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