularis. Untreated, the disease is fatal. The main endemic areas of alveolar echinococcosis (AE) are in Central Europe and Western China.

### *Research motivation*

Early diagnosis is of crucial importance. Imaging techniques play the greatest role here. International comparative studies on imaging are not yet available.

### *Research objectives*

Aim of this study was to evaluate the vascular/biliary involvement of hepatic alveolar echinococcosis and the extrahepatic disease manifestations in a collective of German, French, and Chinese cases.

### *Research methods*

Consecutively, five experienced examiners evaluated contrast-enhanced abdominal

computed tomography (CT) scans for 200 patients with hepatic AE of each of four locations ($n$ = 50) in Germany, France and China according to the echinococcosis multilocularis Ulm classification for *CT* (EMUC-CT). Vascular/biliary involvement of the hepatic disease as well as the presence of distant extrahepatic manifestations were correlated with the EMUC-CT types of liver lesion.

### Research results

Distant extrahepatic AE manifestations were significantly more frequent in China than in Europe ($P$ = 0.0091). A significant relationship was found between the presence of distant extrahepatic disease and AE liver lesion size ($P$ = 0.0075). Vascular/biliary structures were involved by the liver lesions significantly more frequently in China than in Europe ($P$ < 0.0001), and vascular/biliary involvement depended on lesion size. Different morphological types of AE liver lesions led to varying frequencies of vascular/biliary involvement and were associated with different frequencies of distant extrahepatic manifestations. Type IV differed significantly in these associations from types I, II, and III ($P$ < 0.0001). With respect to extrahepatic disease, the primary morphology types IV and V of liver lesions were not associated with any case of distant extrahepatic disease. In contrast, distant extrahepatic manifestations in types I–III were found to varying degrees, with a maximum of 22% for type III.

### Research conclusions

Different CT morphological patterns of hepatic AE lesions influence vascular/biliary involvement and the occurrence of distant extrahepatic manifestations. There are intercontinental differences regarding the characteristics of AE manifestation.

### Research perspectives

The results may give indications about the behavior of this disease in the context of initial manifestation and progression. A morphological classification of AE liver lesions seems therefore not only useful in order to facilitate the initial differential diagnosis but also indicates a direct clinical impact.

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ORIGINAL ARTICLE

**Retrospective Study**

# Multivariate predictive model for asymptomatic spontaneous bacterial peritonitis in patients with liver cirrhosis

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## Abstract

### *BACKGROUND*

Spontaneous bacterial peritonitis (SBP) is a detrimental infection of the ascitic fluid in liver cirrhosis patients, with high mortality and morbidity. Early diagnosis and timely antibiotic administration have successfully decreased the mortality rate to 20%-25%. However, many patients cannot be diagnosed in the early stages due to the absence of classical SBP symptoms. Early diagnosis of asymptomatic SBP remains a great challenge in the clinic.

### *AIM*

To establish a multivariate predictive model for early diagnosis of asymptomatic SBP using positive microbial cultures from liver cirrhosis patients with ascites.

### *METHODS*

A total of 98 asymptomatic SBP patients and 98 ascites liver cirrhosis patients with negative microbial cultures were included in the case and control groups, respectively. Multiple linear stepwise regression analysis was performed to identify potential indicators for asymptomatic SBP diagnosis. The diagnostic performance of the model was estimated using the receiver operating characteristic curve.

### *RESULTS*

Patients in the case group were more likely to have advanced disease stages, cirrhosis related-complications, worsened hematology and ascites, and higher

mortality. Based on multivariate analysis, the predictive model was as follows: y (*P*) = 0.018 + 0.312 × MELD (model of end-stage liver disease) + 0.263 × PMN (ascites polymorphonuclear) + 0.184 × N (blood neutrophil percentage) + 0.233 × HCC (hepatocellular carcinoma) + 0.189 × renal dysfunction. The area under the curve value of the established model was 0.872, revealing its high diagnostic potential. The diagnostic sensitivity was 73.5% (72/98), the specificity was 86.7% (85/98), and the diagnostic efficacy was 80.1%.

*CONCLUSION*

Our predictive model is based on the MELD score, polymorphonuclear cells, blood N, hepatocellular carcinoma, and renal dysfunction. This model may improve the early diagnosis of asymptomatic SBP.

**Key words:** Spontaneous bacterial peritonitis; Asymptomatic; Ascites; Multivariate predictive model; Liver cirrhosis



**Core tip:** This retrospective study established a multivariate diagnostic model for asymptomatic spontaneous bacterial peritonitis in liver cirrhosis patients with ascites. The multivariate predictive model constructed by multiple linear stepwise regression analysis was as follows: y (*P*) = 0.018 + 0.312 × MELD (model of end-stage liver disease) + 0.263 × PMN (ascites polymorphonuclear) + 0.184 × N (blood neutrophil percentage) + 0.233 × HCC (hepatocellular carcinoma) + 0.189 × renal dysfunction. The diagnostic efficacy of the model was 80.1%, sensitivity was 73.5% and specificity was 86.7%. This model may improve the early diagnosis of asymptomatic spontaneous bacterial peritonitis.

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## INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a detrimental infection of the ascitic fluid in liver cirrhosis patients, with a prevalence of 10%-30% among hospitalized patients[1,2]. SBP worsens the outcomes of chronic liver diseases and increases the risk of complications, including renal and hepatic failure and portal hypertension[3,4]. Patients with SBP usually present with fever, shivering, and abdominal pain, but up to 30% of patients can also be asymptomatic[5]. SBP diagnosis follows the European Association for the Study of the Liver and the American Association for the Study of Liver Diseases guidelines, which indicate that ascites polymorphonuclear (PMN) leukocyte counts combined with no intra-abdominal source of infection are suggestive of SBP[6,7]. However, some SBP cases caused by gram-positive cocci often have PMN counts less than 250/mm$^3$[8].

SBP leads to 30%-90% mortality within the first year. However, early diagnosis and timely administration of antibiotics have successfully decreased the mortality rate to 20%-25% in the past three decades[9,10]. In patients with PMN > 250/mm$^3$, antibiotics are promptly administered, while in patients with PMN < 250/mm$^3$, antibiotics are usually administered following typical SBP clinical manifestations[11]. However, many patients cannot be diagnosed in the early stages due to the absence of classical SBP symptoms[12]. Some SBP cases are diagnosed only based on clinical symptoms, leading to possible antibiotic abuse. Therefore, there is an urgent need to establish a more specific and accurate model for early SBP diagnosis, especially for asymptomatic SBP patients. Increasing the diagnosis sensitivity can promote early discovery of SBP, and enhancing the specificity of the current diagnostic tools could inhibit antibiotics abuse.

Accumulating evidence has demonstrated that SBP is regulated by a variety of risk factors, including decreased activity of the reticuloendothelial system, advanced liver dysfunction, medications, and genetic factors[1]. To date, effective multivariate

obtained from the patients for publication of this report and any accompanying images.

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prediction models for asymptomatic SBP are not available. Therefore, the present retrospective cohort study aimed to establish an effective predictive model for early screening of asymptomatic SBP in liver cirrhosis patients with ascites. Early diagnosis of asymptomatic SBP will improve antibiotic management strategies and reduce SBP associated mortality.

## MATERIALS AND METHODS

### Study subjects

A total of 371 cirrhotic patients with ascites who had no SBP symptoms were recruited from the 302 People's Liberation Army Hospital in the Beijing area from January 2015 to December 2018. Liver cirrhosis diagnoses were confirmed *via* clinical, laboratory, histological, and imaging findings. A diagnostic paracentesis was performed to confirm ascites according to standard methodology. This study was approved by the ethics committee of the Beijing 302 People's Liberation Army Hospital. All patients provided informed written consent.

### Inclusion and exclusion criteria

Liver cirrhosis patients with ascites who were enrolled in this study met the following inclusion criteria: (1) Adult population; (2) Ascites PMN count < 250/mm$^3$; (3) Absence of typical SBP symptoms such as fever, abdominal pain, diarrhea, tenderness, or rebound pain; (4) No active infection signs, such as infections of the respiratory tract, digestive tract, urinary tract and central nervous system, *etc*.; (5) No antibiotics administered in the two weeks preceding the study; and (6) Available microbiological results of ascites specimens. The exclusion criteria included incomplete medical record and medication history.

### Grouping

Patients were divided into the case and control groups according to microbiological results. Under aseptic conditions, the ascites specimens were inoculated in aerobic and anaerobic bottles (BACTEC 9120, BD, United States) at the bedside. The bottles were then incubated at 35°C for 5 d. The culture results were confirmed positive if the same type of pathogen was isolated from both the aerobic and anaerobic cultures. The patients with negative microbiological examination results were placed in the control group. Patients with positive cultures were designated as the case group. Moreover, all the individuals in the case group could present signs of disease deterioration, such as ascites PMN > 250/mm$^3$, fever, and other physical signs of infection, or liver and kidney function deterioration, and antibiotic treatments were necessary for these patients. Patients in the control group did not receive antibiotic treatments.

### Demographic features and clinical characteristics

Demographic features and clinical information including age, gender, complications, and etiology of cirrhosis were collected from the initial medical records. Disease severity was estimated using the Child-Pugh stage and model of end-stage liver disease (MELD) score, as described previously[13,14]. Hematological factors included white blood cell (WBC) count, platelet count (PLT), and neutrophil percentage (N). Indicators for ascites included ascites leukocyte and polymorphonuclear percentage, as well as the PMN. Finally, patient condition at discharge was recorded.

### Statistical analysis

The differences between the case and control groups were calculated using the Student's *t* test (normal distribution) or the rank sum test (abnormal distribution). Data (continuous variables) are presented as mean ± SD. Categorical variables are presented as percentages and analyzed using the chi-square test. The predictive model was constructed using the multiple linear stepwise regression method of the logistic regression model. The diagnostic yield of the model was estimated using a receiver operating characteristic (ROC) curve. All analyses were two sided. In the multiple linear analysis, a $P$ value < 0.01 was considered statistically significant, while $P$ < 0.05 was considered significant in other analyses. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Demographic characteristics of the study population

A total of 371 liver cirrhosis patients were initially enrolled in this study. However, during the course of our study, 28 patients dropped out. Thus, 343 patients were included (259 males and 84 females) with an average age of 54.60 ± 12.79 years (Table 1). Among them, 220 (64.14%) patients were diagnosed with cirrhosis due to viral hepatitis, 65 (18.95%) cases were confirmed as having alcoholic cirrhosis, and 27 (7.87%) cirrhosis cases were caused by autoimmune diseases. According to Child-Pugh stages, the majority of patients were at stages B (175, 51.02%) and C (149, 43.44%), and the remaining patients (19, 5.54%) were diagnosed with stage A. The mean MELD score was 12.04 ± 8.87. Liver failure was observed in 51 (14.87%) patients. Additionally, 64 (18.66%) patients were diagnosed with hepatocellular carcinoma (HCC), while hepatic encephalopathy (HE) was detected in 29 (8.45%) patients. A total of 68 (19.82%) patients presented with cirrhosis combined with diabetes, 68 (19.82%) cases presented with renal dysfunction, and 22 (6.41%) cases had upper gastrointestinal bleeding (UGB) (Table 1).

The mean WBC count was 5.19 ± 3.62 × $10^9$/L, the average N was 0.64 ± 0.12 (× 100%), PLT was 89.93 ± 60.52 × $10^9$/L, and the average ratio of WBC/PLT was 0.082 ± 0.013. In the ascites specimens, the leukocyte count was 258.92 ± 247.46 /$mm^3$, polymorphonuclear cell percentage was 0.100 ± 0.098 (× 100%), and PMN was 28.22 ± 39.46 /$mm^3$ (Table 1).

Microbiological investigations demonstrated that 111 (32.26%) patients were positive for pathogens, while no pathogens were isolated from the other 232 (67.64%) patients. Among the patients with positive cultures, gram-positive bacteria were isolated from 80 (72.07%) patients, and gram-negative bacteria were observed in 21 (18.92%) patients. Additionally, 10 (9.01%) infectious ascites cultures were the result of mixed pathogens. Among patients with positive microbial cultures, 13 patients improved without antibiotic administration. At the time of discharge, 297 (86.58%) patients had improved clinical outcomes, 37 (10.79%) patients died, and 9 (2.62%) showed deteriorating symptoms (these 9 patients were discharged upon their request) (Table 1).

### Comparison of baseline characteristics between the case and control groups

Among the study subjects, 98 patients were positive for bacterial pathogens and were administered antibiotics. These patients were designated as the case group. In addition, 98 patients were randomly recruited from the study population into the control group according to the proportion of 1:1 using SPSS 18.0 software. The case and control groups were matched for age and gender ($P > 0.05$ for both).

Next, we compared the baseline characteristics between both groups (Table 2). Compared to the control group, the MELD scores, WBC, N, WBC/PLT, ascites polymorphonuclear and PMN were significantly higher in the case group ($P < 0.05$). Patients in the case group were more likely to develop advanced Child-Pugh stages ($P = 0.004$), liver failure ($P < 0.001$), HCC ($P < 0.001$), HE ($P < 0.001$), renal dysfunction ($P < 0.001$), and UGB ($P < 0.001$). Moreover, the death rate was significantly higher in the case group (33.67% *vs* 3.06%, $P < 0.001$) (Table 2).

### Data processing

ROC curves were plotted for the selected 196 patients according to the continuous variables, including age, MELD scores, WBC, N, PLT, WBC/PLT, ascites leukocyte, polymorphonuclear, and PMN (Figure 1 and Table 3). The area under curve (AUC) values for age and PLT were 0.465 and 0.490, respectively, and their corresponding cut-off values were 71.50 years and 187.50 × $10^9$/L, respectively. The AUC values of MELD scores, WBC, N, WBC/PLT, ascites leukocyte, polymorphonuclear, and PMN were more than 0.5, with the corresponding cut-off values at 15.50, 4.73 × $10^9$/L, 66.15%, 0.0619, 230/$mm^3$, 7.50%, and 10.95/$mm^3$, respectively. Subsequently, based on the ROC cut-off values, these factors were entered into the multiple linear stepwise regression analysis as categorized variables.

### Multivariate analysis and construction of the diagnostic model

Based on the multivariate analysis results (Table 4), blood neutrophil percentage, HCC, MELD, PMN, and renal dysfunction were included in the predictive model. The equation was as follows: y ($P$) = 0.018 + 0.312 × MELD (0: < 15.50; 1: > 15.50) + 0.263 × PMN (0: < 10.95; 1: > 10.95) + 0.184 × N (0: < 0.6615; 1: > 0.6615) + 0.233 × HCC (0: no; 1: yes) + 0.189 × renal dysfunction (0: no; 1: yes).

**Table 1 Demographic characteristics of the study population**

| Parameters | Patients (*n* = 343, %) |
|---|---|
| Age (yr) | 54.60 ± 12.79 |
| Gender | |
| Male | 259 (75.51) |
| Female | 84 (24.49) |
| Clinical characteristics | |
| Etiology of cirrhosis | |
| Viral hepatitis | 220 (64.14) |
| Alcohol | 65 (18.95) |
| Autoimmune | 27 (7.87) |
| Others | 31 (9.04) |
| Child-Pugh | |
| A | 19 (5.54) |
| B | 175 (51.02) |
| C | 149 (43.44) |
| MELD score | 12.04 ± 8.87 |
| Complications | |
| Liver failure | 51 (14.87) |
| HCC | 64 (18.66) |
| HE | 29 (8.45) |
| Diabetes | 68 (19.82) |
| Renal dysfunction | 68 (19.82) |
| UGB | 22 (6.41) |
| Hematological factors | |
| WBC ($\times 10^9$/L) | 5.19 ± 3.62 |
| N (100%) | 0.64 ± 0.12 |
| PLT ($\times 10^9$/L) | 89.93 ± 60.52 |
| WBC/PLT | 0.082 ± 0.013 |
| Ascites examinations | |
| Leukocyte ($mm^3$) | 258.92 ± 247.46 |
| Polymorphonuclear (100%) | 0.10 ± 0.098 |
| PMN ($mm^3$) | 28.22 ± 39.46 |
| Microbiological examinations | |
| Positive | 111 (32.36) |
| Gram-negative | 21 (18.92) |
| Gram-positive | 80 (72.07) |
| Mixture | 10 (9.01) |
| Negative | 232 (67.64) |
| Clinical outcomes | |
| Non-survivors | 37 (10.79) |
| Improved | 297 (86.59) |
| Invalid | 9 (2.62) |

CHILD: Child-Pugh; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; UGB: Upper gastrointestinal bleeding; WBC: White blood cell count; N: Blood neutrophil percentage; PLT: Platelet; PMN: Polymorphonuclear leukocyte count in ascites.

### *ROC analysis for the screening model*

In our study, we also calculated y (*P*) value of each patient based on the original data using the constructed equation. The ROC curve was plotted based on the calculated *P* values (Figure 2). The AUC value was 0.872, revealing the high diagnostic value of the model with diagnostic sensitivity and specificity at 73.5%, and 86.7%, respectively.

In addition, $P = 0.5$ was identified as the optimum cut-off value for asymptomatic SBP diagnosis. The patients with *P* values > 0.5 were confirmed as having asymptomatic SBP. Accordingly, there were 72 patients in the asymptomatic SBP group with $P > 0.5$, while 26 patients in the control group did ($P < 0.001$). Our analysis showed that the diagnostic sensitivity was 73.5% (72/98), the specificity was 86.7% (85/98), and the diagnostic efficacy was 80.1% (Table 5). The Youden's index was 0.602, more than 0.5, suggesting a high application value of the model.

## DISCUSSION

SBP is associated with increased mortality in liver cirrhosis patients[15]. Early diagnosis followed by appropriate antibiotic administration can significantly improve the survival of SBP patients. However, based on the current diagnostic strategies, many asymptomatic SBP cases can be misdiagnosed leading to a delay in treatment or antibiotic abuse[16]. Therefore, in the present study, we established a multivariate predictive model by comparing 98 asymptomatic SBP patients with positive microbial culture (case group) and 98 cirrhotic ascites liver patients with negative microbial culture (control group). We compared the clinical characteristics and hematological factors, as well as ascites examination results between both groups. Our results demonstrated that patients in the case group were more likely to develop advanced Child-Pugh stages and high MELD scores. In agreement with previous reports, our results indicate that liver cirrhosis patients with advanced conditions are more likely to develop SBP. SBP was independently associated with liver disease severity according to Child-Pugh stage and high MELD score[17]. Additionally, the frequencies of liver failure, HCC, HE, renal dysfunction, and UGB were significantly higher in the case group, suggesting a close association of these factors with increased risk of SBP. SBP might not only contribute to the progression of liver disease, but also increase the risk of other complications, such as HE, septic shock, and hepatorenal syndrome. In addition, the WBC levels and the WBC/PLT ratios were significantly increased in asymptomatic SBP cases compared to the controls. The mortality of asymptomatic SBP cases was also higher than that of controls. Our findings are consistent with previous reports suggesting that SBP might aggravate dysregulation of the immune system, thus contributing to disease-related complications and increasing the risk of mortality among cirrhosis patients[18-20].

In this study, multiple linear stepwise regression analysis was performed to identify the potential indicators for early diagnosis of asymptomatic SBP in liver cirrhosis patients. Our results showed that blood neutrophil percentage, HCC, MELD, PMN, and renal dysfunction could be included in the predictive model. Liver cirrhosis patients are more likely to be infected by pathogens due to their compromised immune response. Abnormal stimulation of neutrophils induced by impaired phagocytic and oxidative burst function may be responsible for dysregulation of the immune system in liver cirrhosis patients[21,22]. Therefore, blood neutrophils and PMN in ascitic fluid might be effective factors for early SBP screening in liver cirrhosis patients. MELD is an indicator of disease severity, and its positive association with SBP initiation has been previously reported. Na *et al*[23] demonstrated that patients with SBP had higher MELD scores than those without SBP. Additionally, the MELD score can function as an independent factor for occurrence and clinical outcomes of SBP[24,25]. However, some studies may hold different opinions. In the study by Haddad *et al*[26], MELD was confirmed to have no association with SBP. Although MELD had no direct association with SBP, MELD might be employed as a predictive biomarker for SBP. Moreover, liver disease-related complications may contribute to the development of SBP. Tsung *et al*[27] reported that HCC and renal dysfunction could increase the death rate in liver cirrhosis patients with SBP[27]. Taken together, the predictive model constructed in our study is feasible, and ROC analysis confirmed that the predictive

**Table 2 Comparisons between asymptomatic spontaneous bacterial peritonitis and control groups**

| Characteristics | Asymptomatic SBP (*n* = 98, %) | Control group (*n* = 98, %) | *P* value |
|---|---|---|---|
| Age (yr) | 54.01 ± 13.60 | 53.56 ± 13.47 | 0.817 |
| Gender | | | 0.178 |
| Male | 79 (80.61) | 71 (72.45) | |
| Female | 19 (19.39) | 27 (27.55) | |
| Clinical characteristics | | | |
| Etiology of cirrhosis | | | 0.280 |
| Viral hepatitis | 70 (71.43) | 61 (62.24) | |
| Alcohol | 16 (16.33) | 19 (19.39) | |
| Autoimmune | 5 (5.10) | 12 (12.24) | |
| Others | 7 (7.14) | 6 (6.12) | |
| Child-Pugh | | | 0.004 |
| A | 2 (2.04) | 2 (2.04) | |
| B | 35 (35.71) | 58 (59.18) | |
| C | 61 (62.24) | 38(38.78) | |
| MELD score | 18.20 ± 11.04 | 9.92 ± 5.88 | < 0.001 |
| Complications | | | |
| Liver failure | 28 (28.57) | 7 (7.14) | < 0.001 |
| HCC | 37 (37.75) | 10 (10.20) | < 0.001 |
| HE | 21 (21.43) | 3 (3.06) | < 0.001 |
| Diabetes | 22 (22.45) | 13 (13.26) | 0.093 |
| Renal dysfunction | 39 (39.80) | 10 (10.20) | < 0.001 |
| UGB | 19 (19.39) | 0 (0.00) | < 0.001 |
| Hematological factors | | | |
| WBC (× $10^9$/L) | 7.25 ± 4.66 | 3.97 ± 1.99 | < 0.001 |
| N (100%) | 0.71 ± 0.13 | 0.60 ± 0.13 | 0.007 |
| PLT (×$10^9$/L) | 92.08 ± 71.37 | 82.44 ± 43.27 | 0.244 |
| WBC/PLT | 0.14 ± 0.22 | 0.057 ± 0.04 | 0.001 |
| Ascites examinations | | | |
| Leukocyte (/$mm^3$) | 314.41 ± 351.67 | 254.08 ± 231.51 | 0.151 |
| Polymorphonuclear (100%) | 0.15 ± 0.12 | 0.086 ± 0.10 | < 0.001 |
| PMN (/$mm^3$) | 45.83 ± 52.25 | 21.44 ± 28.71 | < 0.001 |
| Clinical outcomes | | | |
| Non-survivors | 33 (33.67) | 3 (3.06) | < 0.001 |

SBP: Spontaneous bacterial peritonitis; CHILD: Child-Pugh; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; UGB: Upper gastrointestinal bleeding; WBC: White blood cell count; N: Neutrophil percentage; PLT: Platelet; PMN: Polymorphonuclear leukocyte count in ascites.

model had high application potential for early SBP diagnosis. According to Project Leonardo, a health follow-up file may be established for patients with liver cirrhosis, especially those who have ascites. The special care manager will follow up the patients on a regular basis to gain the trust and cooperation of the patients, in order to discover changes in the condition with time. Based on our diagnostic model, time treatments could be supplied for patients to improve the long-term prognosis[28].

There were several limitations in our study. First, the sample size was relatively

**Table 3 Receiver operating characteristic analysis for the continuous variables in 196 selected patients**

| Variables | AUC | Cut-off value |
|---|---|---|
| Age (yr) | 0.489 | 71.50 |
| MELD score | 0.733 | 15.50 |
| WBC (× $10^9$/L) | 0.729 | 4.73 |
| N (100%) | 0.747 | 0.6615 |
| PLT (× $10^9$/L) | 0.494 | 187.50 |
| WBC/PLT | 0.720 | 0.0619 |
| Leukocyte ($mm^3$) | 0.538 | 230.00 |
| Polymorphonuclear (100%) | 0.683 | 0.075 |
| PMN ($mm^3$) | 0.671 | 10.95 |

AUC: Area under curve; MELD: Model for end-stage liver disease; WBC: White blood cell count; N: Blood neutrophil percentage; PLT: Platelet; PMN: Polymorphonuclear leukocyte count in ascites.

**Table 4 Multiple linear stepwise regression analysis of the candidate indicators for asymptomatic spontaneous bacterial peritonitis**

| Parameters | Unstandardized Coefficients | | Standardized Coefficients | *t* | *P* value |
|---|---|---|---|---|---|
| | B | Std. Error | Beta | | |
| Constant | 0.018 | 0.055 | | 0.331 | 0.741 |
| MELD | 0.312 | 0.064 | 0.299 | 4.901 | 0.000 |
| PMN | 0.263 | 0.059 | 0.252 | 4.474 | 0.000 |
| N | 0.184 | 0.062 | 0.183 | 2.963 | 0.003 |
| HCC | 0.233 | 0.067 | 0.199 | 3.480 | 0.001 |
| Renal dysfunction | 0.189 | 0.071 | 0.164 | 2.671 | 0.008 |

SBP: Spontaneous bacterial peritonitis; MELD: Model for end-stage liver disease; HCC: Hepatocellular carcinoma; N: Blood neutrophil percentage; PMN: Polymorphonuclear leukocyte count in ascites.

**Table 5 Predictive results of the constructed model in the original study population**

| Predictive results | Observed results | | Percentage correct |
|---|---|---|---|
| | Asymptomatic SBP | Control group | |
| Asymptomatic SBP | 72 | 26 | 73.5% |
| Control group | 13 | 85 | 86.7% |

SBP: Spontaneous bacterial peritonitis.

small, which may have influenced the accuracy of the established model. SBP patients might not have been admitted to hospital without obvious clinical symptoms; moreover, the positive rate of ascites culture was relatively low. This resulted in a relatively small sample size. Second, the diagnostic performance of the predictive model was only verified in our patient cohort. Only 98 cases met the case conditions; thus, these patients could not be divided into a modeling group and a validation group. Therefore, the validation group was not adopted to verify our model. The cross-validation with a larger sample size will be required to confirm the efficacy and further improve our predictive model. Third, the present study was a retrospective investigation, and some indicators that may have been significant for early diagnosis of SBP could not be obtained. Thus, some potential biomarkers were not taken into consideration in our study, such as inflammatory factors. Therefore, well-designed

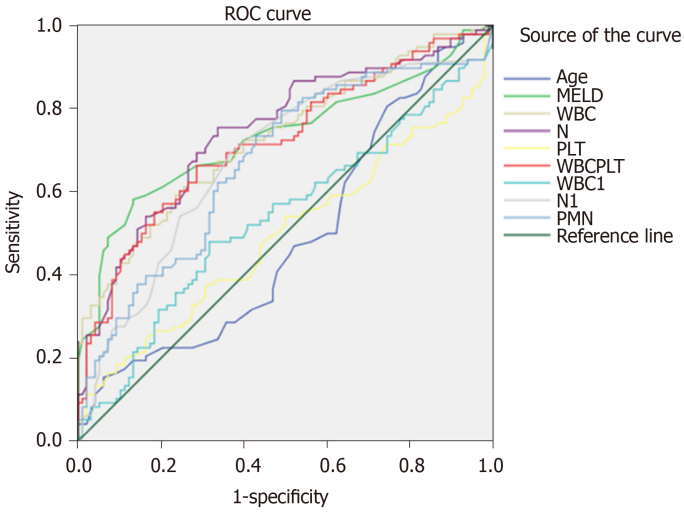


**Figure 1 Receiver operating characteristic analysis of the continuous variables for the selected 196 patients.** ROC: Receiver operating characteristic; MELD: Model for end-stage liver disease; WBC: White blood cell count; N: Blood neutrophil percentage; PLT: Platelet; PMN: Polymorphonuclear.

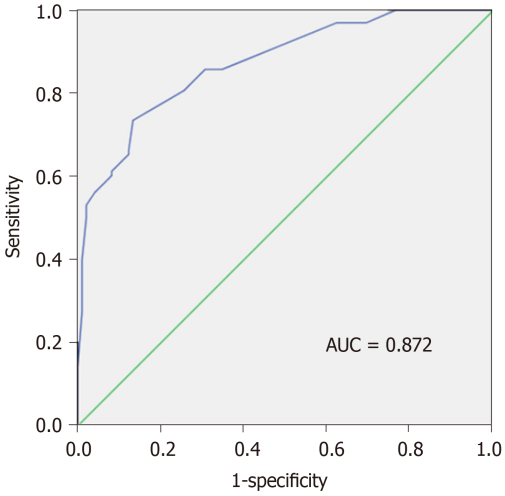


**Figure 2 The receiver operating characteristic curve was plotted based on the *P* values of the patients calculated using the screening model.** Area under curve value was 0.872, with a sensitivity of 73.5% and a specificity of 86.7%. AUC: Area under curve.

prospective studies are required to improve and verify our study.

In conclusion, a multivariate predictive model was established for the early diagnosis of asymptomatic SBP patients with positive microbiological results to determine which patients should be treated with antibiotics. Our predictive model was based on MELD, PMN, blood N, HCC, and renal dysfunction, which may enhance antibiotic treatment in asymptomatic SBP patients.

## ARTICLE HIGHLIGHTS

### *Research background*

Spontaneous bacterial peritonitis (SBP) is a detrimental infection of the ascitic fluid in liver cirrhosis patients, with high mortality and morbidity. Early diagnosis and timely antibiotic administration have successfully decreased the mortality rate to 20%-25%. Early diagnosis of asymptomatic SBP remains a great challenge in the clinic.

### *Research motivation*

Currently, SBP cases are diagnosed based only on clinical symptoms, leading to possible antibiotic abuse. SBP is regulated by a variety of risk factors, including decreased activity of the reticuloendothelial system, advanced liver dysfunction,

medications, and genetic factors. A multivariate predictive model may be effective for early screening of asymptomatic SBP.

### Research objectives

The present retrospective cohort study aimed to establish an effective predictive model for early screening of asymptomatic SBP in liver cirrhosis patients with ascites. Early diagnosis of asymptomatic SBP will improve antibiotic management strategies and reduce SBP-associated mortality.

### Research methods

Liver cirrhosis patients with ascites who had no typical SBP symptoms were included in the current study, and divided into the case (positive cultures) and control (negative cultures) groups according to microbiological results. The demographic features, clinical information, disease activity, hematological and ascites factors were compared between the case and control groups to identify potential indicators of asymptomatic SBP. The multiple linear stepwise regression method of the logistic regression model was adopted to construct the multivariate predictive model. The diagnostic performance of the model was estimated by the receiver operating characteristic curve.

### Research results

Patients in the case group were more likely to have advanced disease stages, cirrhosis related-complications, worsened hematology and ascites, and higher mortality. Based on multivariate analysis, the predictive model was as follows: y (*P*) = 0.018 + 0.312 × MELD (model of end-stage liver disease) + 0.263 × PMN (ascites polymorphonuclear) + 0.184 × N (blood neutrophil percentage) + 0.233 × HCC (hepatocellular carcinoma) + 0.189 × renal dysfunction. The area under curve value of the established model was 0.872, revealing its high diagnostic potential. The diagnostic sensitivity was 73.5% (72/98), the specificity was 86.7% (85/98), and the diagnostic efficacy was 80.1%.

### Research conclusions

The multivariate predictive model based on model of end-stage liver disease, polymorphonuclear, blood neutrophil percentage, hepatocellular carcinoma, and renal dysfunction exerts high diagnostic efficacy which may improve the early diagnosis of asymptomatic SBP.

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ORIGINAL ARTICLE

Retrospective Study

# Clinicopathological characteristics and surgical outcomes of sarcomatoid hepatocellular carcinoma

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**Author contributions:** Wang JP and Liu J designed the study; Yao ZG, Lin CH, Lv BB and Zhang SJ collected and analyzed the pathological data; Ren FX and Wang Y collected and analyzed the imaging data; Liu XH, Sun FK, Meng FY, Zheng SZ and Gong W collected and analyzed the clinical data; Sun YW performed statistical analyses; Wang JP drafted the manuscript; Liu J supervised the study and revised the manuscript.

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## Abstract

***BACKGROUND***

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer and the fourth leading cause of cancer-related death worldwide. Sarcomatoid HCC, which

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**Institutional review board statement:** This study was reviewed and approved by the Ethics Committee of Shandong Provincial Hospital.

**Informed consent statement:** Patients were not required to give informed consent for this study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

**Conflict-of-interest statement:** The authors have no conflict-of interest to disclose.

**Data sharing statement:** No additional data are available.



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contains poorly differentiated carcinomatous and sarcomatous components, is a rare histological subtype of HCC that differs from conventional HCC. It is highly aggressive and has a poor prognosis. Its clinicopathological characteristics, surgical outcomes and underlying mechanisms of its highly aggressive nature have not been fully elucidated.

*AIM*
To examine the clinicopathological characteristics and surgical outcomes of sarcomatoid HCC and explore the histogenesis of sarcomatoid HCC.

*METHODS*
In total, 196 patients [41 sarcomatoid HCC and 155 high-grade (Edmondson-Steiner grade III or IV) HCC] who underwent surgical resection between 2007 and 2017 were retrospectively reviewed. The characteristics and surgical outcomes of sarcomatoid HCC were compared with those of patients with high-grade HCC. The histological composition of invasive and metastatic sarcomatoid HCCs was evaluated.

*RESULTS*
Sarcomatoid HCC was more frequently diagnosed at an advanced stage with a larger tumor and higher rates of nonspecific symptom, adjacent organ invasion and lymph node metastasis than high-grade HCC (all $P < 0.05$). Compared with high-grade HCC patients, sarcomatoid HCC patients are less likely to have typical dynamic imaging features of HCC (44.4% *vs* 72.7%, $P = 0.001$) and elevated serum alpha-fetoprotein levels (> 20 ng/mL; 36.6% *vs* 78.7%, $P < 0.001$). The sarcomatoid group had a significantly shorter median recurrence-free survival (5.6 mo *vs* 16.4 mo, log-rank $P < 0.0001$) and overall survival (10.5 mo *vs* 48.1 mo, log-rank $P < 0.0001$) than the high-grade group. After controlling for confounding factors, the sarcomatoid subtype was identified as an independent predictor of poor prognosis. Pathological analyses indicated that invasive and metastatic lesions were mainly composed of carcinomatous components.

*CONCLUSION*
Sarcomatoid HCC was associated with a more advanced stage, atypical dynamic imaging, lower serum alpha-fetoprotein levels and a worse prognosis. The highly aggressive nature of sarcomatoid HCC is perhaps mediated by carcinomatous components.

**Key words:** Sarcomatoid hepatocellular carcinoma; Histological composition; Liver resection; Overall survival; Recurrence-free survival



**Core tip:** Sarcomatoid hepatocellular carcinoma is a rare malignancy. Patients usually present with an advanced stage of the disease and have a poor prognosis. Histopathological examination plays an important role in diagnosis because serologic and radiologic examinations cannot help in distinguishing this disease from conventional hepatocellular carcinoma or other intrahepatic masses. Most patients have a history of chronic liver disease; thus, regular ultrasound screening of such patients can help detect tumors at an early stage and reduce the risk of death. Moreover, we discovered that carcinomatous components occupied the predominant proportion of invasions and metastases, and we developed a hypothesis regarding the occurrence of the disease.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer and the fourth leading cause of cancer-related death worldwide[1]. Sarcomatoid HCC is a histological subtype of HCC that differs from conventional HCC and presents with an unusual characteristic: sarcomatoid HCCs contain variable proportions of sarcomatous and carcinomatous components, wherein the sarcomatous component usually consists of spindle-shaped cells that form interlacing bundles and show a partial storiform pattern, and the carcinomatous component commonly comprises poorly differentiated [Edmondson-Steiner (ES) grade III or IV] conventional HCC cells[2,3]. Previous studies have indicated that certain anticancer therapies, such as transcatheter arterial chemoembolization (TACE), can lead to more frequent sarcomatous changes in HCC[4,5]. However, an increasing number of sarcomatoid HCC cases without previous anticancer therapy have been reported[6-8]. To date, the pathogenesis of the sarcomatous change has not been elucidated.

Sarcomatoid HCC is a rare malignancy, with an incidence of 1.7%-1.9% among surgically resected HCC cases and 3.9%-9.4% among autopsied HCC patients[3,4,8-11]. A few studies have reported that sarcomatoid HCC is associated with a higher recurrence rate, more frequent metastasis and poorer survival than conventional HCC[2,3,8,12]. However, these studies did not further stratify conventional HCC into low- (ES grade I and II) and high-grade (ES grade III and IV) HCC; in particular, high-grade HCC is considered similar to sarcomatoid HCC in terms of histological differentiation, more aggressive nature and poor prognosis[2,3,13,14]. In addition, although sarcomatoid HCC has a high incidence of adjacent organ invasion and metastasis[8], the underlying mechanisms remain unknown. One previous study reported that most portal venous invasions and metastases had sarcomatous components, indicating that the sarcomatous component is responsible for metastasis[3].

In this study, we comprehensively compared the clinicopathological characteristics and surgical outcomes of sarcomatoid HCC and high-grade HCC patients. We also analyzed the histological composition of metastatic and invasive sarcomatoid HCCs to study the relative importance of sarcomatous and carcinomatous components in highly aggressive behavior.

## MATERIALS AND METHODS

### *Patient selection*

From January 2007 to December 2017, a total of 2287 patients underwent surgical resection for HCC at Shandong Provincial Hospital. We retrospectively reviewed the pathological records of these patients, of which 186 were diagnosed with high-grade HCC defined as grade III or IV differentiated HCC according to the ES classification[15]. In addition, 45 patients who underwent surgical resection for sarcomatoid HCC were identified from the pathology database of Shandong Provincial Hospital. We excluded patients who had previous interventions (including TACE, radiofrequency ablation and previous surgical resection) or missing data. A total of 41 sarcomatoid HCC and 155 high-grade HCC patients were included in the final analysis. The flowchart of patient selection is shown in Figure 1.

### *Data collection*

The medical records of the included patients were retrospectively reviewed. A standardized record form was used to collect clinical information, including age, sex, symptoms, alcoholism, hepatitis virus B or C infection, liver cirrhosis, laboratory test results, Child-Pugh classification and tumor-specific characteristics, such as tumor size, tumor number and macrovascular invasion. Tumor staging was classified according to the Barcelona Clinic Liver Cancer staging system and the American Joint Committee on Cancer (AJCC) staging system (8$^{th}$ edition, 2017). Computed tomography (CT) and magnetic resonance imaging (MRI) images were also reviewed.

### *Postoperative follow-up*

Routine follow-ups were conducted during the 1$^{st}$ and 3$^{rd}$ mo after resection and subsequently every 2 to 3 mo during the first postoperative year and every 3 to 6 mo thereafter. At each follow-up, serum alpha-fetoprotein (AFP) levels and liver function were assessed, and abdominal ultrasonography was completed. An abdominal CT or MRI was performed at an interval of 6 to 12 mo depending on the postoperative time. If recurrence was suspected, an additional CT or MRI scan was performed

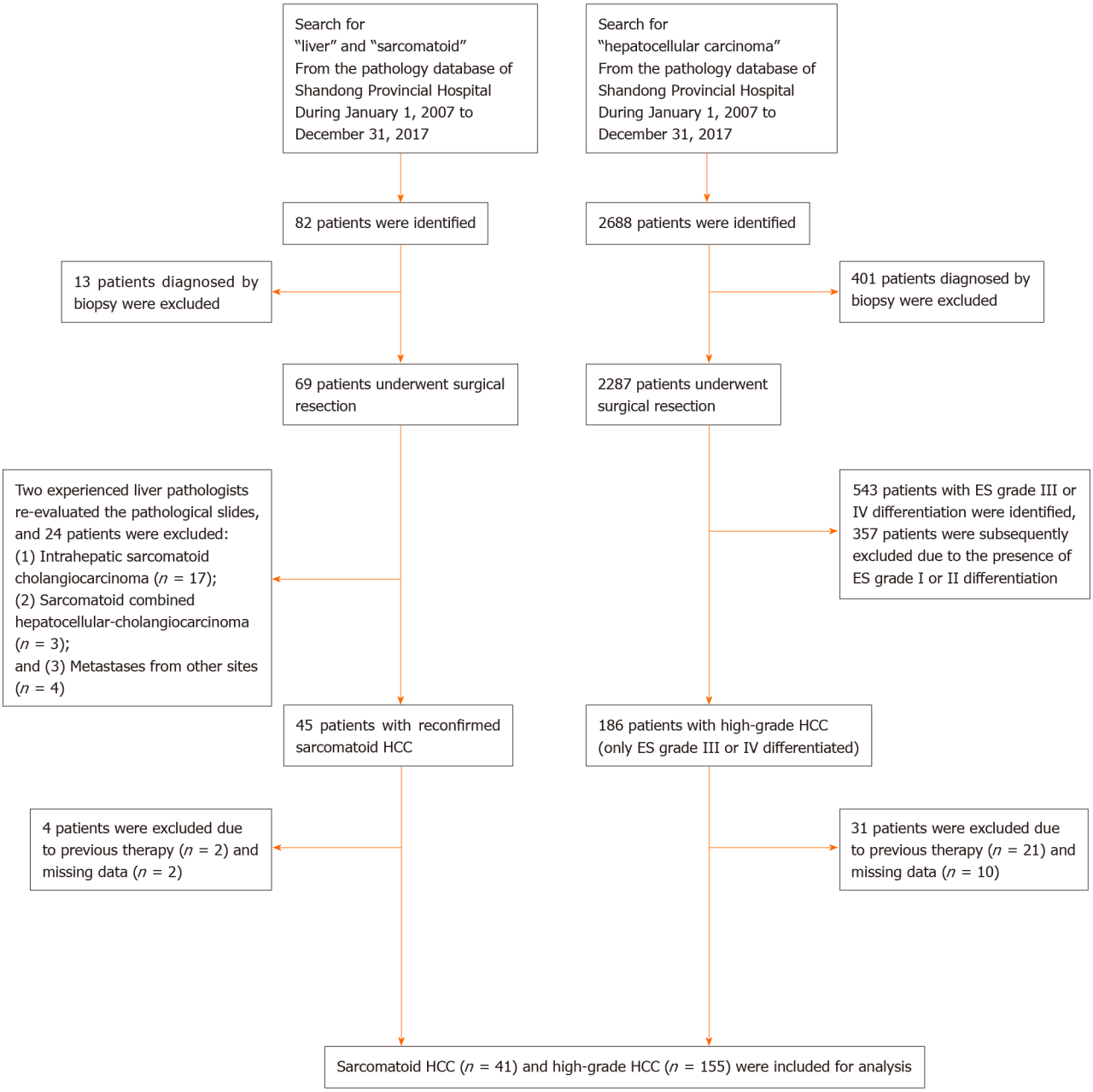


**Figure 1 Flowchart of patient selection.** ES: Edmondson-Steiner; HCC: Hepatocellular carcinoma.

immediately. Overall survival (OS) was defined as the time from the date of surgery to the date of death or last follow-up. Recurrence was defined as the appearance of a new lesion as confirmed by CT or MRI during follow-up. Recurrence-free survival (RFS) was calculated from the date of surgery to the date of the first documented recurrence, death or last follow-up.

### *Statistical analysis*

Numerical data are presented as the median (range) or mean ± standard deviation. Differences between groups were compared using Pearson's $\chi^2$ test or the two-tailed Fisher's exact test for categorical data and the Mann-Whitney *U* test for numerical data. OS and RFS were determined using the Kaplan-Meier method, and differences between groups were assessed by the log-rank test. To evaluate further the impact of histological subtype on prognosis, univariate analyses of prognostic factors were performed using univariate Cox regression analysis. Among the parameters with $P < 0.1$ in the univariate analyses, age, serum AFP level, AJCC stage, differentiation grade of the carcinomatous component and histological subtype were used to build a basic

multivariate Cox proportional hazard model since they reflect multiple aspects of a patient's condition. We also adjusted for variables that changed the matched hazard ratio of the histological subtype by at least 5% upon addition into the model[16]. A $P$ value < 0.05 was considered to indicate statistical significance. Kaplan-Meier curves were generated and analyzed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, United States). All other statistical analyses were performed using IBM SPSS 23.0 software (SPSS Inc., Armonk, NY, United States).

## RESULTS

### Clinical and laboratory characteristics of the studied patients

A total of 196 patients, including 41 sarcomatoid and 155 high-grade HCC patients, were included in this study, and the clinical and laboratory characteristics are shown in Table 1. In both the sarcomatoid and high-grade HCC groups, the majority of the patients were men (80.5% and 86.5%, $P$ = 0.339), with a median age of 54 years and 55 years ($P$ = 0.942), respectively. During the first hospital visit, sarcomatoid HCC patients had a higher incidence of concomitant symptoms than high-grade HCC patients (78.0% *vs* 55.5%, $P$ = 0.009), including symptoms of epigastric discomfort (63.4% *vs* 43.2%), weight loss (34.1% *vs* 18.1%) and fever (22% *vs* 2.6%). The etiology of hepatopathy, presence of cirrhosis, Child-Pugh classification and laboratory test results were comparable between the two groups (all $P$ > 0.05). However, serum AFP levels were significantly lower in the sarcomatoid group than in the high-grade group (5.8 ng/mL *vs* 348.0 ng/mL, $P$ < 0.001).

### Tumor-specific characteristics of the studied patients

The tumor-specific characteristics are shown in Table 2. At the time of diagnosis, the frequency of sarcomatoid HCC patients presenting with typical dynamic image patterns (arterial phase enhancement and portal and delayed phase washout) was significantly lower than that of patients with high-grade HCC (44.4% *vs* 72.7%, $P$ = 0.001). Some sarcomatoid HCCs might be misdiagnosed as intrahepatic cholangiocarcinoma (iCCA) or a hepatic abscess (Figure 2). Compared to high-grade HCC patients, sarcomatoid HCC patients had larger tumors, a lower incidence of tumor encapsulation and higher frequencies of tumor necrosis, adjacent organ invasion, lymph node metastasis and advanced AJCC stage (all $P$ < 0.05). In addition, the two groups showed significant differences in the differentiation grades of carcinomatous components ($P$ < 0.05). Patients in the sarcomatoid HCC group showed a trend towards a higher postoperative recurrence rate than those in the high-grade HCC group, but the difference was not significant ($P$ = 0.091).

### Sarcomatoid HCC patients have a worse prognosis than high-grade HCC patients

The sarcomatoid HCC patients had a shorter median OS than high-grade HCC patients (10.5 mo *vs* 48.1 mo, $P$ < 0.0001, Figure 3A). The 1-, 3- and 5-year OS rates were 48.8%, 17.3% and 11.5% for the sarcomatoid group and 85.2%, 53.4% and 41.1% for the high-grade group, respectively. Moreover, the sarcomatoid HCC group had a shorter median RFS than the high-grade HCC group (5.6 mo *vs* 16.4 mo, $P$ < 0.0001, Figure 3B). The RFS rates post resection were 49.7% and 83.1% at 6 mo, 20.9% and 60.2% at 1 year and 6.3% and 31.8% at 3 years for the sarcomatoid and high-grade groups, respectively. Even after stratification by AJCC stage (Figure 4) or differentiation grade of the carcinomatous component (Figure 5), the sarcomatoid HCC patients still had worse OS and shorter RFS than the high-grade HCC patients in each subgroup (all $P$ < 0.05).

### Sarcomatoid subtype is an independent predictor of poor prognosis

Cox regression analysis was used to verify whether the sarcomatoid type is an independent prognostic factor for HCC patients. Our univariate analysis showed that larger tumors, multiple tumors, a lack of tumor encapsulation, tumor necrosis, macro- and microvascular invasion, adjacent organ invasion, lymph node metastasis, advanced AJCC stage, poorer differentiation of the carcinomatous component and sarcomatoid subtype were significantly associated with increased mortality (Table 3) and recurrence (Table 4) in the total population (all $P$ < 0.05). In addition, age < 55 years, Child-Pugh B and lower serum AFP levels were significantly related to increased mortality (all $P$ < 0.05, Table 3). After controlling for confounding factors, sarcomatoid subtype was identified as an independent predictor of poorer OS and RFS

**Table 1 Clinical and laboratory characteristics of the studied patients, *n* (%)**

| Variables | Sarcomatoid HCC, *n* = 41 | High-grade HCC, *n* = 155 | *P* value |
|---|---|---|---|
| Age at diagnosis, median (range), yr | 54 (15-74) | 55 (28-81) | 0.942 |
| Sex (male/female) | 33/8 | 134/21 | 0.339 |
| Follow-up duration, median (range), mo | 10.5 (1.7-81.7) | 27.7 (2.1-138.5) | < 0.001 |
| Symptom at diagnosis | 32 (78.0) | 86 (55.5) | 0.009 |
| Alcoholism | 10 (24.4) | 38 (24.5) | 0.987 |
| HBsAg(+) | 29 (70.7) | 124 (80.0) | 0.202 |
| HCV-Ab(+) | 3 (7.3) | 5 (3.2) | 0.368 |
| Liver cirrhosis | 32 (78.0) | 133 (85.8) | 0.226 |
| Child-Pugh class, A/B | 36/5 | 143/12 | 0.359 |
| AFP, median (range), ng/mL | 5.8 (1.0-16660.0) | 348.0 (2.0-63208.5) | < 0.001 |
| AFP level | | | < 0.001 |
| ≤ 20 ng/mL | 26 (63.4) | 33 (21.3) | |
| > 20, < 400 ng/mL | 10 (24.4) | 47 (30.3) | |
| ≥ 400 ng/mL | 5 (12.2) | 75 (48.4) | |
| Creatinine, median (range), mg/dL | 0.77 (0.50-1.11) | 0.76 (0.49-1.58) | 0.475 |
| ALT, median (range), U/L | 26 (10-145) | 32 (8-301) | 0.204 |
| Total bilirubin, median (range), mg/dL | 0.85 (0.33-2.45) | 0.95 (0.33-10.29) | 0.095 |
| Albumin, mean ± SD, g/dL | 3.9 ± 0.5 | 4.0 ± 0.5 | 0.082 |
| INR, median (range) | 1.11 (0.96-1.53) | 1.08 (0.84-1.50) | 0.066 |

AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen; HCC: Hepatocellular carcinoma; HCV-Ab: Hepatitis C virus antibody; INR: International normalized ratio; SD: Standard deviation.

in the multivariable analysis (all $P < 0.05$, Table 3 and Table 4).

### *Invasive and metastatic sarcomatoid HCCs mainly comprise carcinomatous components*

Sarcomatoid HCC is composed of both sarcomatous and carcinomatous components (Figure 6); however, it remains unknown which component is the primary contributor to the highly aggressive nature of sarcomatoid HCC. To explore further this, the histological composition of lymph node metastases, macrovascular invasions, bile duct invasions and multiple liver tumor lesions of sarcomatoid HCCs were analyzed. Nine patients showed a total of 33 lymph node metastases. Of these, 26 (78.8%) metastases contained purely carcinomatous components, two (6.1%) were purely sarcomatous and five (15.1%) had mixed carcinomatous and sarcomatous components. Seven macrovascular invasions and two bile duct invasions were observed in eight patients (one patient had both macrovascular and bile duct invasion). Six of these invasions were evaluated by pathological examination, and only two (33.3%) had sarcomatous components. A total of 16 patients were confirmed to have multiple liver tumors (10 patients with multinodular HCC and six with satellite nodules). Of the 10 patients with multinodular HCC, only two (20.0%) had concurrent sarcomatoid HCC, whereas the others had simultaneous sarcomatoid and conventional HCC. In addition, of the six patients with satellite nodules, sarcomatous changes were found in only two patients (33.3%).

### *Proportion of sarcomatous components in sarcomatoid HCC does not predict survival*

Next, we divided the sarcomatoid HCC patients into three subgroups according to the proportion of sarcomatous components in the tumor: (1) Mixed subgroup ≤ 50% ($n$ = 14 patients); (2) Mixed subgroup > 50% ($n$ = 16); and (3) Pure subgroup ($n$ = 11). The OS and RFS were similar among these subgroups (Figure 7).

**Table 2 Tumor-specific characteristics of the studied patients, *n* (%)**

| Variables | Sarcomatoid HCC, *n* = 41 | High-grade HCC, *n* = 155 | *P* value |
|---|---|---|---|
| Tumor size, median (range), cm | 8.6 (1.5-24.0) | 5.5 (1.3-19.0) | 0.007 |
| Tumor number, single/multiple | 25/16 | 108/47 | 0.443 |
| Tumor location | | | 0.549 |
| Left lobe | 11 (26.8) | 34 (21.9) | |
| Right lobe | 28 (68.3) | 106 (68.4) | |
| Both lobes | 2 (4.9) | 15 (9.7) | |
| Tumor encapsulation | 11 (26.8) | 77 (49.7) | 0.009 |
| Tumor necrosis | 35 (85.4) | 53 (34.2) | < 0.001 |
| Spontaneous rupture | 4 (9.8) | 9 (5.8) | 0.477 |
| Adjacent organ invasion | 9 (22.0) | 13 (8.4) | 0.024 |
| Satellite nodules | 6 (14.6) | 25 (16.1) | 0.816 |
| Macrovascular invasion | 7 (17.1) | 34 (21.9) | 0.496 |
| Microvascular invasion | 16 (39.0) | 63 (40.6) | 0.851 |
| Lymph node metastasis | 9 (22.0) | 9 (5.8) | 0.004 |
| Differentiation grade of the carcinomatous component | | | < 0.001 |
| II | 3 (7.3) | 0 (0) | |
| III | 19 (46.4) | 116 (74.8) | |
| IV | 8 (19.5) | 39 (25.2) | |
| NA[1] | 11 (26.8) | 0 (0) | |
| AJCC stage | | | 0.006 |
| Stage I | 10 (24.4) | 68 (43.9) | |
| Stage II | 7 (17.0) | 25 (16.1) | |
| Stage III | 15 (36.6) | 53 (34.2) | |
| Stage IV | 9 (22.0) | 9 (5.8) | |
| BCLC stage | | | 0.543 |
| 0 | 0 (0) | 2 (1.3) | |
| A | 19 (46.4) | 95 (61.3) | |
| B | 6 (14.6) | 15 (9.7) | |
| C | 16 (39.0) | 43 (27.7) | |
| Recurrence | 34 (82.9) | 108 (69.7) | 0.091 |
| Recurrence pattern | 34 | 108 | 0.246 |
| Intrahepatic | 14 (41.2) | 62 (57.4) | |
| Extrahepatic | 8 (23.5) | 17 (15.7) | |
| Both | 12 (35.3) | 29 (26.9) | |
| Imaging data at diagnosis | 36 | 139 | |
| Typical dynamic image patterns[2] | 16 (44.4) | 101 (72.7) | 0.001 |

[1]The tumor consists purely of sarcomatous components.
[2]Arterial phase enhancement, portal and delayed phase washout. AJCC: American Joint Committee on Cancer; BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; NA: Not available.

**Table 3 Univariable and multivariable analyses of overall survival of the studied patients**

| Variables | HR (95%CI) | *P* value | HR (95%CI) | *P* value |
|---|---|---|---|---|
| Age, ≥ 55 yr *vs* < 55 yr | 0.687 (0.477-0.991) | 0.044 | 0.977 (0.654-1.460) | 0.910 |
| Sex, male *vs* female | 0.972 (0.579-1.630) | 0.913 | | |
| Hepatitis virus infection, yes *vs* no | 1.140 (0.718-1.811) | 0.579 | | |
| Alcoholism, yes *vs* no | 1.101 (0.723-1.677) | 0.653 | | |
| Liver cirrhosis, yes *vs* no | 1.027 (0.620-1.703) | 0.917 | | |
| Child-Pugh, B *vs* A | 2.287 (1.281-4.083) | 0.005 | Excluded[1] | Excluded |
| AFP, > 20 ng/mL *vs* ≤ 20 ng/mL | 0.560 (0.386-0.813) | 0.002 | 0.555 (0.377-0.817) | 0.003 |
| Tumor size, > 5 cm *vs* ≤ 5 cm | 2.718 (1.806-4.089) | < 0.001 | 1.871 (1.146-3.054) | 0.012 |
| Tumor number, multiple *vs* single | 1.647 (1.129-2.402) | 0.010 | Excluded | Excluded |
| Tumor encapsulation, yes *vs* no | 0.443 (0.302-0.651) | < 0.001 | Excluded | Excluded |
| Tumor necrosis, yes *vs* no | 2.237 (1.548-3.233) | < 0.001 | 0.973 (0.622-1.523) | 0.904 |
| Macrovascular invasion, yes *vs* no | 3.403 (2.256-5.133) | < 0.001 | 2.308 (1.438-3.702) | 0.001 |
| Microvascular invasion, yes *vs* no | 1.615 (1.115-2.338) | 0.011 | Excluded | Excluded |
| Adjacent organ invasion, yes *vs* no | 2.329 (1.407-3.857) | 0.001 | Excluded | Excluded |
| Lymph node metastasis, yes *vs* no | 2.413 (1.371-4.249) | 0.002 | Excluded | Excluded |
| AJCC stage, III + IV *vs* I + II | 3.221 (2.200-4.715) | < 0.001 | 1.763 (1.085-2.864) | 0.022 |
| Histological subtype, sHCC *vs* hgHCC | 3.460 (2.283-5.243) | < 0.001 | 3.140 (2.032-4.851) | < 0.001 |
| Differentiation grade of carcinomatous component | | $P_{trend}$ < 0.001 | | $P_{trend}$ = 0.421 |
| NA[2] | 1 (reference) | | 1 (reference) | |
| II | 0.383 (0.083-1.758) | 0.217 | 0.297 (0.062-1.422) | 0.129 |
| III | 0.226 (0.115-0.444) | < 0.001 | 0.644 (0.281-1.475) | 0.298 |
| IV | 0.327 (0.159-0.670) | 0.002 | 0.739 (0.311-1.755) | 0.494 |

[1]Because of changing the matched hazard ratio of histological subtype less than 5%.
[2]The tumor consists purely of sarcomatous components. AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; CI: Confidence interval; hgHCC: High-grade hepatocellular carcinoma; HR: Hazard ratio; NA: Not available; sHCC: Sarcomatoid hepatocellular carcinoma.

## DISCUSSION

Sarcomatoid HCC is a rare histological subtype of HCC, with an incidence of approximately 2% of surgically resected cases[3,8]. A few studies have reported that sarcomatoid HCC is more aggressive than conventional HCC and associated with a worse prognosis[2,3,8,12]. However, sarcomatoid HCC usually has a worse differentiation grade[12], which is regarded as a prognostic factor for HCC, and this fact can lead to inaccurate comparisons between sarcomatoid HCC and conventional HCC. In this study, high-grade HCC, which is thought to be similar to sarcomatoid HCC in terms of histological differentiation, was used as the control in a detailed examination of the clinicopathological characteristics and surgical outcomes of sarcomatoid HCC. Moreover, this study analyzed the histological composition of metastatic and invasive sarcomatoid HCCs.

Our results demonstrate that sarcomatoid HCC is more frequently diagnosed at an advanced AJCC stage with relatively larger tumors and higher rates of adjacent organ invasion and lymph node metastasis. During the first visit to the hospital, sarcomatoid HCC patients have a higher incidence of epigastric discomfort, weight loss and fever than high-grade HCC patients. In particular, the incidence of fever was nearly 10 times higher in the sarcomatoid HCC group than in the high-grade HCC group, perhaps due to the higher frequency of tumor necrosis caused by the relatively larger size and faster progression of sarcomatoid HCC tumors. Imaging plays a critical role in HCC diagnosis. There are typical dynamic image patterns considered specific for HCC[17]. However, more than 60% of sarcomatoid HCCs show MRI features more similar to

**Table 4 Univariable and multivariable analyses of recurrence-free survival of the studied patients**

| Variables | HR (95% CI) | *P* value | HR (95% CI) | *P* value |
|---|---|---|---|---|
| Age, ≥ 55 yr *vs* < 55 yr | 0.743 (0.533-1.035) | 0.079 | 0.857 (0.605-1.214) | 0.386 |
| Sex, male *vs* female | 0.771 (0.493-1.206) | 0.254 | | |
| Hepatitis virus infection, yes *vs* no | 1.369 (0.879-2.133) | 0.165 | | |
| Alcoholism, yes *vs* no | 0.940 (0.634-1.394) | 0.759 | | |
| Liver cirrhosis, yes *vs* no | 1.283 (0.790-2.084) | 0.314 | | |
| Child-Pugh, B *vs* A | 1.699 (0.938-3.078) | 0.081 | Excluded[1] | Excluded |
| AFP, > 20 ng/mL *vs* ≤ 20 ng/mL | 0.710 (0.501-1.006) | 0.054 | 0.680 (0.469-0.986) | 0.042 |
| Tumor size, > 5 cm *vs* ≤ 5 cm | 2.398 (1.685-3.413) | < 0.001 | 1.680 (0.469-0.986) | 0.015 |
| Tumor number, multiple *vs* single | 2.058 (1.460-2.902) | < 0.001 | 1.210 (0.824-1.777) | 0.331 |
| Tumor encapsulation, yes *vs* no | 0.561 (0.400-0.786) | 0.001 | Excluded | Excluded |
| Tumor necrosis, yes *vs* no | 1.726 (1.239-2.404) | 0.001 | Excluded | Excluded |
| Macrovascular invasion, yes *vs* no | 2.922 (1.961-4.354) | < 0.001 | Excluded | Excluded |
| Microvascular invasion, yes *vs* no | 1.646 (1.178-2.300) | 0.003 | Excluded | Excluded |
| Adjacent organ invasion, yes *vs* no | 2.329 (1.445-3.753) | 0.001 | Excluded | Excluded |
| Lymph node metastasis, yes *vs* no | 2.327 (1.356-3.992) | 0.002 | Excluded | Excluded |
| AJCC stage, III + IV *vs* I + II | 3.416 (2.420-4.821) | < 0.001 | 2.752 (1.879-4.032) | < 0.001 |
| Histological subtype, sHCC *vs* hgHCC | 2.842 (1.913-4.222) | < 0.001 | 2.355 (1.564-3.546) | < 0.001 |
| Differentiation grade of carcinomatous component | | $P_{trend}$ = 0.036 | | $P_{trend}$ = 0.194 |
| NA[2] | 1 (reference) | | 1 (reference) | |
| II | 1.001 (0.264-3.790) | 0.999 | 0.650 (0.162-2.603) | 0.543 |
| III | 0.445 (0.215-0.922) | 0.029 | 1.605 (0.672-3.832) | 0.287 |
| IV | 0.642 (0.298-1.380) | 0.256 | 2.025 (0.822-4.988) | 0.125 |

[1]Because of changing the matched hazard ratio of histological subtype less than 5%.
[2]The tumor consists purely of sarcomatous components. AFP: Alpha-fetoprotein; AJCC: American Joint Committee on Cancer; CI: Confidence interval; hgHCC: High-grade hepatocellular carcinoma; HR: hazard ratio; NA: Not available; sHCC: Sarcomatoid hepatocellular carcinoma.

iCCA than to HCC[18]. In our study, only 44.4% of sarcomatoid HCC patients presented with typical dynamic image patterns of HCC, and some patients with sarcomatoid HCC may be misdiagnosed with iCCA or a hepatic abscess. Consistent with the results of previous studies[8,12], our findings show that the majority of patients with sarcomatoid HCC had a history of chronic viral hepatitis and liver cirrhosis, which are key risk factors for the development of HCC[19]. Regular ultrasound screening of these high-risk patients could help detect tumors at an early stage and reduce the risk of death[19,20]. Of note, only 36.6% of sarcomatoid HCC patients had serum AFP levels > 20 ng/mL in the current study. Therefore, serum AFP tests may have no use for the early detection of sarcomatoid HCC. Moreover, both serologic and radiologic examinations could not help in distinguishing the sarcomatoid subtype from conventional HCC or other intrahepatic masses, and this might be problematic when selecting a potential recipient of liver transplantation because the sarcomatoid subtype usually predicts a poor prognosis[2].

Previous studies have demonstrated that patients with resected sarcomatoid HCC have a worse RFS than those with resected conventional HCC[2,8]. Similar to the findings of previous studies, we found that the median RFS of sarcomatoid HCC patients was significantly shorter than that of high-grade HCC patients (5.6 mo *vs* 16.4 mo, $P < 0.0001$). A previous study from Taiwan indicated that sarcomatoid HCC is more prone to extrahepatic metastasis than conventional HCC after curative therapy[12]. Nevertheless, in our study, there was no difference in the recurrence pattern between the sarcomatoid and high-grade HCC groups, perhaps due to their similar histological differentiation. Several previous studies have also indicated that

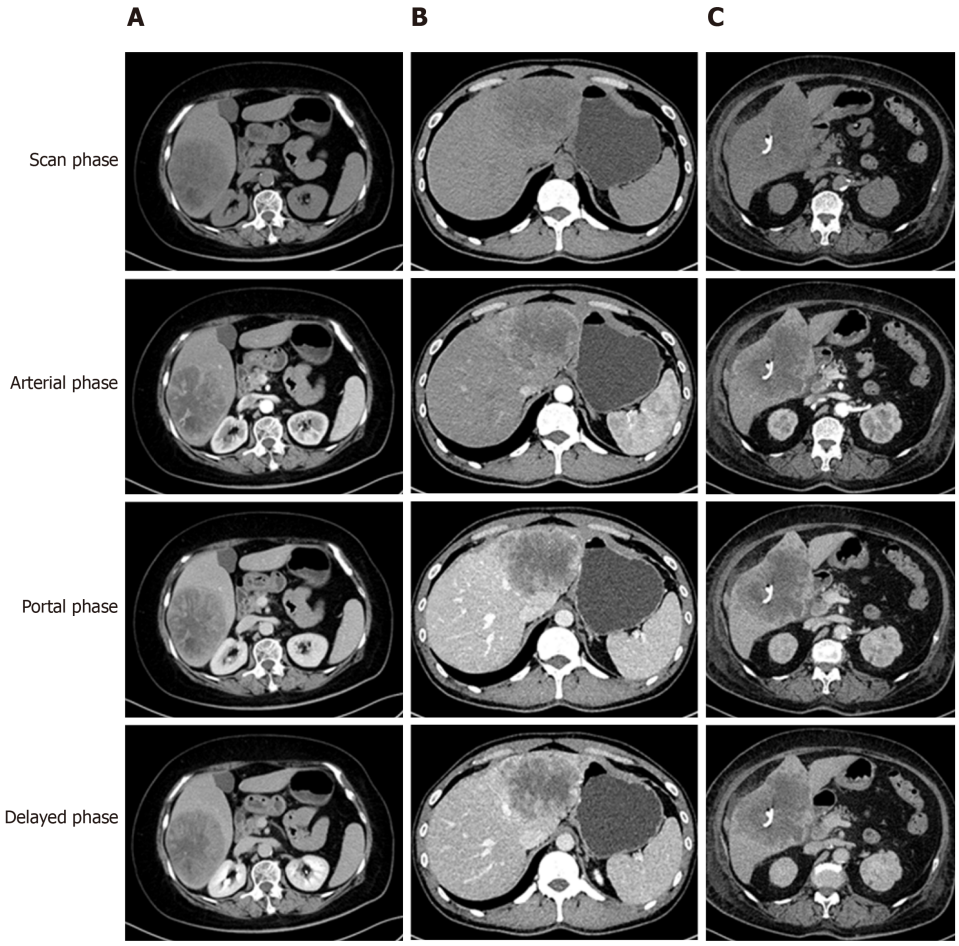


**Figure 2 Imaging findings of sarcomatoid hepatocellular carcinoma in patients with various initial diagnoses based on radiologic findings.** A: Hepatocellular carcinoma; B: Intrahepatic cholangiocarcinoma; C: Hepatic abscess.

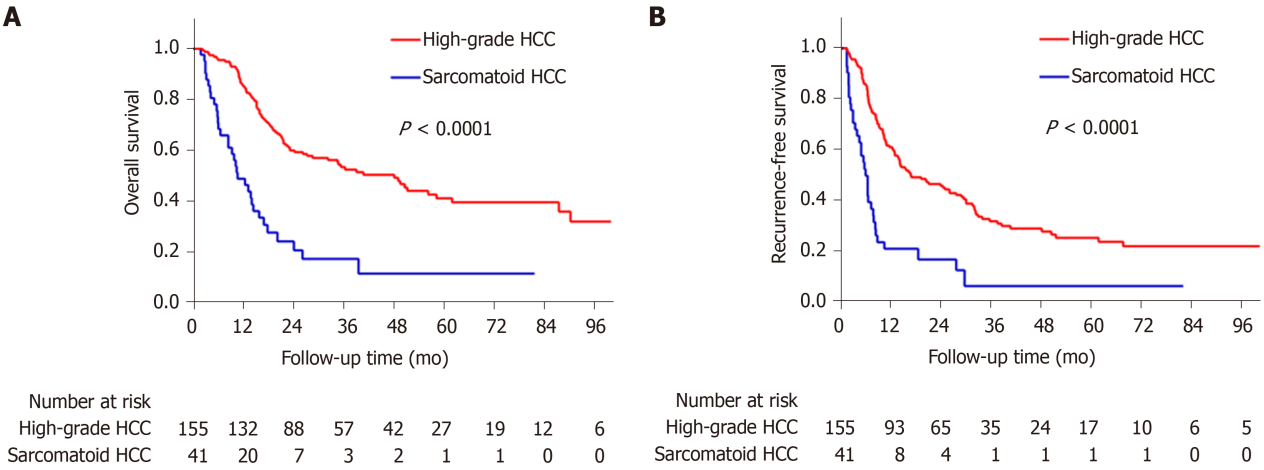


**Figure 3 Kaplan-Meier estimated overall and recurrence-free survival curves.** A: Sarcomatoid hepatocellular carcinoma (HCC) is associated with worse overall survival (log-rank $P < 0.0001$); B: Sarcomatoid HCC is associated with worse recurrence-free survival (log-rank $P < 0.0001$).

the OS of sarcomatoid HCC patients is significantly worse than that of conventional HCC patients, with a 3-year OS rate ranging from 8.0% to 17.5%[2,8,12,21]. Consistent with previous studies, the current study showed that patients with resected sarcomatoid HCC had an elevated risk of death, with an abysmal 3-year OS compared with the prognosis of resected high-grade HCC patients (17.3% *vs* 53.4%, $P < 0.0001$). Even after controlling for confounding factors, the association between sarcomatoid HCC and worse RFS and OS persisted. These results strongly suggest that sarcomatoid HCC is

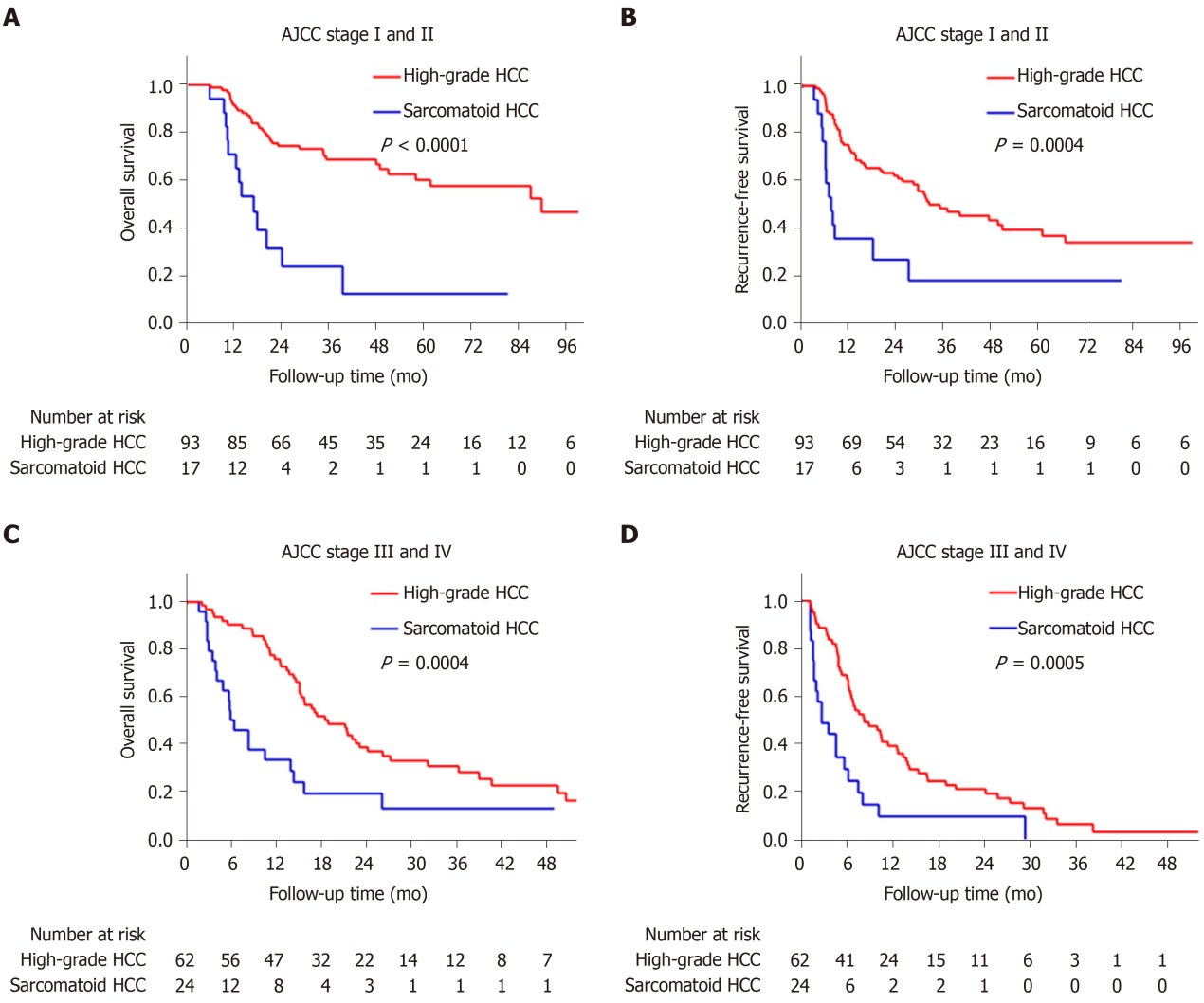


**Figure 4 Kaplan-Meier curves of the estimated overall survival and recurrence-free survival of patients with sarcomatoid or high-grade hepatocellular carcinoma stratified by American Joint Committee on Cancer stage.** A: Sarcomatoid hepatocellular carcinoma (HCC) is associated with worse overall survival (OS) in patients with American Joint Committee on Cancer (AJCC) stage I-II disease (log-rank $P < 0.0001$); B: Sarcomatoid HCC is associated with worse recurrence-free survival (RFS) in patients with AJCC stage I-II disease (log-rank $P = 0.0004$); C: Sarcomatoid HCC is associated with worse OS in patients with AJCC stage III-IV disease (log-rank $P = 0.0004$); D: Sarcomatoid HCC is associated with worse RFS in patients with AJCC stage III-IV disease (log-rank $P = 0.0004$).

more aggressive than high-grade HCC, although they are similar in terms of histological differentiation.

The histogenesis of sarcomatous tissue in cancers, including HCC, has not yet been elucidated. The most widely accepted theory is the conversion theory, which postulates that the sarcomatous element derives from the carcinoma during tumor evolution[3,9,22-25]. Several recent studies indicate that epithelial-mesenchymal transition is an important mechanism underlying the sarcomatous change, which further confirms the conversion theory[26-28]. Sarcomatoid HCCs comprise variable proportions of sarcomatous and carcinomatous components. A previous study reported no survival difference between mixed and pure sarcomatoid HCC patients[12]. However, the proportion of sarcomatous tissue in some patients could not be precisely evaluated because of pathological diagnosis by biopsy[12]. In our study, all patients with sarcomatoid HCC underwent surgical resection and were divided into three subgroups according to the proportion of the sarcomatous component. Our results indicated that the OS and RFS were similar among the three subgroups, suggesting that the proportion of the sarcomatoid component is not associated with prognosis.

Several previous studies demonstrated that sarcomatoid HCC is associated with a high frequency of portal venous invasion and metastasis[3,11,29,30]; our study showed a higher rate of lymph node metastasis but not of venous invasion. However, few studies have explored the related mechanisms, and only Maeda *et al*[3] have reported the composition of portal venous invasions and metastases. They studied 13 cases of

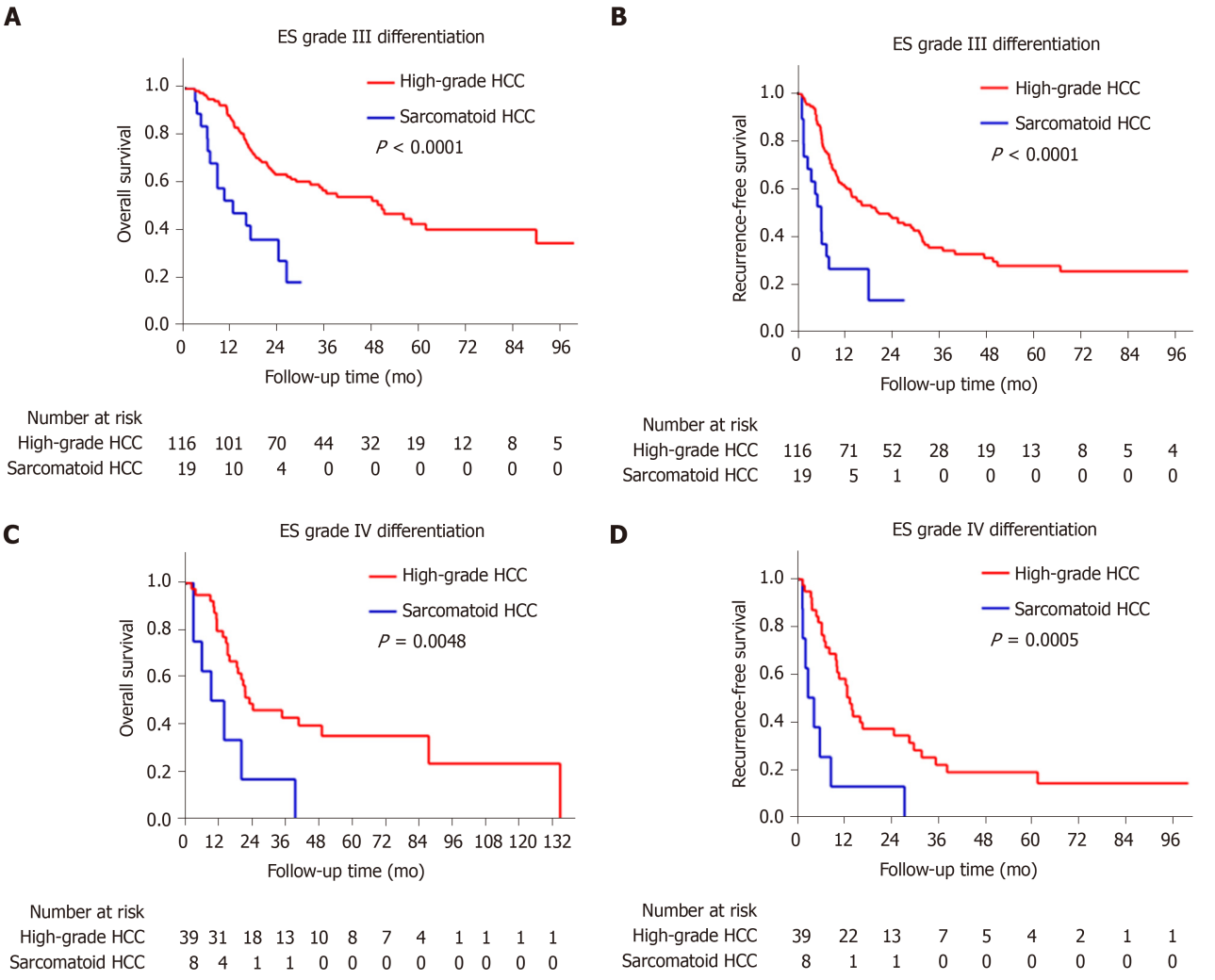


**Figure 5 Kaplan-Meier curves of the estimated overall survival and recurrence-free survival of patients with sarcomatoid or high-grade hepatocellular carcinoma stratified by differentiation grade of the carcinomatous component.** A: Sarcomatoid hepatocellular carcinoma (HCC) is associated with worse overall survival (OS) in patients with Edmondson-Steiner (ES) grade III differentiation (log-rank $P < 0.0001$); B: Sarcomatoid HCC is associated with worse recurrence-free survival (RFS) in patients with ES grade III differentiation (log-rank $P < 0.0001$); C: Sarcomatoid HCC is associated with worse OS in patients with ES grade IV differentiation (log-rank $P = 0.0048$); D: Sarcomatoid HCC is associated with worse RFS in patients with ES grade IV differentiation (log-rank $P = 0.0005$).

sarcomatoid HCC treated by surgical resection and found that most portal venous invasions and metastases had sarcomatous components, which were postulated to be responsible for metastasis. Contrary to their results, our study demonstrated that the majority of lymph node metastases, macrovascular/bile duct invasions and satellite nodules were composed of carcinomatous components. This apparent inconsistency may be because in Maeda's study, five (38%) patients underwent preoperative treatment, such as TACE, and their analysis included an autopsied case with extensive postoperative metastases comprising sarcomatoid components[3]. In line with our findings, however, they showed that the metastatic lymph nodes in two patients comprised carcinomatous components. In addition, some invasions and metastases had mixed carcinomatous and sarcomatous components. Based on these findings, we speculate that the carcinomatous component might be the dominant factor mediating the highly aggressive nature of sarcomatoid HCC. Furthermore, the carcinomatous components might exist in a "presarcomatoid" state[31], in which the tumor cells have cellular morphology typical of conventional HCC but greatly enhanced invasive ability. As a result, sarcomatous changes might occur after invasion or metastasis in some cases (Figure 8). This could also explain the phenomenon of sarcomatoid HCC being associated with more frequent lymph node metastasis and poorer prognosis than high-grade HCC even when they are similar in terms of histological differentiation. Further studies focusing on the underlying molecular pathogenesis of sarcomatoid HCC are urgently needed to understand better its highly aggressive

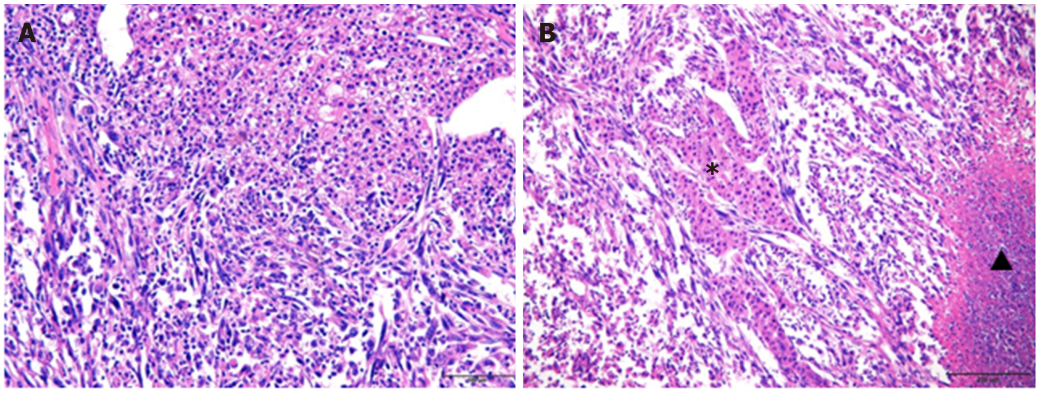


**Figure 6 Pathological findings of sarcomatoid hepatocellular carcinoma.** A: The lower left area of the image shows the sarcomatous change, with spindle-shaped cells forming interlacing bundles. The upper right region represents conventional hepatocellular carcinoma, with tumor cells at Edmondson-Steiner (ES) grade II differentiation (hematoxylin & eosin staining, × 200 magnification). (B) Scattered patchy carcinomatous components with ES grade III differentiation in sarcomatous regions (Hematoxylin and eosin staining, × 100 magnification). Star: Carcinomatous components, Triangle: Tumor necrosis.

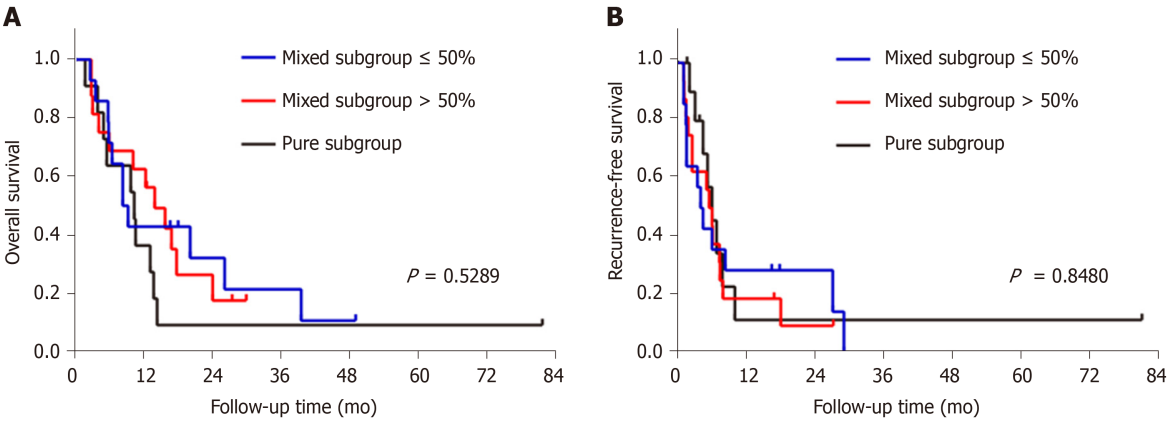


**Figure 7 Kaplan-Meier curves of the estimated overall survival and recurrence-free survival of patients with sarcomatoid hepatocellular carcinoma stratified by the proportion of the sarcomatous component in the tumor.** Sarcomatoid hepatocellular carcinoma patients were divided into three subgroups based on the proportion of the sarcomatous component in the tumor: (1) mixed subgroup ≤ 50% (*n* = 14); (2) mixed subgroup > 50% (*n* = 16); and (3) pure subgroup (*n* = 11). Kaplan-Meier analyses of (A) overall survival and (B) recurrence-free survival showed no differences among the three subgroups.

biological features.

There are limitations of our study. First, this was a retrospective study conducted in a single center, and randomized studies should be performed in multiple centers. Second, although this study presented an analysis of one of the largest series of sarcomatoid HCC cases, the relatively small number of patients may have influenced the accuracy of the results, and additional studies with more cases should be performed. Third, in this study, we discovered that carcinomatous components occupied the predominant proportion of invasions and metastases and developed a hypothesis regarding the occurrence of sarcomatoid HCC. The related mechanisms were not further explored, and future studies should address these issues.

In conclusion, compared with high-grade HCC, sarcomatoid HCC is associated with more advanced AJCC stage, an atypical dynamic image pattern and lower serum AFP levels. Patients with sarcomatoid HCC have significantly worse RFS and OS than those with high-grade HCC. Furthermore, the highly aggressive nature of sarcomatoid HCC seems to be mediated by its carcinomatous components.

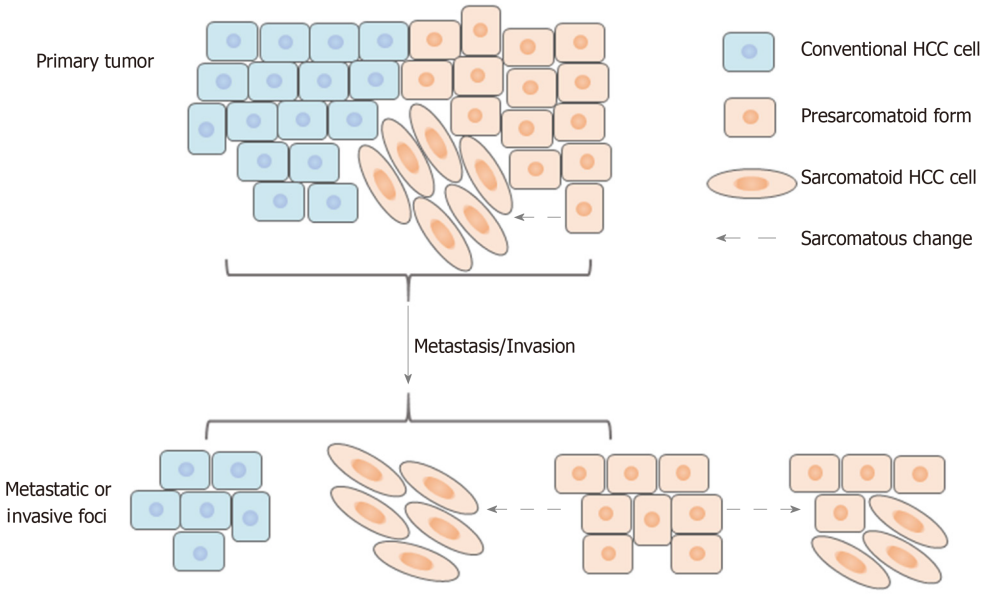


**Figure 8 Schematic of the hypothesis.** HCC: Hepatocellular carcinoma.

## ARTICLE HIGHLIGHTS

### *Research background*

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer and the fourth leading cause of cancer-related death worldwide. Sarcomatoid HCC, which contains poorly differentiated carcinomatous and sarcomatous components, is a rare histological subtype of HCC that differs from conventional HCC. It is highly aggressive and has a poor prognosis. Its clinicopathological characteristics, surgical outcomes and underlying mechanisms of its highly aggressive nature have not been fully elucidated.

### *Research motivation*

A few studies have reported that sarcomatoid HCC is associated with a higher recurrence rate, more frequent metastasis and poorer survival than conventional HCC. However, these studies did not further stratify conventional HCC into low- [Edmondson-Steiner (ES) grade I and II] and high-grade (ES grade III and IV) HCC; in particular, high-grade HCC is considered similar to sarcomatoid HCC in terms of histological differentiation, more aggressive nature and poor prognosis. In addition, although sarcomatoid HCC has a high incidence of adjacent organ invasion and metastasis, the underlying mechanisms remain unknown. One previous study reported that most portal venous invasions and metastases had sarcomatous components, indicating that the sarcomatous component is responsible for metastasis. However, in the study, five (38%) patients underwent preoperative treatment, such as TACE, and their analysis included an autopsied case with extensive postoperative metastases comprising sarcomatoid components. Therefore, the results might be biased. In view of the deficiencies of previous studies, we will conduct further studies on sarcomatoid HCC.

### *Research objectives*

To examine the clinicopathological characteristics and surgical outcomes of sarcomatoid HCC and explore the histogenesis of sarcomatoid HCC.

### *Research methods*

In total, 196 patients [41 sarcomatoid HCC and 155 high-grade (ES grade III or IV) HCC] who underwent surgical resection between 2007 and 2017 were retrospectively reviewed. The characteristics and surgical outcomes of sarcomatoid HCC were compared with those of patients with high-grade HCC. The histological composition of invasive and metastatic sarcomatoid HCCs was evaluated.

### Research results

Sarcomatoid HCC was more frequently diagnosed at an advanced stage with a larger tumor and higher rates of nonspecific symptom, adjacent organ invasion and lymph node metastasis than high-grade HCC (all $P < 0.05$). Compared with high-grade HCC patients, sarcomatoid HCC patients are less likely to have typical dynamic imaging features of HCC (44.4% *vs* 72.7%, $P = 0.001$) and elevated serum alpha-fetoprotein levels (> 20 ng/mL; 36.6% *vs* 78.7%, $P < 0.001$). The sarcomatoid group had a significantly shorter median recurrence-free survival (5.6 mo *vs* 16.4 mo, log-rank $P < 0.0001$) and overall survival (10.5 mo *vs* 48.1 mo, log-rank $P < 0.0001$) than the high-grade group. After controlling for confounding factors, the sarcomatoid subtype was identified as an independent predictor of poor prognosis. Pathological analyses indicated that invasive and metastatic lesions were mainly composed of carcinomatous components.

### Research conclusions

Sarcomatoid HCC was associated with a more advanced stage, atypical dynamic imaging, lower serum alpha-fetoprotein levels and a worse prognosis. The highly aggressive nature of sarcomatoid HCC is perhaps mediated by carcinomatous components.

### Research perspectives

Studies focusing on the underlying molecular pathogenesis of sarcomatoid HCC are urgently needed to understand better its highly aggressive biological features.

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ORIGINAL ARTICLE

**Observational Study**

# Patients' perspectives on smoking and inflammatory bowel disease: An online survey in collaboration with European Federation of Crohn's and Ulcerative Colitis Associations

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**Author contributions:** Le Berre C and Loy L was the conception and design of the study, interpretation of data, and drafting the article; Lönnfors S and Avedano L was the acquisition of data, making critical revisions related to important intellectual content of the manuscript; Piovani D was the analysis and interpretation of data, drafting the article; All authors read and approved the final version of the article to be published.

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**Informed consent statement:** This was an online survey. Before answering the questions, patients were informed of the objectives of the study and gave their consent

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## Abstract

### *BACKGROUND*

Smoking has detrimental effects on Crohn's disease (CD) activity while data on ulcerative colitis (UC) are conflicting. Little is known about the use and impact of alternative smoking products in inflammatory bowel diseases (IBD).

### *AIM*

To understand the patients' perceptions of the impact of smoking on their IBD and to assess differences between CD and UC patients.

### *METHODS*

The questionnaire was developed by Philip Morris Products SA in cooperation with European Federation of Crohn's and Ulcerative Colitis Associations. The final survey questionnaire consisted of 41 questions divided in 8 categories: (1) Subject screener; (2) Smoking history; (3) Background information; (4) IBD disease background; (5) Current disease status; (6) Current therapeutics and medications; and (7) Current nicotine/cigarettes use and awareness of the impacts of smoking on IBD. The questionnaire was submitted online from 4th November 2019 to 11th

online.

**Conflict-of-interest statement:** The authors declare no conflicts of interest relevant to this work.

**Data sharing statement:** No additional data are available.

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March 2020 through the European Federation of Crohn's and Ulcerative Colitis Associations website to IBD patients who were current smokers or had a history of smoking.

*RESULTS*

In total 1050 IBD patients speaking nine languages participated to the survey. Among them, 807 (76.9%) patients declared to have ever smoked or consumed an alternative smoking product, with a higher proportion of current cigarette smokers among CD patients (CD: 63.1% *vs* UC: 54.1%, $P = 0.012$). About two-thirds of the participants declared to have ever stopped cigarette smoking and restarted (67.0%), with a significantly higher proportion among UC patients compared to CD patients (73.1% *vs* 62.0%, $P = 0.001$). We also found significant differences between CD and UC patients in the awareness of the health consequences of smoking in their disease and in the perceived impact of smoking on disease activity, for both cigarettes and alternative smoking products.

*CONCLUSION*

This survey found significant differences between CD and UC patients in both awareness and perception of the impact of smoking on their disease. Further efforts should be done to encourage smoking cessation for all IBD patients, including UC patients.

**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Cigarettes; Alternative smoking products; Tobacco; Nicotine; Marijuana



**Core tip:** We performed a European-wide online survey to understand the patients' perceptions on how smoking has impacted their inflammatory bowel diseases (IBD). In total 1050 IBD patients [427 with Crohn's disease (CD), 355 with ulcerative colitis (UC)] participated to the survey, with a higher proportion of cigarette smokers among CD patients. About two-thirds of the participants declared to have ever stopped cigarette smoking and restarted, with a higher proportion among UC compared to CD patients (73.1% *vs* 62.0%, $P = 0.001$). There were also differences between CD and UC patients in the awareness of the health consequences of smoking and in the perceived impact of smoking on disease activity.

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## INTRODUCTION

Environmental factors are probably primarily responsible for the growing incidence of inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), around the globe over the last decades[1-3]. Accumulating evidence supports an association between IBD and several environmental factors, including smoking, diet, drugs, geographical and social status, stress, microbial agents, intestinal permeability and appendectomy[4,5]. Among these factors, smoking is the one for which the most solid evidence is currently available[5]. Smoking increases the risk of CD and worsens its clinical course[6,7], especially after digestive surgery[8], but has a protective effect in UC[6,9]. Thus, smoking habits are much more frequent in CD patients than in UC patients, except in Jewish patients in Israel in whom the stronger genetic tendency in CD may contribute to this discrepancy[10].

In CD, the odds of flare of disease activity, flare after surgery, need for first and second surgery among ex-smokers diminish upon smoking cessation and become comparable to non-smokers[11]. In UC, nicotine has been tested as a therapeutic agent in

the form of chewing gum, transdermal patches, and nicotine-based enemas[12-17], with conflicting results and variable efficacy in the induction of remission when compared to placebo and conventional treatments[18-20]. However, it is clear that the protective effect of cigarette smoking in UC is temporary, since the relative risk of developing the disease increases after smoking cessation compared with patients who have never smoked[21].

Until now, there has been little patient-centered research aiming at assessing the perceived impact of smoking or nicotine use on IBD symptoms by patients who are current adult smokers and/or nicotine-containing products users. Few studies demonstrated that a high proportion of patients with IBD are unaware of the effects of tobacco on their disease, but these limited available data are solely based on small-scale studies[22-26]. Yet, making patients aware of the impact of nicotine use on the course of their IBD is essential to expect smoking cessation and improve the management of their disease.

Thus, the objectives of this European-wide online survey developed in collaboration with European Federation of Crohn's and Ulcerative Colitis Association (EFCCA) were to understand the patients' perceptions on the impact of smoking on their IBD and to assess differences of these perceptions between CD and UC patients. The results of this study will be shared with the IBD community through EFCCA in order to facilitate the management of smoking cessation among patients.

## MATERIALS AND METHODS

### *Questionnaire development*

The questionnaire was developed by Philip Morris Products SA in cooperation with EFCCA and reviewed prior to use by health informaticians. Le Berre C and Loy L also participated in the design of the questionnaire. The final survey questionnaire consisted of 41 questions divided in 8 categories: (1) Subject screener; (2) Smoking history; (3) Background information; (4) IBD disease background; (5) current disease status; (6) current therapeutics and medications; and (7) Current nicotine/cigarettes use and awareness of the impacts of smoking on IBD (Supplemental document).

In most questions, participants were allowed to tick one applicable option. In some questions, such as those concerning the type of alternative smoking products that were used, several options could be chosen. In others, such as questions concerning the duration of use of nicotine products, therapy change due to smoking habits or use of alternative smoking products, or the perceived impact of nicotine use on disease activity, participants were given multiple categorical options.

The survey was made available in English, French, German, Spanish, Portuguese, Italian, Greek, Finnish and Slovenian. Translations were made by translators that EFCCA has previously worked with. They were proofread by native speakers from EFCCA's member associations and revised, if necessary. Philip Morris International did final checks and revisions on the translations in order to have a double validation before the survey was launched.

### *Questionnaire administration*

The questionnaire was submitted through EFCCA to IBD patients who were current smokers or had a history of smoking. Patients were not pre-screened and were eligible for inclusion if they were aged over 18 years and had a confirmed diagnosis of IBD, including CD or UC. Patients gave their consent and were not paid for participating.

The online survey was open from 4$^{th}$ November 2019 to 11$^{th}$ March 2020 on the EFCCA website. National associations were encouraged to promote the survey online by providing their members with an online link on their website. Information on this survey was also displayed in posters and flyers at the clinic of each participating investigator. The survey was closed after having enrolled more than 1000 participants, whatever their characteristics in terms of gender or nationality.

As this was a non-interventional survey, ethics committee approval was not required. Data was collected anonymously online, and participation was entirely optional. Since it was an anonymous survey, there was no data or user tracking. EFCCA strictly follows the General Data Protection Regulation and does not own the data that was transferred in a secured way to the biostatistician.

### *Statistical analysis*

Descriptive statistics included means with standard deviations and medians with interquartile ranges (IQR) for continuous variables, and frequency analyses

(percentages) for categorical variables. The association between categorical variables was investigated with the Fisher's exact test. Stata 15.0 software was used for all the analyses (Stata Corp, College Station, TX, United States). *P* values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

## RESULTS

This survey enrolled 1180 IBD patients speaking nine different languages. However, 130 patients only selected their language and did not answer the first question (*i.e.*, if they have ever smoked or consumed an alternative smoking product). This group of patients was excluded, and we considered 1050 patients as participants. The most represented countries were Italy (20.3%), Finland (13.9%) and Portugal (13.9%). Among them, 807 (76.9%) patients declared to have ever smoked or consumed an alternative smoking product and proceeded to complete the rest of the questionnaire.

### Baseline characteristics and treatmen

Participants were mainly females (65.4%) with a median age of 40 (IQR: 32-51) years old. Most patients were diagnosed with CD (53.5%) with a disease duration of 11 (IQR: 5-20) years. Patients diagnosed with UC (44.5%) had a shorter median disease duration (4 years; IQR: 1-8). About one quarter of the participants perceived their disease as severe (26.2%) and 28.8% had undergone surgery. The most common type of surgery was ileocaecal resection (11.8%). The most common drug treatment regimens were oral aminosalicylates (39.8%), anti-TNF agents (28.2%) and immunomodulators (26.7%). Baseline characteristics reported by the patients are detailed in (Table 1 and Supplemental Table 1) compares these baseline characteristics by type of IBD diagnosis.

### Smoking habits and use of alternative smoking products

Among patients declaring themselves as having ever smoked cigarettes or having ever used an alternative smoking product, more than half were current cigarette smokers (59.0%). This proportion was significantly higher in CD than in UC patients (63.1% *vs* 54.1%, $P = 0.012$) (Table 2). Most ever smokers smoked cigarettes for more than 10 years (63.4%) and declared to have consumed on average more than 10 cigarettes per day (50.6%). The extent of cigarette smoking was significantly higher in CD than in UC patients ($P < 0.001$). The most commonly used products were cigarettes (91.6%), followed by e-cigarettes (19.2%) and marijuana (17.0%). There were no significant differences in the use of any nicotine-containing product between CD and UC patients.

The vast majority of patients declared to be aware of the adverse health consequences of smoking (97.0%) and started cigarette smoking before IBD diagnosis (88.7%). This proportion was significantly higher in CD than in UC patients (92.0% *vs* 84.6%, $P = 0.001$). About two-thirds of the participants declared to have ever stopped cigarette smoking and restarted (67.0%), with a significantly higher proportion among UC patients compared to CD patients (73.1% *vs* 62.0%, $P = 0.001$). Most UC patients stopped cigarette smoking before diagnosis (59.1%), while this was true for only about one-third of CD patients (34.0%).

Among patients declaring to have ever smoked cigarettes or used an alternative smoking product, 20.0% were current users of an alternative smoking product. Differently from cigarette smokers, most users of these products declared a duration of use of less than one year (56.1%). Most users of alternative smoking products started using them (57.0%) or switched from cigarettes to these products after IBD diagnosis (59.6%).

### Impact of cigarette smoking or use of alternative smoking products on IBD

Most cigarette smoking patients perceived that smoking significantly or moderately worsened disease activity (60.9%), while a lower proportion of patients using alternative smoking products had the same impression (15.3%). A much lower proportion of patients believed their habit had no impact on disease activity among cigarette smokers (8.3%) than among users of alternative smoking products (66.4%).

Most patients did not have any therapy change due to smoking habits or use of alternative smoking products (66.7% after starting smoking, 79.2% after restarting smoking, 75.2% after stopping smoking, 90.1% after switching to alternative smoking products and 89.7% after starting to use an alternative smoking product).

The perception of the impact of cigarette smoking significantly differed between CD and UC patients ($P < 0.001$) (Table 3). More than three-quarters (79.2%) of CD patients

**Table 1 Baseline characteristics reported by the participants (*n* = 1050)**

| Questionnaire items | mean ± SD; median (IQR) or *n*(%) |
|---|---|
| **Demographic characteristics** | |
| Age (*n* = 796) | 41.9 ± 12.4; 40.0 (32.0–51.0) |
| Gender | |
| Female | 522/798 (65.4) |
| Male | 276/798 (34.6) |
| Language | |
| Italian | 213 (20.3) |
| Finnish | 146 (13.9) |
| Portuguese | 146 (13.9) |
| Slovenian | 128 (12.2) |
| Spanish | 120 (11.4) |
| Greek | 102 (9.7) |
| English | 83 (7.9) |
| German | 57 (5.4) |
| French | 55 (5.3) |
| IBD characteristics and treatments | |
| Diagnosis | |
| Crohn's disease | 427/798 (53.5) |
| Ulcerative colitis | 355/798 (44.5) |
| No gastrointestinal condition | 5/798 (0.6) |
| Other gastrointestinal condition | 11/798 (1.4) |
| Crohn's disease duration (yr) | 13.6 ± 10.6; 11 (5–20) |
| Ulcerative colitis duration (yr) | 7.52 ± 9.12; 4 (1–8) |
| Self-perceived disease activity | |
| Inactive | 155/770 (20.1) |
| Mildly active | 261/770 (33.9) |
| Moderately active | 251/770 (32.6) |
| Severely active | 76/770 (9.9) |
| Not sure | 27/770 (3.5) |
| Self-perceived disease severity since diagnosis | |
| Mild | 173/768 (22.5) |
| Moderate | 360/768 (46.9) |
| Severe | 201/768 (26.2) |
| Not sure | 34/768 (4.4) |
| Intestinal surgery | 230/798 (28.8) |
| Type of surgery | |
| Partial colectomy | 41/798 (5.1) |
| Full colectomy | 29/798 (3.6) |
| Small-bowel resection | 48/798 (6.0) |
| Ileocaecal resection | 94/798 (11.8) |
| Other | 79/798 (9.9) |

| | |
|---|---|
| Current IBD-related medications | |
| Antibiotics | 41/798 (5.1) |
| Oral aminosalicylate | 318/798 (39.8) |
| Topical aminosalicylate | 91/798 (11.4) |
| Topical steroid | 47/798 (5.9) |
| Systemic steroid | 78/798 (9.8) |
| Budesonide | 28/798 (3.5) |
| Immunomodulator | 213/798 (26.7) |
| Anti-TNF | 225/798 (28.2) |
| Combination of anti-TNF and immunomodulator | 69/798 (8.6) |
| Anti-integrin | 51/798 (6.4) |
| Tofacitinib | 6/798 (0.8) |
| Ustekinumab | 28/798 (3.5) |
| None | 76/798 (9.5) |
| Other | 135/798 (16.9) |
| Duration of current medical therapy (yr) | |
| < 1 | 240/765 (31.4) |
| 1-5 | 262/765 (34.2) |
| > 5 | 263/765 (34.4) |
| Concomitant non-IBD drug treatment | |
| Yes | 282/767 (63.2) |
| No | 485/767 (36.8) |

The survey had a hierarchical structure, meaning that only patients who answered to certain items could answer to other following questions. For this reason, the denominator for several questions is different. In some cases, the patient did not answer and this can result in a missing value. We have transparently declared in our tables the denominator we have used to calculate the proportion of patients giving certain answers. IBD: Inflammatory bowel diseases; IQR: Interquartile ranges.

perceived that smoking significantly or moderately worsened disease activity versus 34.0% of UC patients. Similarly, the perceived impact of using alternative smoking products on disease activity significantly differed between CD and UC patients ($P$ = 0.004), even though the magnitude of the effect was less strong (Table 3).

### *Discussion with own physician about the effect of smoking on IBD*

Among patients having ever smoked cigarettes, 31.7% did not receive any information from their physician on the effect of smoking on disease activity, while 45.4% of them received the information that smoking is detrimental to disease activity. These proportions were significantly different in CD than in UC patients (not discussed: 21.6% *vs* 44.6%; detrimental: 69.4% *vs* 14.6%; $P < 0.001$) (Table 4).

Among patients having ever used alternative smoking products, 56.8% did not discuss the effect of these products on disease activity, and 25.2% of them received the information that using them is detrimental to disease activity. These proportions were significantly different in CD than in UC patients (not discussed: 51.8% *vs* 62.5%; detrimental: 37.4% *vs* 11.1%; $P < 0.001$) (Table 4).

## DISCUSSION

Our first objective was to assess patients' perceptions about the impact of smoking on their disease, and next to assess differences of these perceptions between CD and UC patients, with the aim to understand how different smoker profiles perceived the impact of smoking on their IBD.

Most patients were aware of the adverse health consequences of smoking and

**Table 2 Smoking habits and use of alternative smoking products based on type of diagnosis**

| Questionnaire items | Crohn's disease | Ulcerative colitis | *P* value[1] |
|---|---|---|---|
| Smoking habits and use of an alternative smoking product | | | |
| Current cigarettes smoker | | | |
| Yes | 268/425 (63.1) | 187/346 (54.1) | 0.012 |
| No | 157/425 (36.9) | 159/346 (45.9) | |
| Duration of cigarette smoking (yr) | | | |
| < 1 | 22/410 (5.4) | 26/317 (8.2) | 0.13 |
| 1-5 | 46/410 (11.2) | 47/317 (14.8) | |
| 6-10 | 69/410 (16.8) | 56/317 (17.7) | |
| > 10 | 273/410 (66.6) | 188/317 (59.3) | |
| Extent of cigarette smoking (cigarettes per day) | | | |
| < 1 | 12/425 (2.8) | 22/341 (6.5) | < 0.001 |
| 1-5 | 51/425 (12.0) | 67/341 (19.6) | |
| 6-10 | 120/425 (28.3) | 106/341 (31.1) | |
| 11-20 | 179/425 (42.1) | 113/341 (33.1) | |
| > 20 | 63/425 (14.8) | 33/341 (9.7) | |
| Current use of an alternative smoking product | | | |
| Yes | 343/427 (80.3) | 282/355 (79.4) | 0.79 |
| No | 84/427 (19.7) | 73/355 (20.6) | |
| Type of smoking/nicotine product ever used | | | |
| Cigarettes | 409/427 (95.8) | 330/355 (93.0) | 0.11 |
| E-cigarettes | 84/427 (19.7) | 71/355 (20.0) | 0.93 |
| Heat not burn tobacco product | 19/427 (4.5) | 25/355 (7.0) | 0.12 |
| Nicotine gum | 37/427 (8.7) | 36/355 (10.1) | 0.54 |
| Nicotine patches | 21/427 (4.9) | 23/355 (6.5) | 0.35 |
| Chewing tobacco/snus/snuff | 10/427 (2.3) | 15/355 (4.2) | 0.16 |
| Cigars | 44/427 (10.3) | 31/355 (8.7) | 0.47 |
| Pipes | 13/427 (3.0) | 8/355 (2.3) | 0.66 |
| Marijuana | 81/427 (19.0) | 56/355 (15.8) | 0.26 |
| Any other combustion/smoking product | 11/427 (2.6) | 5/355 (1.4) | 0.31 |
| None of the above | 2/427 (0.5) | 7/355 (2.0) | 0.09 |
| Ever stopped cigarette smoking and restarted | | | |
| Yes | 264/426 (62.0) | 253/346 (73.1) | 0.001 |
| No | 162/426 (38.0) | 93/346 (26.9) | |
| Ever user of an alternative smoking product | | | |
| Yes | 215/427 (50.4) | 196/354 (55.4) | 0.17 |
| No | 212/427 (49.6) | 158/354 (44.6) | |
| Current use of an alternative smoking product | | | |
| Yes | 84/427 (19.7) | 73/355 (20.6) | 0.79 |
| No | 343/427 (80.3) | 282/355 (79.4) | |
| Duration of use of alternative smoking products | | | |

| | | | |
|---|---|---|---|
| < 1 | 44/76 (55.3) | 46/81 (56.7) | 0.33 |
| 1-5 | 23/76 (30.3) | 16/81 (19.8) | |
| 6-10 | 10/76 (13.1) | 16/81 (19.8) | |
| > 10 | 1/76 (1.3) | 3/81 (3.7) | |
| Started cigarette smoking | | | |
| Before IBD diagnosis | 392/426 (92.0) | 290/343 (84.6) | 0.001 |
| After IBD diagnosis | 34/426 (8.0) | 53/343 (15.4) | |
| Stopped cigarette smoking | | | |
| Before IBD diagnosis | 85/250 (34.0) | 146/247 (59.1) | < 0.001 |
| After IBD diagnosis | 165/250 (66.0) | 101/247 (40.9) | |
| Restarted cigarette smoking | | | |
| Before IBD diagnosis | 89/260 (34.2) | 66/243 (27.2) | 0.10 |
| After IBD diagnosis | 171/260 (65.8) | 177/243 (72.8) | |
| Started using an alternative smoking product | | | |
| Before IBD diagnosis | 85/210 (40.5) | 85/185 (46.0) | 0.31 |
| After IBD diagnosis | 125/210 (59.5) | 100/185 (54.0) | |
| Switched from cigarette smoking to using an alternative smoking product | | | |
| Before IBD diagnosis | 77/207 (37.2) | 81/184 (44.0) | 0.18 |
| After IBD diagnosis | 130/207 (62.8) | 103/184 (56.0) | |

The survey had a hierarchical structure, meaning that only patients who answered to certain items could answer to other following questions. For this reason, the denominator for several questions is different. In some cases, the patient did not answer and this can result in a missing value. We have transparently declared in our tables the denominator we have used to calculate the proportion of patients giving certain answers.
[1]Fisher's exact test. IBD: Inflammatory bowel diseases.

started cigarette smoking before IBD diagnosis. However, CD and UC patients showed different awareness about the impact of smoking cigarettes on their disease activity. Indeed, most CD patients were aware of a detrimental effect of smoking, and a large proportion of UC patients was aware of possible beneficial impact of smoking on their disease activity. Interestingly, most UC patients did not discuss this topic with their own physician. This is in line with previous studies conducted on this issue[25,26]. Both Wahed *et al*[25] and Ducharme-Bénard *et al*[26] reported informed rates of 52% and 57.7% in patients with CD, whilst this was the case in only 21% and 13% of patients with UC Saadoune *et al*[27] Reported about two-thirds of smoking CD patients were aware of the harmful effects on the course of disease, whereas all UC patients were aware of its protective role[27]. The detrimental effects of smoking on CD are well-established in the literature. On the contrary, health professionals might be reticent to explain the apparent and less-proven benefit of smoking to UC patients, by fear of discouraging them to stop[28-30].

We also can presume that a different perception of smoking on IBD activity between CD and UC patients could influence the need to be informed about risks and benefits of smoking habits. In our cohort, the large majority of CD patients perceived their disease activity significantly or moderately worsened by cigarette smoking, while 59.7% of UC patients perceived a positive effect of cigarette smoking on their disease activity. Patients' perceptions in our cohort thus confirm previous results in literature on this topic[11,18-21]. It was not surprising to find different smoking profiles between CD an UC patients based on timing of IBD diagnosis. Most CD patients stopped cigarette smoking after IBD diagnosis, whereas most UC patients stopped cigarettes before IBD diagnosis. Among participants declaring to have ever stopped cigarette smoking and restarted, we expected to find a large majority of UC patients restarting smoking after IBD diagnosis. Quite surprisingly, the same result was observed in the CD group of patients while smoking is known to worsen the course of CD. In a study conducted by the Nancy group, similar results were reported, with about one-third of IBD patients who had already stopped smoking to prevent flares with a significant difference

**Table 3 Perceived effect of smoking on inflammatory bowel diseases based on type of diagnosis**

| Questionnaire items | Crohn's disease | Ulcerative colitis | *P* value[1] |
|---|---|---|---|
| Perceived impact of cigarette smoking on disease activity | | | |
| Significantly worsened | 12/279 (4.3) | 2/191 (1.0) | < 0.001 |
| Moderately worsened | 209/279 (74.9) | 63/191 (33.0) | |
| No impact | 27/279 (9.7) | 12/191 (6.3) | |
| Moderately improved | 25/279 (9.0) | 58/191 (30.4) | |
| Significantly improved | 6/279 (2.2) | 56/191 (29.3) | |
| Perceived impact of using alternative smoking products on disease activity | | | |
| Significantly worsened | 4/211 (1.9) | 12/187 (6.4) | 0.004 |
| Moderately worsened | 31/211 (14.7) | 14/187 (7.5) | |
| No impact | 146/211 (69.2) | 118/187 (63.1) | |
| Moderately improved | 23/211 (10.9) | 28/187 (15.0) | |
| Significantly improved | 7/211 (3.3) | 15/187 (8.0) | |

The survey had a hierarchical structure, meaning that only patients who answered to certain items could answer to other following questions. For this reason, the denominator for several questions is different. In some cases, the patient did not answer and this can result in a missing value. We have transparently declared in our tables the denominator we have used to calculate the proportion of patients giving certain answers.
[1]Fisher's exact test.

**Table 4 Extent of discussing with own physician the effect of smoking on inflammatory bowel diseases based on type of diagnosis**

| Questionnaire items | Crohn's disease | Ulcerative colitis | *P* value[1] |
|---|---|---|---|
| Cigarette smoking | | | |
| Not discussed | 89/412 (21.6) | 143/321 (44.6) | < 0.001 |
| Detrimental | 286/412 (69.4) | 47/321 (14.6) | |
| Possibly beneficial | 37/412 (9.0) | 131/321 (40.8) | |
| Use of alternative smoking products | | | |
| Not discussed | 43/83 (51.8) | 45/72 (62.5) | < 0.001 |
| Detrimental | 31/83 (37.4) | 8/72 (11.1) | |
| Possibly beneficial | 9/83 (10.8) | 19/72 (26.4) | |

The survey had a hierarchical structure, meaning that only patients who answered to certain items could answer to other following questions. For this reason, the denominator for several questions is different. In some cases, the patient did not answer and this can result in a missing value. We have transparently declared in our tables the denominator we have used to calculate the proportion of patients giving certain answers.
[1]Fisher's exact test.

between CD and UC patients[27], whereas we did not observe any significant difference among CD and UC patients restarting smoking to prevent flares.

Evidence from literature underlines the importance of informing patients with CD about the negative influence of smoking on their disease, as this will directly influence their intent to quit smoking and positively impact clinical outcomes if they succeed[26,31,32]. Education on smoking is probably insufficiently considered for the management of our patients, particularly in CD. Hilsden *et al*[33] suggested that patients with CD are not more refractory to smoking cessation compared to the general population of active smokers and factors unrelated to CD may be more important in their decision to smoke than CD-related factors[33].

Furthermore, recent data from the Epi-IBD cohort also highlights the economic impact of smoking on IBD, especially in CD, where smoking cessation has medical and economic benefits[34].

To our knowledge, the present study represents the most complete survey

evaluating the consumption of any type of nicotine-containing products in IBD patients. The available literature previously addressed the topic of smoking habits focusing on cigarettes, which are the most commonly used nicotine-containing products[7,9,11,20]. Our survey represents a further effort to fill the gap about former or present use in IBD patients also considering alternative smoking products, like e-cigarettes.

Despite the absence of any significant differences in the use of any nicotine-containing product between CD and UC patients, the two groups had a different perception about the impact of using alternative smoking products on their disease activity, with a trend in favor of worsening in CD patients and a trend in favor of improvement in UC patients; however, in both groups most patients perceived no change in their disease activity. Additionally, a higher proportion of CD patients discussed with their physician about a detrimental effect of these products, while a higher proportion of UC patients never discussed about this subject.

Despite the study design did not allow to stratify our data for the specific type of nicotine-alternative products used, it is interesting to appreciate a growing interest regarding the consumption of electronic cigarettes and its possible impact in IBD populations. A recently published study conducted in United Kingdom showed the proportion of e-cigarette users among IBD patients was marginally lower than in the general population, with no significant difference between CD and UC patients. However, due to the small number of cases, the authors failed to demonstrate a significant different rate of disease-related complications in e-cigarettes users (higher in UC, lower in CD) compared with cigarette users[35].

Regarding e-cigarettes, that deliver nicotine *via* aerosol formed through the heating of a mixture of liquid usually made up of nicotine, propylene glycol or glycerol (glycerine) and flavouring chemicals, their use among European population has been growing steadily since they entered the European market in 2006[36]. The impact of e-cigarettes remains unknown. The lack of evidence about safety requires to remain vigilant over potential adverse effects; however, current available research also suggests the potential benefits of e-cigarettes as a harm reduction model for those who use combustible cigarettes, and e-cigarettes may have an important role to play in preventing death and disability from tobacco use[37,38]. Those devices may theoretically have less impact on the course of IBD because of lower nicotine concentrations, but the latter vary considerably. Despite that, the safety of e-cigarettes in diseases such as IBD remains unknown. Thus, further research is warranted to assess whether e-cigarettes could be an effective smoking cessation tool, and to evaluate both short- and long-term health effects of e-cigarettes.

In this large, European, multicenter survey of over 1000 IBD patients, we assessed the level of knowledge of patients with IBD regarding the impact of any type of nicotine-containing products in both CD and UC. We found significant differences between CD and UC patients in both awareness and perception of the impact of smoking on their disease. Furthermore, despite most CD patients were aware of a detrimental effect of smoking, and a large part of UC patients was aware of possible beneficial impact of smoking in their disease, further efforts should be done to encourage smoking cessation for all IBD patients, including UC patients, because of the well-established beneficial effects of smoking cessation on general health. In light of the increasing use of alternative nicotine-containing products, like e-cigarettes, further studies are mandatory to explore the safety and impact of these products in patients with IBD.

## ARTICLE HIGHLIGHTS

### *Research background*

Environmental factors are probably primarily responsible for the growing incidence of inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), around the globe over the last decades. Among these factors, smoking is the one for which the most solid evidence is currently available. Smoking increases the risk of CD and worsens its clinical course but has a protective effect in UC.

### *Research motivation*

Until now, there has been little patient-centered research aiming at assessing the perceived impact of smoking or nicotine use on IBD symptoms by patients who are

current adult smokers and/or nicotine-containing products users. Few studies demonstrated that a high proportion of patients with IBD are unaware of the effects of tobacco on their disease, but these limited available data are solely based on small-scale studies. Yet, making patients aware of the impact of nicotine use on the course of their IBD is essential to expect smoking cessation and improve the management of their disease.

### Research objectives

To understand the patients' perceptions on the impact of smoking on their IBD and to assess differences of these perceptions between CD and UC patients.

### Research methods

This was a European-wide online survey developed by Philip Morris Products SA in collaboration with European Federation of Crohn's and Ulcerative Colitis Association. The final survey questionnaire consisted of 41 questions divided in 8 categories: (1) Subject screener; (2) Smoking history; (3) Background information; (4) IBD disease background; (5) Current disease status; (6) Current therapeutics and medications; and (7) Current nicotine/cigarettes use, and awareness of the impacts of smoking on IBD. The survey was made available in English, French, German, Spanish, Portuguese, Italian, Greek, Finnish and Slovenian. The online survey was open from 4$^{th}$ November 2019 to 11$^{th}$ March 2020 on the European Federation of Crohn's and Ulcerative Colitis Association website.

### Research results

This survey enrolled 1050 IBD patients speaking nine different languages. Among them, 807 declared to have ever smoked or consumed an alternative smoking product. More than half were current cigarette smokers (59.0%). This proportion was significantly higher in CD than in UC patients. There were no significant differences in the use of any nicotine-containing product between CD and UC patients. The perception of the impact of cigarette smoking significantly differed between CD and UC patients. Similarly, the perceived impact of using alternative smoking products on disease activity significantly differed between CD and UC patients. Among patients having ever smoked cigarettes, 31.7% did not receive any information from their physician on the effect of smoking on disease activity, while 45.4% of them received the information that smoking is detrimental to disease activity. These proportions were significantly different in CD and in UC patients.

### Research conclusions

We found significant differences between CD and UC patients in both awareness and perception of the impact of smoking on their disease. Further efforts should be done to encourage smoking cessation for all IBD patients, including UC patients, because of the well-established beneficial effects of smoking cessation on general health.

### Research perspectives

In light of the increasing use of alternative nicotine-containing products, like e-cigarettes, further studies are mandatory to explore the safety and impact of these products in patients with IBD.

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META-ANALYSIS

# Dysregulation of microRNA in cholangiocarcinoma identified through a meta-analysis of microRNA profiling

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## Abstract

### *BACKGROUND*

In the past decades, the potential of microRNA (miRNA) in cancer diagnostics and prognostics has gained a lot of interests. In this study, a meta-analysis was conducted upon the pooled miRNA microarray data of cholangiocarcinoma (CCA).

### *AIM*

To identify differentially expressed (DE) miRNAs and perform functional analyses in order to gain insights to understanding miRNA-target interactions involved in tumorigenesis pathways of CCA.

### *METHODS*

Raw data from 8 CCA miRNA microarray datasets, consisting of 443 samples in total, were integrated and statistically analyzed to identify DE miRNAs *via* comparison of levels of miRNA expression between CCA and normal bile duct samples using *t*-tests ($P < 0.001$). The 10-fold cross validation was performed in order to increase the robustness of the *t*-test results.

### *RESULTS*

This study was written according to the PRISMA 2009 Checklist.



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Our data showed 70 up-regulated and 48 down-regulated miRNAs in CCA. Gene Ontology and pathway enrichment analyses revealed that mRNA targets of DE miRNAs were significantly involved in several biological processes. The most prominent dysregulated pathways included phosphatidylinositol-3 kinases/Akt, mitogen-activated protein kinase and Ras signaling pathways.

*CONCLUSION*
DE miRNAs found in our meta-analysis revealed dysregulation in major cancer pathways involved in the development of CCA. These results indicated the necessity of understanding the miRNA-target interactions and the significance of dysregulated miRNAs in terms of diagnostics and prognostics of cancers.

**Key words:** Cholangiocarcinoma; Microarray; MicroRNA; Meta-analysis



**Core tip**: At present, there is an accumulating mass of cholangiocarcinoma microRNA (miRNA) profiling data, however, it is challenging to gain the maximal information from these data because the experimental designs in each study tend to focus on only a few specific research questions. This work therefore integrates and inter-validates the cholangiocarcinoma miRNA expression profiles from multiple independent datasets to identify the differential dysregulation of miRNA and their corresponding downstream pathways underlying mechanism of pathogenesis. The significant merit of our findings offers a valuable reference for future studies and further investigation of these miRNA/genes and their interactions will eventually lead to the identification of genes and pathways important to the overall mechanism of the dysregulated processes in cholangiocarcinoma development.

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## INTRODUCTION

Cholangiocarcinoma (CCA), first described by Durand-Fardel in 1840, is a form of malignant tumor that originate from biliary epithelial cells in the liver and/or extrahepatic bile ducts[1,2]. CCA accounts for 10%-15% of hepato-biliary neoplasm. Thus, it is the second most common primary hepato-biliary malignancy after hepatocellular carcinoma (HCC)[3]. Moreover, the incidence and mortality rate of CCA have been reportedly increasing worldwide over the past three decades[2,4-6]. However, the prevalence of CCA vary greatly among different geographical regions of the world. Incidence of CCA in most Western countries ranges from 2 to 6 cases per 100000 people per year[7]. There is a higher prevalence of CCA in Asia and in people of Asian descent, which has been attributed to endemic chronic parasitic infestation[6,7]. Primary sclerosing cholangitis, inflammation that causes scars within the bile ducts, is the most common known predisposing factor for CCA[6]. In East and Southeast Asia, where the disease is common, CCA has been pathogenically associated with liver fluke infestation, particularly the endemic *Clonorchis sinensis*[8] and Opisthorcis viverrini[9]. In addition, hepatitis C virus infection and liver cirrhosis have been suggested as potential risk factors for CCA[6].

MicroRNAs (miRNAs) are a family of endogenous, non-coding RNAs found in plants, animals, and some viruses[10-13]. miRNA genes are highly-conserved and may be located either within the introns or exons of protein-coding genes (70%) or in intergenic areas (30%)[10,14]. A miRNA in its single-stranded functional form is usually 21-22 nucleotides long (though it can vary from 19-25 nucleotides)[10,13]. In present, the latest release of miRBase (version 22) (http://www.mirbase.org/) contains 38589 hairpin precursors and 48860 mature miRNA from 271 organisms[15]. Several hundreds of miRNA genes in the human genome have been discovered in the last decade[10,13,16].

However, it is estimated that the human genome may encode over 1000 miRNAs in total[17,18]. The primary function of miRNAs is to control gene expression at post-transcriptional level. miRNAs suppress the target mRNA expression, mostly through interaction with the 3′ untranslated region[13,19], resulting in inhibition of target mRNA translation activity and, to a lesser extent, targeting mRNA cleavage[11,16,20]. Each miRNA may be responsible for regulation of the expression of hundreds of gene targets[19].

Although the functions of dysregulated miRNAs in human cancers remain largely a mystery, multiple miRNAs and their corresponding target genes have been reported to be associated with tumor initiation and progression[18,21,22]. Many transcriptional profiling data demonstrated that miRNA expression profiling efficiently classified different tumor types more reliably than did mRNA profiling[23,24]. Systematic expression analyses using miRNA microarray technology have been performed in several types of cancer; for example, hepatic[25], colorectal[26], lung[27], and breast cancers[28].

However, due to insufficient control of false positives and the small sample sizes relative to the large sets of microarray probes, individual microarray-based studies are often deficient in terms of statistical robustness[29,30]. Meta-analysis is the use of statistical techniques to combine results from independent but related studies, hence it is one of the most preferable ways to increase the statistical power of the readily available microarray data. Moreover, it is relatively inexpensive in terms of financial and time investments[31].

Our meta-analysis of miRNA microarray datasets was aimed to identify the differentially expressed (DE) miRNA in various CCA samples compared to the non-cancerous counterparts. The results from the robust statistical tests would provide insights to understanding the regulatory potential of miRNAs in tumorigenesis pathways of CCA.

## MATERIALS AND METHODS

### *miRNA microarray data collection*

The miRNA microarray datasets were retrieved from the public repository database Gene Expression Omnibus (http: //ncbi.nlm.nih.gov/geo) *via* the computerized search using combinations of relevant keywords, including (miRNA OR microRNA) AND (cholangiocarcinoma OR CCA OR CCC). All dataset search hits were initially checked whether raw data were provided. Datasets without raw data were promptly omitted. A matrix series tables and raw data package(s) of each available dataset were downloaded and extracted. Eight miRNA microarray datasets from independent research studies were employed in our meta-analysis. Six out of these 8 datasets (GSE32958, GSE47764, GSE50894, GSE51429, GSE53870, GSE53992) were conducted using biopsied tissue samples. One study (GSE59856) used serum miRNA and another (GSE47396) used miRNA samples from cell lines.

### *Data processing*

After extraction and background-correction, the GPR or TXT raw data files of each dataset were converted to comma-separated values file format and then imported as a data frame into R software version 3.1.2[32]. Before pooling samples of all datasets, the identification name of every miRNA probe was checked against identification entries registered in miRBase database (www.mirbase.org/)[15] and was subsequently renamed in accordance with miRBase identification, in order to avoid confusion from naming system of different microarray platforms. $Log_2$ transformation was applied to all intensity values. The transformed data were then normalized following 2 steps, *i.e.*, within-array normalization using a median centering method, followed by between-array normalization using a quantile normalization method. Hence normalized data would exhibit normal distribution with the same standard deviation across datasets which is an essential assumption of parametric statistical tests.

### *Statistical analysis*

In order to identify DE miRNA in CCA, the pooled samples were grouped into 2 groups: "CCA" and "Normal". The normalized intensity values of each miRNA were compared between these two groups using *t*-tests conducted in MultiViewer Experiment version 4.6 in TM4 software suite[33]. *P* value threshold was set at below 0.001 ($P < 0.001$). As clinical validation in our study is restricted, *k*-fold cross validation was applied to verify the integrity of significant DE miRNAs from *t*-test analysis. The

10-fold cross validation was performed by randomly assigning samples into 10 different sets and repeating *t*-test statistical analysis with the same parameter ($P < 0.001$) for 10 rounds. In each round, one set was chosen as the validation set whereas the rest were test sets. DE miRNA which showed statistical significance among test sets and validation sets from all 10 rounds were collected as validated DE miRNAs for further analysis.

### Bioinformatics analysis and visualization of miRNA-target interactions

In order to assess the biological functions of the gene targets of DE miRNAs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were employed using DIANA miRPath version 3.0[34]. The lists of up- and down-regulated miRNAs were categorized and separately uploaded as inputs to DIANA miRPath. The human KEGG and GO analyses were selected with P-value threshold at 0.01. The KEGG pathways, which were the most enriched by target genes of DE miRNAs, were selected to analyze the miRNA-target interactions. Unique targets of each DE miRNA in the selected pathways were filtered into the list, which was then used in the network visualizer software. Visualized miRNA-target interaction networks were constructed using Cytoscape version 3.3.0[35] with CyTargetLinker plugin version 2.1[36].

## RESULTS

### Differentially-expressed miRNA

A framework of our meta-analysis approach is depicted in Figure 1. In total, there were eligible 246 CCA and 197 normal samples from 8 independent miRNA microarray datasets, which included tissue samples, sera and cell lines as detailed in Table 1. Initial *t*-tests results identified 224 DE miRNAs consisting 114 up-regulated and 110 down-regulated miRNAs. Following statistical validation of these results using *k*-fold cross validation, the numbers of up-regulated and down-regulated miRNAs were confined to 70 (Table 2) and 48 (Table 3), respectively. The overall expression profiling of DE miRNAs is presented in Figure 2.

### Enrichment analyses

To explore the biological functions of DE miRNAs found in the meta-analysis results, GO and KEGG pathway enrichment analyses were performed using DIANA miRPath v3.0 with *P* value threshold at 0.01 and MicroT score threshold at 0.8. The miRPath v.3.0 results indicated that there were 4407 and 7236 predicted target genes of up-regulated and down-regulated miRNAs, respectively, involved in GO biological processes. GO enrichment analysis showed 72 biological processes associated with up-regulated miRNAs (Supplementary Table 1), whereas 95 biological processes were identified to be associated with down-regulated miRNAs (Supplementary Table 2).

KEGG pathway enrichment analysis showed that gene targets of up-regulated miRNAs were significantly involved ($P < 0.01$) in 48 molecular biological processes ( Supplementary Table 3) while gene targets of down-regulated miRNAs were significantly involved ($P < 0.01$) in 32 processes (Supplementary Table 4). Figure 3 represents the most prominent dysregulated pathways of up-regulated miRNAs including phosphatidylinositol-3 kinases/Akt (PI3K/Akt) signaling pathway (215 gene targets of 57 miRNAs, $P = 0.00424$), mitogen-activated protein kinase (MAPK) signaling pathway (169 gene targets of 55 miRNAs, P = 0.00034), and Ras signaling pathway (159 gene targets of 58 miRNAs, $P = 2.34E-06$). These three pathways were also the most prominent dysregulated pathways of down-regulated miRNAs, which were PI3K/Akt signaling pathway (209 gene targets of 42 miRNAs, $P = 1.91E-05$), MAPK signaling pathway (147 gene targets of 39 miRNAs, $P = 0.0079$), and Ras signaling pathway (147 gene targets of 39 miRNAs, $P = 2.03E-06$).

### Visualization of miRNA-target interaction networks

According to the results of KEGG pathway analysis, PI3K/Akt, MAPK and Ras signaling pathways were the most enriched biological processes with which target genes of DE miRNAs were associated. For up-regulated miRNAs associated with these three pathways, 7 miRNAs including miR-330-5p, miR-519d-3p, miR-548a-5p, miR-548d-3p, miR-1207-3p, miR-1304-5p, and miR-2113 were chosen as representative miRNAs of this group. For down-regulated miRNAs, 9 miRNAs including let-7b-5p, let-7c-3p, let-7f-5p, miR-195-5p, miR-20a-5p, miR-26b-5p, miR-27b-3p, miR-29b-3p, and

**Table 1 Summary of microRNA microarray datasets used in this study**

| Dataset | Sample type | Number of samples | | Microarray platform |
|---|---|---|---|---|
| | | CCA | Normal | |
| GSE32958 | Tissue | 19 | 5 | Nanostring nCounter human miRNA expression |
| GSE47396 | Cell lines | 2 | | miRCURY LNA miRNA Array, 5th generation |
| GSE47764 | Tissue | 3 | 3 | Agilent-021827 human miRNA microarray |
| GSE50894 | Tissue | 16 | | Exiqon miRCURY LNA miRNA array version 10.0 extended and version 11.0 (condensed version) |
| GSE51429 | Tissue | 29 | | Applied Biosystems human miRNA array version 2.0 |
| GSE53870 | Tissue | 63 | 9 | State Key Laboratory human miRNA array 1104 |
| GSE53992 | Tissue | 16 | 30 | Agilent-031181 unrestricted human miRNA version 16.0 |
| GSE59856 | Serum | 98 | 150 | 3D-Gene human miRNA version 20_1.0.0 |
| Total | | 246 | 197 | |

CCA: Cholangiocarcinoma; miRNA: MicroRNA.

miR-330-3p were chosen as representatives. Using regulatory interaction networks data from TargetScan and miRTarBase, 35 and 46 unique target genes of up- and down-regulated miRNAs, respectively, were identified to be involved in these three pathways (Figure 4).

## DISCUSSION

At present, a global view of miRNA roles in the development of cancers remains incomplete. Due to widespread usage of microarray technology, there has been an enormous expansion of publicly available datasets[37], which could be integrated and analyzed with a statistically robust meta-analysis approach. In our meta-analysis, miRNA microarray datasets from multiple independent studies were analyzed with highly stringent statistics and cross validation, leading to identification of DE miRNAs in CCA compared to non-cancerous cells across the pooled samples. The lack information on whether the samples were from primary or metastatic sites would pose as one of the limitations of this study.

Many DE miRNAs observed in our study are significantly related to 3 major cancer signaling pathways, namely the MAPK signaling pathway, PI3K/Akt signaling pathway, and Ras signaling pathway. Among the up-regulated miRNAs found in our meta-analysis, miR-519d and miR-330 are of particular interest as dysregulated expression of these miRNAs have been reported in several types of cancer. miR-519d belongs to the chromosome 19 miRNA cluster, which is the largest human miRNA cluster described so far[17]. Although there is no direct evidence on the relationship of miR-519d and CCA development, miR-519d has been shown to be up-regulated in HCC patient's tissues, exerting oncogenic activity by inhibiting the tumor suppressor proteins such as CDKN1A/p21, PTEN, AKT3 and TIMP2[38]. In contrast, overexpression of miR-519d in a human HCC cell line QGY-7703 has been shown to block cell proliferation. On the contrary, down-regulation of miR-519d has been reported to promote cell proliferation in many cancers, including cervical cancer, breast cancer, and ovarian cancer[39-42].

Another up-regulated miRNA identified in our study includes miR-330. This miRNA has been previously reported to be up-regulated in glioblastoma[43], colorectal cancer[44], non-small cell lung cancer[45], and esophageal cancer[46] whereas its down-regulated expression has been demonstrated in prostate cancer[47-49]. miR-330 has been shown to promote cancer cell proliferation *via* suppression of *CDC42*, a Rho GTPase-associated with MAPK signaling pathway[44]. Besides, hypoxia-induced upregulation of integrin-alpha 5, a predicted target of miR-330 and a critical receptor in PI3K/Akt signaling pathway, has been shown to enhance cell proliferation, metastasis and apoptosis resistance of CCA cell lines[50-52]. Paradoxically, miR-330 has been reported to induce apoptosis in prostate cancer cells through E2F1-mediated suppression of Akt phosphorylation in prostate cancer cells[47].

**Table 2 List of 10-fold-cross-validated differentially-expressed microRNAs which were up-regulated in cholangiocarcinoma**

| miRNA | FC | Abs. *t* value | DF | Adj. *P* value | FDR |
|---|---|---|---|---|---|
| hsa-let-7b-3p | 1.482 | 6.603 | 74 | 0.000 | 0.000 |
| hsa-miR-1193 | 1.579 | 7.409 | 112 | 0.000 | 0.000 |
| hsa-miR-1207-3p | 1.516 | 10.533 | 120 | 0.000 | 0.000 |
| hsa-miR-1224-3p | 5.164 | 7.024 | 66 | 0.000 | 0.000 |
| hsa-miR-1227-3p | 2.039 | 7.963 | 117 | 0.000 | 0.000 |
| hsa-miR-1237-3p | 1.594 | 5.7 | 355 | 0.000 | 0.000 |
| hsa-miR-1247-5p | 1.781 | 7.406 | 126 | 0.000 | 0.000 |
| hsa-miR-1249-3p | 3.877 | 9.138 | 137 | 0.000 | 0.000 |
| hsa-miR-1267 | 2.433 | 7.758 | 119 | 0.000 | 0.000 |
| hsa-miR-1269a | 1.647 | 7.209 | 72 | 0.000 | 0.000 |
| hsa-miR-1273d | 1.665 | 7.159 | 80 | 0.000 | 0.000 |
| hsa-miR-1284 | 1.606 | 7.019 | 127 | 0.000 | 0.000 |
| hsa-miR-1304-5p | 1.651 | 6.863 | 89 | 0.000 | 0.000 |
| hsa-miR-1322 | 1.731 | 9.986 | 132 | 0.000 | 0.000 |
| hsa-miR-147b | 2.727 | 8.195 | 52 | 0.000 | 0.000 |
| hsa-miR-149-3p | 3.022 | 8.11 | 88 | 0.000 | 0.000 |
| hsa-miR-181a-2-3p | 1.476 | 7.004 | 90 | 0.000 | 0.000 |
| hsa-miR-185-3p | 5.044 | 9.684 | 113 | 0.000 | 0.000 |
| hsa-miR-1908-5p | 9.384 | 7.977 | 150 | 0.000 | 0.000 |
| hsa-miR-1909-3p | 2.19 | 6.502 | 129 | 0.000 | 0.000 |
| hsa-miR-1913 | 2.222 | 6.861 | 137 | 0.000 | 0.000 |
| hsa-miR-1914-5p | 1.575 | 6.615 | 140 | 0.000 | 0.000 |
| hsa-miR-1972 | 2.043 | 8.605 | 111 | 0.000 | 0.000 |
| hsa-miR-2113 | 1.634 | 7.74 | 358 | 0.000 | 0.000 |
| hsa-miR-2116-3p | 1.656 | 9.782 | 115 | 0.000 | 0.000 |
| hsa-miR-219a-2-3p | 1.6 | 6.436 | 125 | 0.000 | 0.000 |
| hsa-miR-2355-5p | 2.762 | 9.902 | 113 | 0.000 | 0.000 |
| hsa-miR-30c-1-3p | 1.976 | 7.144 | 114 | 0.000 | 0.000 |
| hsa-miR-3131 | 2.446 | 6.211 | 114 | 0.000 | 0.000 |
| hsa-miR-3147 | 1.371 | 7.617 | 115 | 0.000 | 0.000 |
| hsa-miR-3150a-3p | 1.894 | 7.004 | 112 | 0.000 | 0.000 |
| hsa-miR-3151-5p | 1.387 | 6.831 | 115 | 0.000 | 0.000 |
| hsa-miR-3153 | 1.994 | 9.741 | 115 | 0.000 | 0.000 |
| hsa-miR-3178 | 4.752 | 8.999 | 97 | 0.000 | 0.000 |
| hsa-miR-3180 | 4.161 | 9.232 | 92 | 0.000 | 0.000 |
| hsa-miR-3184-5p | 4.809 | 6.949 | 81 | 0.000 | 0.000 |
| hsa-miR-3185 | 3.193 | 8.217 | 96 | 0.000 | 0.000 |
| hsa-miR-3186-3p | 1.446 | 6.611 | 104 | 0.000 | 0.000 |
| hsa-miR-3197 | 4.454 | 13.341 | 95 | 0.000 | 0.000 |
| hsa-miR-325 | 1.498 | 5.994 | 168 | 0.000 | 0.000 |
| hsa-miR-330-5p | 1.808 | 8.061 | 161 | 0.000 | 0.000 |

| | | | | | |
|---|---|---|---|---|---|
| hsa-miR-412-3p | 1.933 | 7.414 | 144 | 0.000 | 0.000 |
| hsa-miR-423-3p | 1.784 | 8.826 | 81 | 0.000 | 0.000 |
| hsa-miR-4258 | 3.644 | 8.72 | 99 | 0.000 | 0.000 |
| hsa-miR-4270 | 3.705 | 6.216 | 113 | 0.000 | 0.000 |
| hsa-miR-4288 | 2.393 | 7.991 | 84 | 0.000 | 0.000 |
| hsa-miR-4292 | 2.93 | 7.318 | 83 | 0.000 | 0.000 |
| hsa-miR-4301 | 2.558 | 8.404 | 93 | 0.000 | 0.000 |
| hsa-miR-4310 | 2.22 | 8.196 | 99 | 0.000 | 0.000 |
| hsa-miR-4312 | 1.462 | 7.004 | 115 | 0.000 | 0.000 |
| hsa-miR-4323 | 1.915 | 9.85 | 109 | 0.000 | 0.000 |
| hsa-miR-512-5p | 1.484 | 6.579 | 172 | 0.000 | 0.000 |
| hsa-miR-515-3p | 1.833 | 7.931 | 97 | 0.000 | 0.000 |
| hsa-miR-517c-3p | 1.635 | 6.22 | 186 | 0.000 | 0.000 |
| hsa-miR-519d-3p | 1.833 | 7.499 | 164 | 0.000 | 0.000 |
| hsa-miR-520a-5p | 1.627 | 6.71 | 151 | 0.000 | 0.000 |
| hsa-miR-520d-5p | 5.392 | 7.514 | 342 | 0.000 | 0.000 |
| hsa-miR-520g-3p | 2.047 | 6.687 | 119 | 0.000 | 0.000 |
| hsa-miR-548a-5p | 1.79 | 6.719 | 131 | 0.000 | 0.000 |
| hsa-miR-548d-3p | 2.938 | 6.573 | 62 | 0.000 | 0.000 |
| hsa-miR-612 | 1.79 | 6.952 | 376 | 0.000 | 0.000 |
| hsa-miR-615-5p | 1.691 | 7.028 | 116 | 0.000 | 0.000 |
| hsa-miR-625-3p | 3.763 | 8.618 | 104 | 0.000 | 0.000 |
| hsa-miR-637 | 2.081 | 7.404 | 159 | 0.000 | 0.000 |
| hsa-miR-668-3p | 1.437 | 6.341 | 153 | 0.000 | 0.000 |
| hsa-miR-765 | 2.014 | 7.266 | 338 | 0.000 | 0.000 |
| hsa-miR-891a-5p | 2.297 | 13.332 | 105 | 0.000 | 0.000 |
| hsa-miR-891b | 2.052 | 7.342 | 117 | 0.000 | 0.000 |
| hsa-miR-92b-5p | 2.802 | 6.499 | 106 | 0.000 | 0.000 |
| hsa-miR-938 | 1.556 | 8.445 | 155 | 0.000 | 0.000 |

The *P* value was set to lower than 0.001 ($P < 0.001$). miRNA: MicroRNA; FC: Fold change (ratio of mean signal intensities of cholangiocarcinoma to those of normal samples); DF: Degree of freedom; Abs. *t* value: Absolute *t* value; Adj. *P* value: Adjusted *P* value; FDR: False discovery rate.

One of the down-regulated miRNAs identified in our study was miR-20a, one of the mature miRNA products of the miR-17-92 cluster pri-miRNA. Down-regulation of miR-20a has been shown to mediate cellular differentiation and growth arrest induced by HIF-1 in acute myeloid leukemia cells by targeting p21 and STAT3[52], demonstrating an oncogenic role of miR-20a. In addition, the miR-17-92 cluster miRNAs have been shown to be up-regulated and play oncogenic roles in many types of cancer including CCA[53]. In contrast, down-regulation of miR-20a has been shown to promote HCC cell proliferation *via* upregulation of Mcl-1, an antiapoptotic member of the Bcl-2 family, suggesting a tumor-suppressor role[54].

Besides, our meta-analysis of CCA miRNA microarrays has revealed down-regulation of several members of the let-7 miRNA family (let-7b, -7c, -7f), whose members are estimated to comprise 1%-5% of the mammalian genome[10,17]. This miRNA family has been shown to generally play a tumor suppressor role[55], where ectopic expression of the let-7 family inhibits cell proliferation through down-regulation of c-Myc in nasopharyngeal carcinoma cells, whereas down-regulation of let-7 promotes cancer cell growth by increasing the activity of Ras protein, in lung cancer[56] and liver cancer[57] cells.

**Table 3 List of 10-fold-cross-validated differentially-expressed microRNAs which were down-regulated in cholangiocarcinoma**

| miRNA | FC | Abs. *t* value | DF | Adj. *P* value | FDR |
|---|---|---|---|---|---|
| hsa-let-7b-5p | 0.336 | 6.655 | 186 | 0.000 | 0.000 |
| hsa-let-7c-3p | 0.686 | 9.519 | 138 | 0.000 | 0.000 |
| hsa-let-7f-5p | 0.226 | 6.65 | 89 | 0.000 | 0.000 |
| hsa-miR-100-5p | 0.424 | 7.984 | 144 | 0.000 | 0.000 |
| hsa-miR-10a-5p | 0.458 | 5.943 | 196 | 0.000 | 0.000 |
| hsa-miR-10b-3p | 0.352 | 8.834 | 72 | 0.000 | 0.000 |
| hsa-miR-1225-5p | 0.097 | 6.448 | 88 | 0.000 | 0.000 |
| hsa-miR-127-3p | 0.62 | 6.167 | 84 | 0.000 | 0.000 |
| hsa-miR-1288-3p | 0.394 | 7.17 | 61 | 0.000 | 0.000 |
| hsa-miR-1305 | 0.311 | 7.255 | 66 | 0.000 | 0.000 |
| hsa-miR-130a-3p | 0.459 | 7.775 | 101 | 0.000 | 0.000 |
| hsa-miR-136-5p | 0.51 | 7.184 | 191 | 0.000 | 0.000 |
| hsa-miR-139-3p | 0.626 | 7.227 | 95 | 0.000 | 0.000 |
| hsa-miR-145-5p | 0.359 | 7.026 | 129 | 0.000 | 0.000 |
| hsa-miR-150-3p | 0.51 | 7.109 | 139 | 0.000 | 0.000 |
| hsa-miR-17-3p | 0.638 | 7.108 | 115 | 0.000 | 0.000 |
| hsa-miR-181c-3p | 0.602 | 6.651 | 133 | 0.000 | 0.000 |
| hsa-miR-181d-5p | 0.598 | 7.741 | 138 | 0.000 | 0.000 |
| hsa-miR-192-5p | 0.267 | 7.582 | 69 | 0.000 | 0.000 |
| hsa-miR-194-5p | 0.283 | 8.454 | 90 | 0.000 | 0.000 |
| hsa-miR-195-5p | 0.449 | 7.441 | 101 | 0.000 | 0.000 |
| hsa-miR-20a-5p | 0.386 | 6.929 | 99 | 0.000 | 0.000 |
| hsa-miR-215-5p | 0.271 | 8.404 | 70 | 0.000 | 0.000 |
| hsa-miR-26b-5p | 0.305 | 7.657 | 121 | 0.000 | 0.000 |
| hsa-miR-27b-3p | 0.317 | 8.02 | 146 | 0.000 | 0.000 |
| hsa-miR-29b-3p | 0.432 | 6.216 | 110 | 0.000 | 0.000 |
| hsa-miR-29c-3p | 0.25 | 9.578 | 93 | 0.000 | 0.000 |
| hsa-miR-29c-5p | 0.755 | 6.521 | 122 | 0.000 | 0.000 |
| hsa-miR-3120-3p | 0.673 | 6.341 | 90 | 0.000 | 0.000 |
| hsa-miR-330-3p | 0.691 | 6.605 | 99 | 0.000 | 0.000 |
| hsa-miR-339-3p | 0.746 | 7.662 | 90 | 0.000 | 0.000 |
| hsa-miR-342-3p | 0.516 | 8.228 | 193 | 0.000 | 0.000 |
| hsa-miR-345-5p | 0.531 | 8.154 | 69 | 0.000 | 0.000 |
| hsa-miR-374a-5p | 0.439 | 8.25 | 99 | 0.000 | 0.000 |
| hsa-miR-374b-5p | 0.568 | 6.771 | 117 | 0.000 | 0.000 |
| hsa-miR-378b | 0.489 | 7.181 | 101 | 0.000 | 0.000 |
| hsa-miR-4294 | 0.471 | 7.279 | 96 | 0.000 | 0.000 |
| hsa-miR-4326 | 0.69 | 6.513 | 115 | 0.000 | 0.000 |
| hsa-miR-451a | 0.143 | 7.636 | 85 | 0.000 | 0.000 |
| hsa-miR-483-5p | 0.412 | 7.272 | 166 | 0.000 | 0.000 |
| hsa-miR-505-3p | 0.625 | 6.612 | 105 | 0.000 | 0.000 |

| | | | | | |
|---|---|---|---|---|---|
| hsa-miR-575 | 0.311 | 7.35 | 62 | 0.000 | 0.000 |
| hsa-miR-614 | 0.341 | 7.8 | 412 | 0.000 | 0.000 |
| hsa-miR-627-5p | 0.601 | 6.504 | 190 | 0.000 | 0.000 |
| hsa-miR-650 | 0.252 | 9.369 | 148 | 0.000 | 0.000 |
| hsa-miR-671-5p | 0.594 | 6.372 | 402 | 0.000 | 0.000 |
| hsa-miR-922 | 0.323 | 8.522 | 148 | 0.000 | 0.000 |
| hsa-miR-99a-5p | 0.328 | 9.611 | 117 | 0.000 | 0.000 |

The *P* value was set to lower than 0.001 ($P < 0.001$). miRNA: MicroRNA; FC: Fold change (ratio of mean signal intensities of cholangiocarcinoma to those of normal samples); DF: Degree of freedom; Abs. *t* value: Absolute *t* value; Adj. *P* value: Adjusted *P* value; FDR: False discovery rate.

Altogether, the meta-analysis of miRNA microarray datasets with highly stringent statistical methodology provides new insights into the role of miRNA and its dysregulations in CCA. Our findings of miRNA dysregulations in the cancer signaling pathways including PI3K/Akt pathway, MAPK pathway, and Ras pathway give clues into underlying miRNA-mRNA interplays of CCA. However, the analyses reported herein were based on the different origins of miRNAs, validations of such findings are warranted. Of clinical relevance, since many miRNAs have been reported as highly specific biomarkers for several types of cancer[58-60], the identified miRNA in this study may have predictive values for CCA cases. Also, there are conflicting results in adjuvant settings for CCA[61], the detection of a specific miRNA may be associated with an increased risk of recurrence after surgery. Further investigation of the miRNAs reported herein will bring about the novel knowledge of the dysregulated processes in CCA development at post-transcriptional level which could offer novel diagnostic and therapeutic approaches in the future.

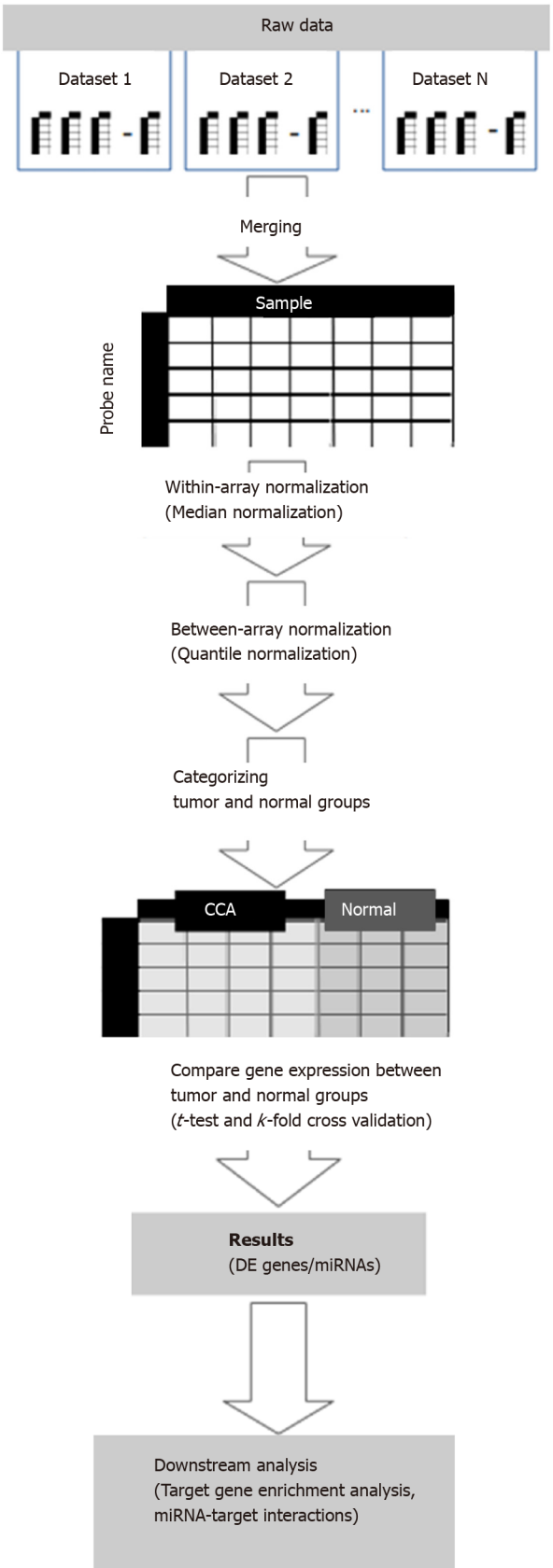


**Figure 1 Overview of a meta-analysis approach in this study.** CCA: Cholangiocarcinoma; DE: Differentially expressed; miRNA: MicroRNA.

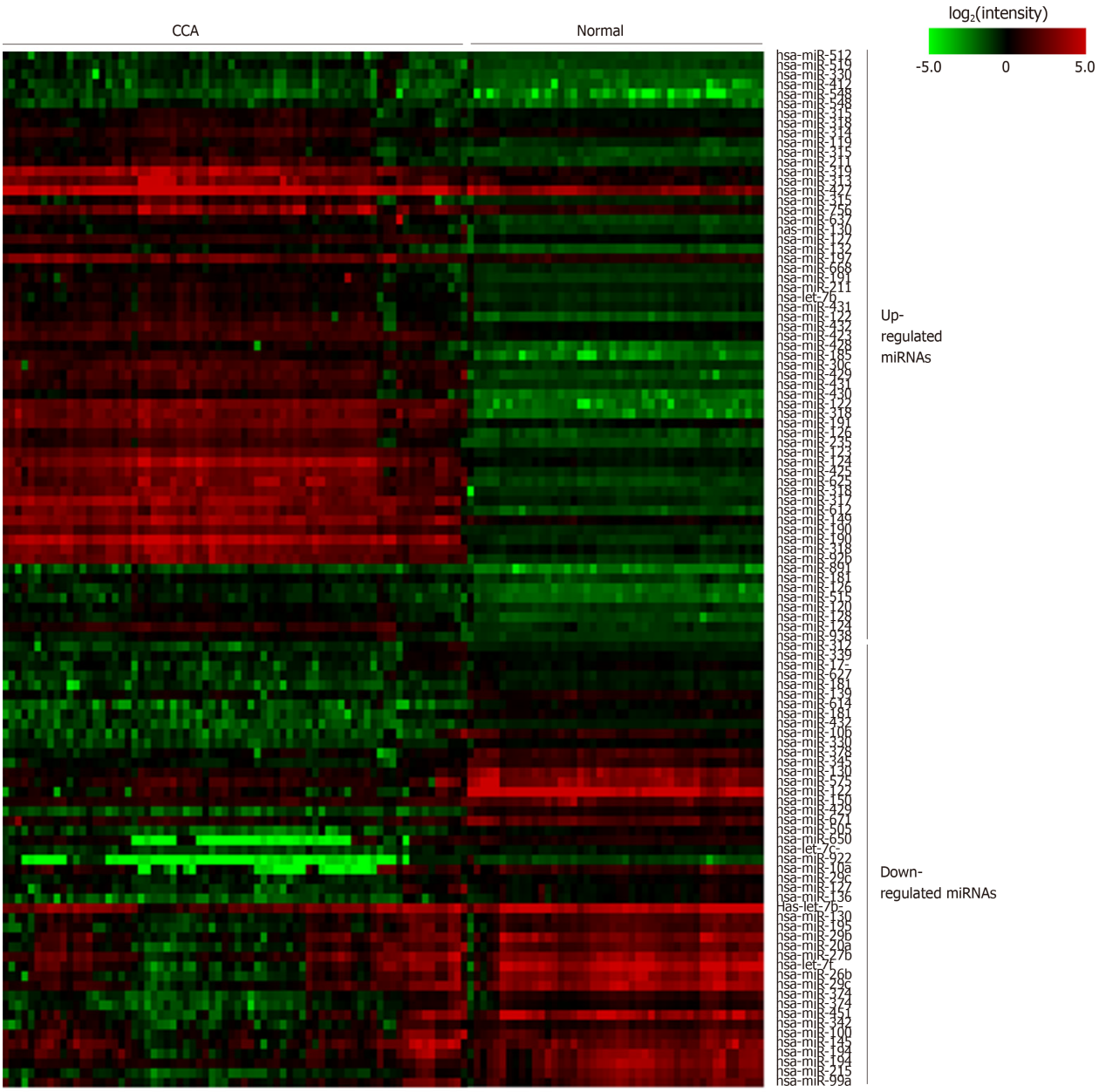


**Figure 2 Heatmap of microRNA expression in cholangiocarcinoma and normal samples.** The color gradient of each cell represents the $\log_2$ of normalized intensity value of microRNA microarray spot. CCA: Cholangiocarcinoma; miRNA: MicroRNA.

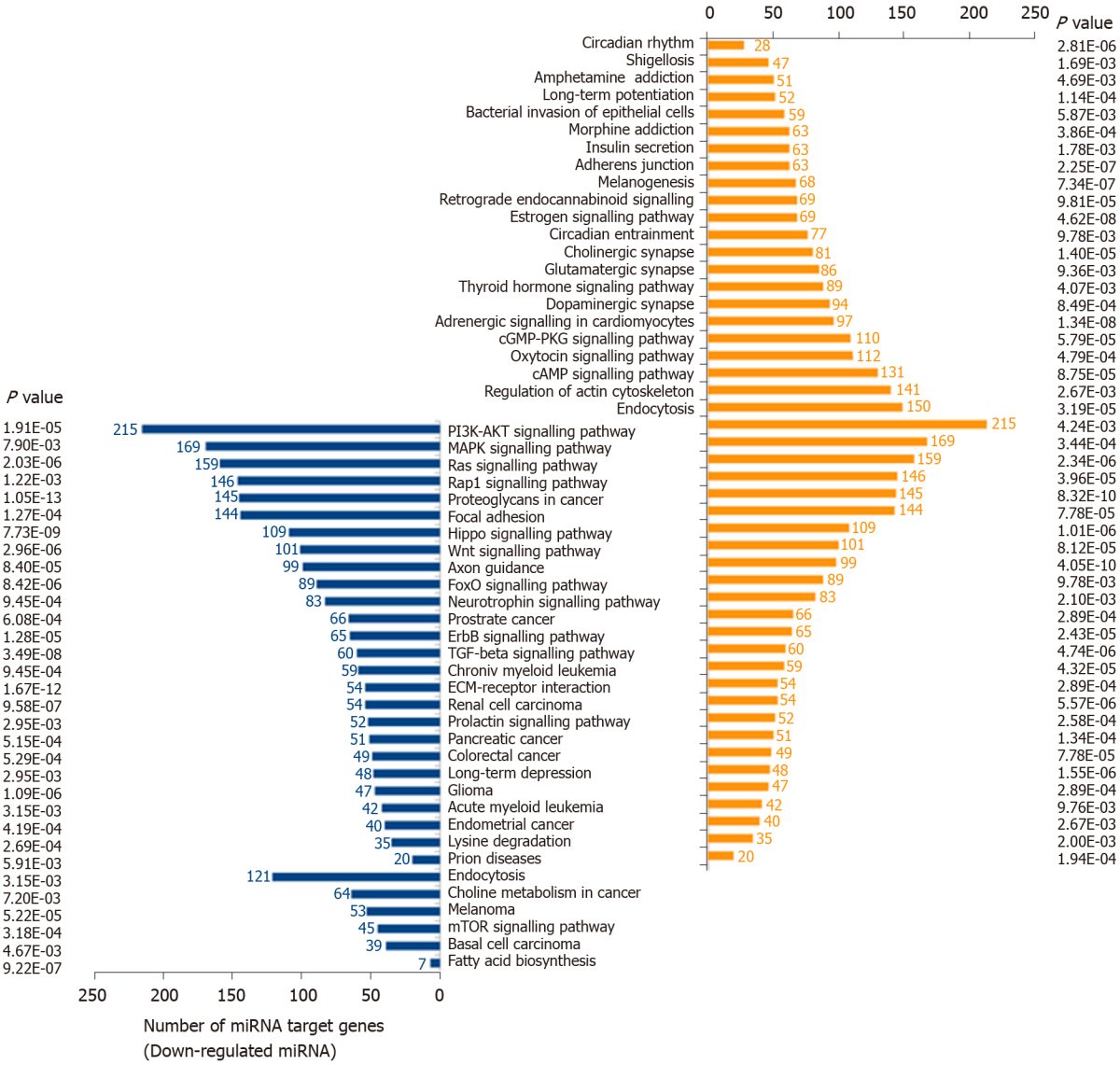


**Figure 3 Pathway enrichment analyses of predicted target genes of differentially expressed microRNAs.** The differentially expressed microRNAs obtained from meta-analysis were input to DIANA miRPath version 3.0. *P* value thresholds were set at 0.01. PI3K: Phosphatidylinositol-3 kinases; MAPK: Mitogen-activated protein kinase.

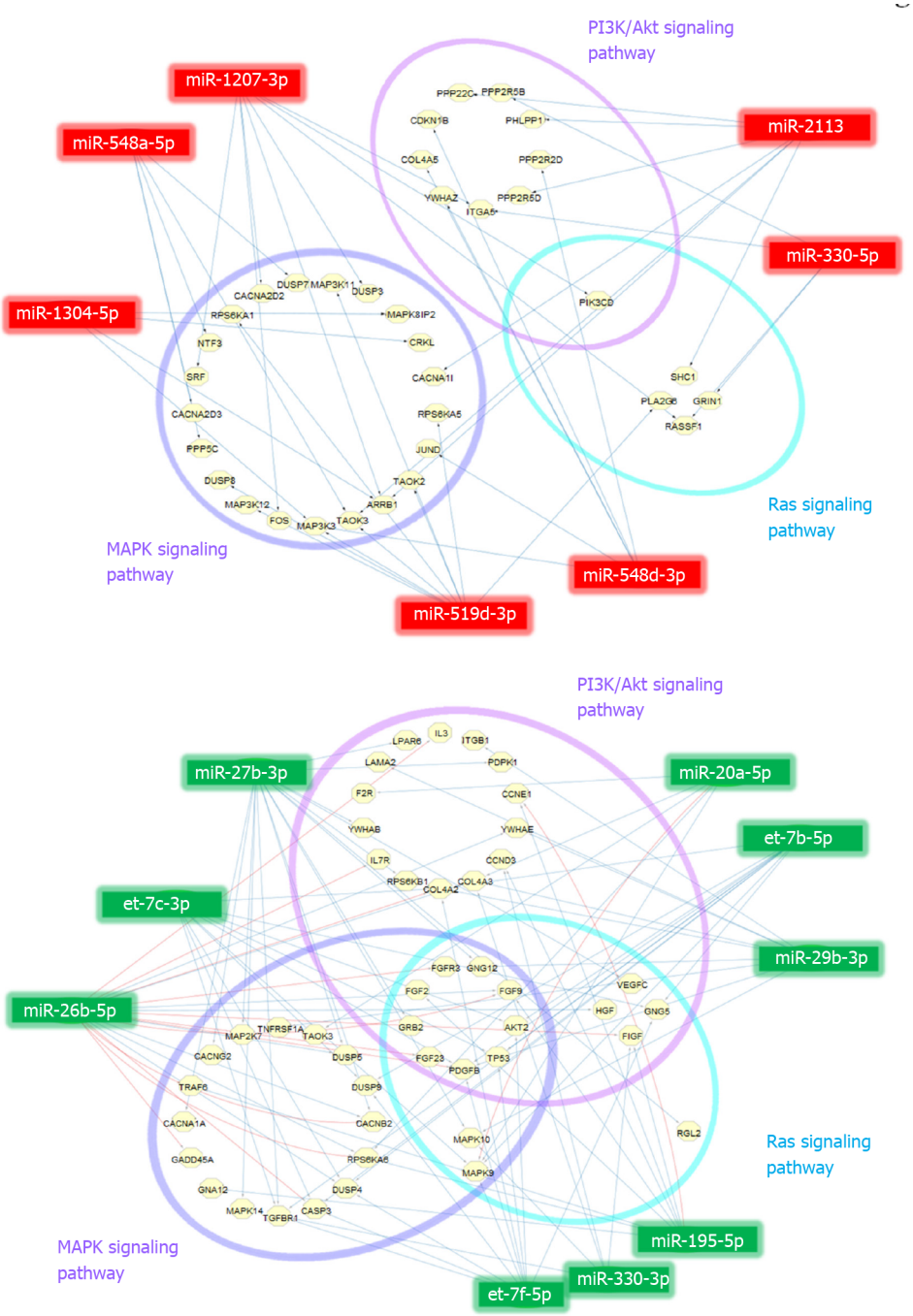


**Figure 4 MicroRNA-target interaction networks.** A: Up-regulated; B: Down-regulated. MicroRNA (miRNA)-target interaction networks of 7 up-regulated and 9 down-regulated miRNAs associating in phosphatidylinositol-3 kinases/Akt, mitogen-activated protein kinase, and Ras signaling pathways based on pathway enrichment analysis via DIANA miRPath version 3.0. Blue and red lines indicate the miRNA-target prediction or information based on TargetScan and miRTarBase databases, respectively. PI3K: Phosphatidylinositol-3 kinases; MAPK: Mitogen-activated protein kinase.

## ARTICLE HIGHLIGHTS

### *Research background*

The incidence of cholangiocarcinoma (CCA) is alarmingly elevating in many countries. Patients with CCA usually have poor prognosis as there is still no effective screening and treatment available. Therefore, it is essential to identify biomarkers for CCA.

### *Research motivation*

Differential expression profiles of microRNA (miRNA) have been reported for many different types of cancer. Thus, a growing number of miRNA microarray data can be a valuable resource for the discovery of biomarkers to tackle challenges in the clinical management of CCA.

### Research objectives

This work integrates and intervalidates the CCA miRNA expression profiles from multiple independent datasets to identify the differential dysregulation of miRNA and their corresponding downstream pathways underlying mechanism of pathogenesis.

### Research methods

Eight independent CCA miRNA profiling microarray datasets, including 246 CCA and 197 normal samples were assimilated into a meta-analysis and cross-validation to identify a cohort of miRNA that were significantly dysregulated in CCA.

### Research results

Of 118 dysregulated miRNA identified in our study, 70 were up-regulated and 48 were down-regulated miRNAs in CCA. Bioinformatic analyses revealed that mRNA targets of differentially expressed miRNAs were significantly distributed across various biological processes. The most prominent dysregulated pathways included phosphatidylinositol-3 kinases/Akt, mitogen-activated protein kinase and Ras signaling pathways.

### Research conclusions

This current study represents the meta-analysis of miRNA microarray datasets with highly stringent statistical methodology and provides new insights into the role of miRNA and its dysregulations in CCA.

### Research perspectives

The merit of our findings offers a valuable reference for future studies and further investigation of these miRNA/genes and their interactions will eventually lead to the identification of genes and pathways important to the overall mechanism of the dysregulated processes in CCA development.

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CASE REPORT

# Multifocal gastrointestinal epithelioid angiosarcomas diagnosed by endoscopic mucosal resection: A case report

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**Author contributions:** Chen YW was the patient's doctor, who performed the gastroscopy, colonoscopy and endoscopic mucosal resection; Dong J was responsible for collecting medical history of the patient and drafting the paper; Chen WY was a pathologist who provided the pathological results; Dai YN reviewed the literature and revised the manuscript; all authors issued final approval for the version to be submitted.

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## Abstract

*BACKGROUND*

Epithelioid angiosarcoma is a vascular neoplasm that is among the most aggressive subtypes of sarcomas. Its involvement in the gastrointestinal tract is rare. We here report a case of multifocal gastrointestinal epithelioid angiosarcomas presenting with gastrointestinal bleeding.

*CASE SUMMARY*

A 77-year-old woman was admitted because of melena and dizziness for three months. Gastroscopy and colonoscopy were performed, revealing a centrally ulcerated hemorrhagic polypoid lesion in the gastric body and multiple polypoid lesions with blood clots and hemorrhagic tendency in the colon. Histopathological examination of routine endoscopic biopsy samples showed inflammation in the gastric mucosa and tubular adenomas in the colon. The polypoid lesions were removed by endoscopic mucosal resection. Immunohistochemistry suggested a final diagnosis of epithelioid angiosarcomas. The patient refused chemotherapy and died after three months.

*CONCLUSION*

Epithelioid angiosarcomas are characterized by highly vascular nature and tendency to cause gastrointestinal bleeding. Efforts to obtain histological findings using endoscopic mucosal resection are of great importance.

**Key words:** Epithelioid angiosarcoma; Gastrointestinal tract; Endoscopic mucosal

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**Core tip:** Gastrointestinal epithelioid angiosarcoma is extremely rare, which is only described in individual case reports and case series. Most reported cases appeared as centrally ulcerated, hemorrhagic, highly erythematous or purpuric nodules. Histopathology is the golden standard for diagnosis. Endoscopic biopsy often obtains insufficient specimens, while endoscopic mucosal resection of suspected lesions is satisfactory for histopathological examination. We here report a case of gastrointestinal bleeding which was finally diagnosed as multifocal epithelioid angiosarcomas in the gastrointestinal tract.

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## INTRODUCTION

Epithelioid angiosarcoma is a sporadic, highly aggressive malignancy that originates from a variety of locations, most commonly from the skin and soft tissue[1]. This type of tumor occasionally occurs in the liver, spleen, ovary, breast and adrenal gland[2]. Primary gastrointestinal angiosarcoma is extremely rare[3,4]. Here, we present a case of multifocal epithelioid angiosarcomas involved in the gastrointestinal tract.

## CASE PRESENTATION

### *Chief complaints*

A previous healthy 77-year-old woman presented with a 3-mo history of melena and dizziness and was admitted to our hospital.

### *Physical and accessory examinations*

The physical examination was unremarkable except for pale conjunctiva. Her hemoglobin level was 65 g/L. The platelet count, coagulation function, and blood biochemistry were within normal limits. The antinuclear antibody titre was 1:1000, while the anti-SSA (Ro60) antibodies were weakly positive. Autoimmune diseases were ruled out by rheumatologists because there were no related symptoms. Abdominal computed tomography angiography revealed nothing remarkable.

### *Endoscopy and histopathology*

Gastroscopy demonstrated a centrally ulcerated haemorrhagic polypoid lesion in the gastric body (Figure 1A). Colonoscopy revealed multiple polypoid lesions with blood clots and hemorrhagic tendency in the ileocecum, ascending colon, transverse colon and sigmoid colon (Figure 1B-D). Routine endoscopic biopsies showed inflammatory infiltration in the gastric mucosa and tubular adenomas with low-grade intraepithelial neoplasia and local eosinophilic changes in the colon. Nevertheless, the pathologic findings did not conform to the endoscopic appearance of the foci. Subsequently, the lesions were resected *en bloc* by endoscopic mucosal resection for further diagnosis.

Microscopically, hematoxylin and eosin staining showed areas of epithelioid cells with abundant eosinophilic cytoplasm, which was morphologically similar to undifferentiated carcinoma or poorly differentiated adenocarcinoma (Figure 2). Immunohistochemical staining (Figure 3) demonstrated that the tumor cells were positive for pan-cytokeratin (AE1/AE3), CD31, CD34, EMA, vimentin and Ki67 (60% positive) and negative for hepatocytes, S100, CD117, DOG1, CD56, SYN, CgA, LCA, desmin or ALK. The final pathological diagnosis was epithelioid angiosarcoma.

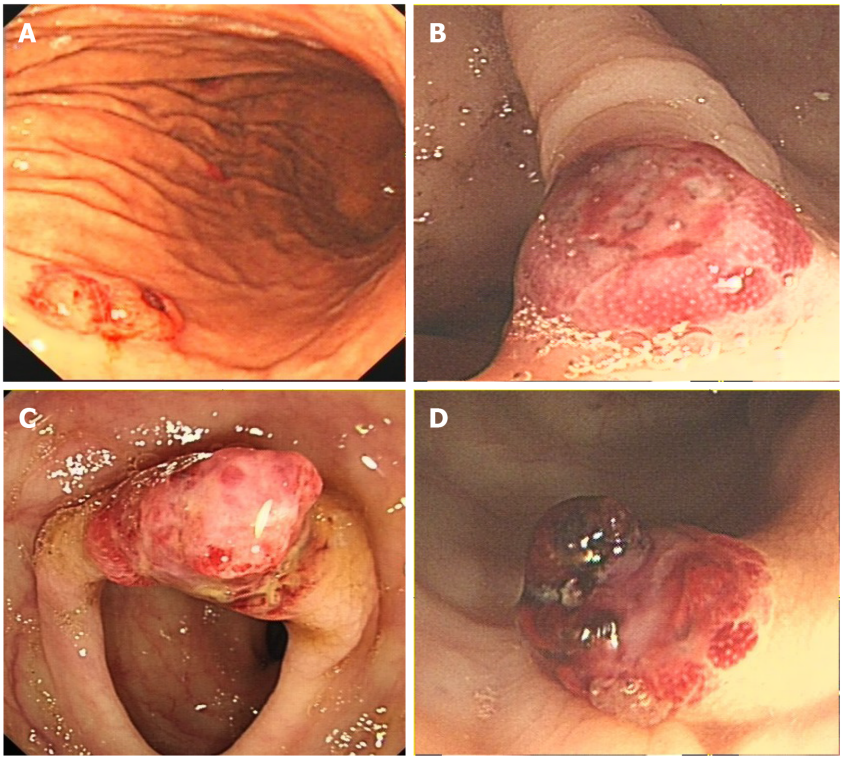


**Figure 1 Endoscopy.** A: Gastroscopy revealed a 0.5 cm × 0.6 cm centrally ulcerated polypoid lesion in the gastric body; B: A 0.8 cm × 0.8 cm polypoid lesion in the ascending colon; C: A 1.2 cm × 1.0 cm hyperaemic mass in the transverse colon; and D: A 0.8 cm × 0.8 cm polypoid nodule with hemorrhagic tendency and blood clots in the sigmoid colon.

### *Positron emission tomography/computed tomography*

To determine whether distant metastasis occurred, additional F-18 fluorodeoxyglucose positron emission tomography/computed tomography (PET/CT) was performed. PET/CT showed tubular hypermetabolic lesions in the colon, suggesting malignancy. Furthermore, numerous hypermetabolic hilar and mediastinal lymph nodes, as well as multiple node-like foci with active F-18 fluorodeoxyglucose metabolism in the bones, were observed, revealing lymphatic and osseous metastasis (Figure 4).

## FINAL DIAGNOSIS

Multifocal gastrointestinal epithelioid angiosarcomas with lymphatic and osseous metastasis.

## OUTCOME AND FOLLOW-UP

The patient refused chemotherapy and died after three months due to gastrointestinal bleeding.

## DISCUSSION

Angiosarcomas may arise in any part of the body, but commonly occur in skin and superficial soft tissues, with its predilection sites in the head and neck[5]. Epithelioid angiosarcoma of the gastrointestinal tract is an extremely rare neoplasm, which is only described in individual case reports and case series[6-9], with a lack of demographic morbidity data. According to literatures, vascular tumors of the stomach constitute only 0.9%-3.3% of all gastric cancers[10], while colorectal angiosarcomas represent less than 0.001% of all colorectal neoplasms[11].

Epithelioid angiosarcoma is characterized by an extremely aggressive course, leading to a very poor prognosis[12]. Even for localized disease, patients without any

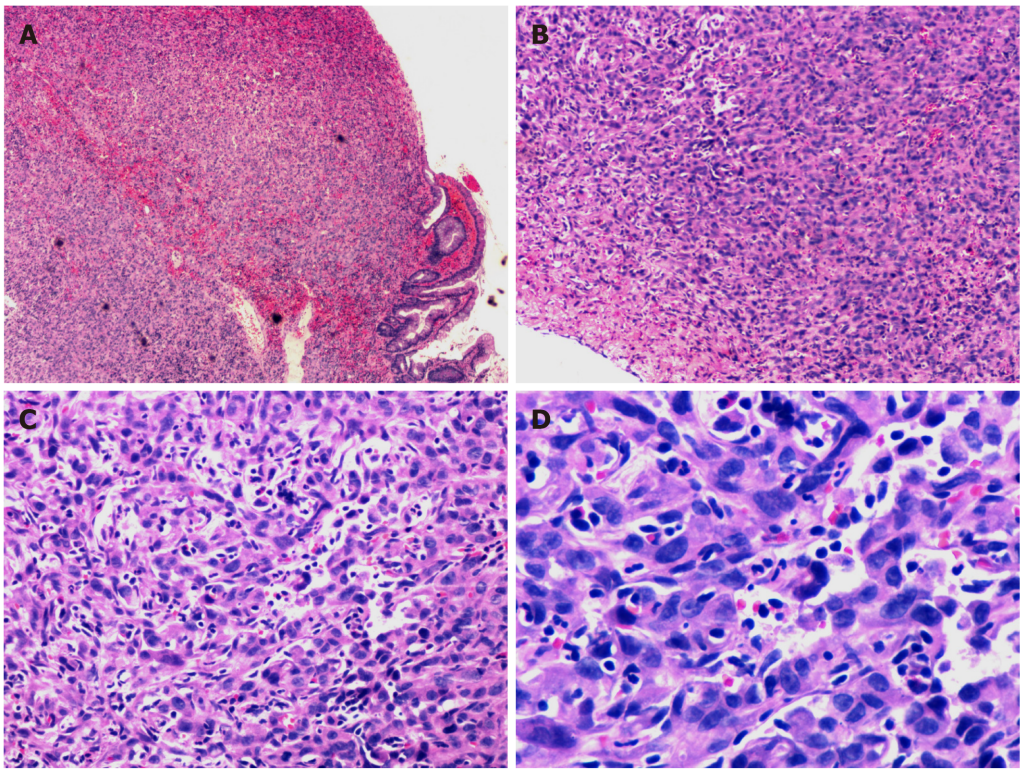


**Figure 2 Histopathological findings.** Poorly differentiated neoplasms consisting of large cells with pleomorphic nuclei and abundant eosinophilic cytoplasm were observed. A: Haematoxylin and eosin (HE) staining section × 40; B: HE staining section (× 100); C: HE staining section (× 200); and D: HE staining section (× 400).

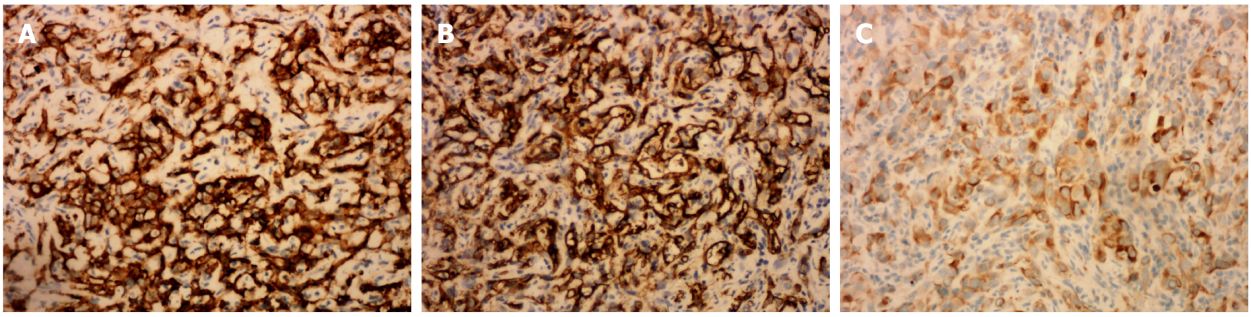


**Figure 3 Immunohistochemical staining.** A: Immunostaining for CD31; B: Immunostaining for CD34; and C: Immunostaining for pan-cytokeratin.

therapy had a 0% survival rate at 2 years[5]. Due to the infiltrative nature of this neoplasm, recurrence and metastasis are frequent after surgical resection, even for localized disease. Cytotoxic chemotherapy can be effective in a subset of patients for a limited period, but metastatic angiosarcomas remain incurable and even fatal[5]. Targeted therapy with tyrosine kinase inhibitors usually resulted in significant responses, but these tumors are prone to developing resistance[5].

In most cases of gastrointestinal angiosarcoma, the symptoms are nonspecific, including abdominal pain, weight loss and anorexia. Overt bleeding with melena or hematochezia is less common. The patient in the present report had positive antinuclear antibody and anti-SSA, which might be secondary to the tumor. Nevertheless, until now, there is no evidence that the positivity of autoimmune antibodies has association with angiosarcomas. Further studies are needed to clarify their relationships.

Almost all reported lesions that occurred in the gastrointestinal tract appeared endoscopically as centrally ulcerated, hemorrhagic, highly erythematous or purpuric nodules/masses[13]. Endoscopy with direct visualization and biopsy is crucial for diagnosis. However, endoscopic biopsy often obtains insufficient specimens and leads to the possibility of a missed diagnosis[14], similar to this case. It is well known that

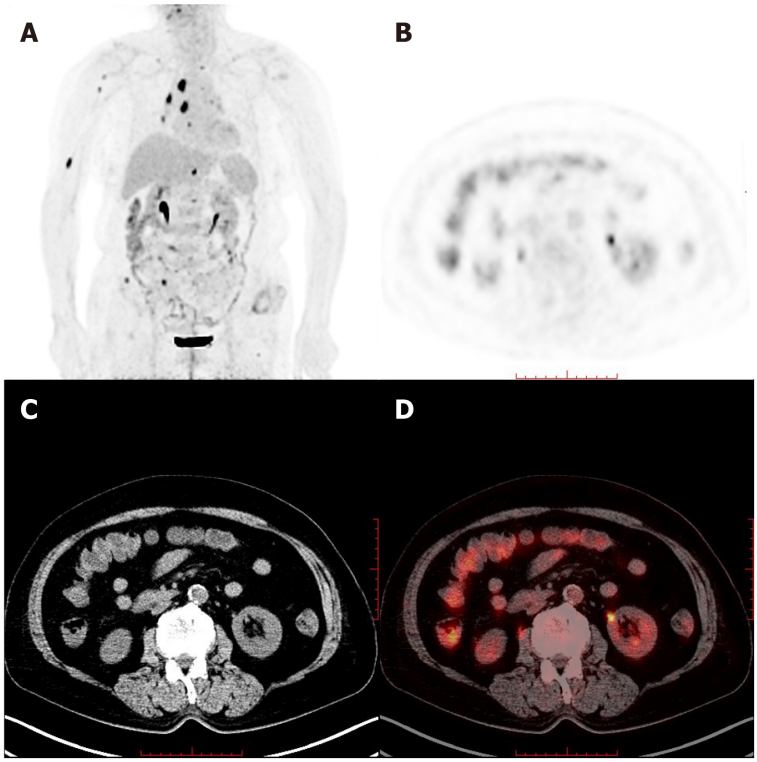


**Figure 4 Whole-body maximum intensity projection 18F- fluorodeoxyglucose and positron emission tomography image.** A: Remarkably increased fluorodeoxyglucose metabolism in the colon and mediastinal lymph nodes, as well as the right humerus; B: Positron emission tomography; C: Computed tomography; and D: Positron emission tomography/computed tomography in axial projection showed multiple tubular hypermetabolic lesions in the colon.

invasive carcinomas are likely to be covered by normal epithelium or intraepithelial neoplasm. Consequently, findings of the biopsy on their surface usually do not reflect the invasive components. Possible efforts to obtain additional histopathological findings must be made. In the present case, *en bloc* endoscopic mucosal resection of the suspected lesions in the colon was satisfactory for the histopathological examination. Due to the confirmation of malignant angiosarcomas, the patient refused to have another large-scale biopsy of the gastric lesion, in which the endoscopic performance was similar and typical.

Epithelioid angiosarcoma mimics carcinoma morphologically and contains numerous vasoformative structures. Because of the high architectural and cytological variability, pathological diagnosis is difficult. Immunoreactivity for endothelial and epithelial cell markers can confirm the diagnosis. This innate characteristic of epithelioid angiosarcoma corresponds to the tendency of gastrointestinal bleeding. PET/CT has been reported to detect primary uterine and pulmonary epithelioid angiosarcoma[15,16] and is of great value in identifying metastases.

## CONCLUSION

We report a case of gastrointestinal bleeding that was finally diagnosed as multifocal epithelioid angiosarcomas involved in the gastrointestinal tract. The endoscopic characteristics of the lesions were highlighted and corresponded to the propensity of gastrointestinal bleeding. *En bloc* endoscopic mucosal resection contributed to the final histopathological diagnosis.

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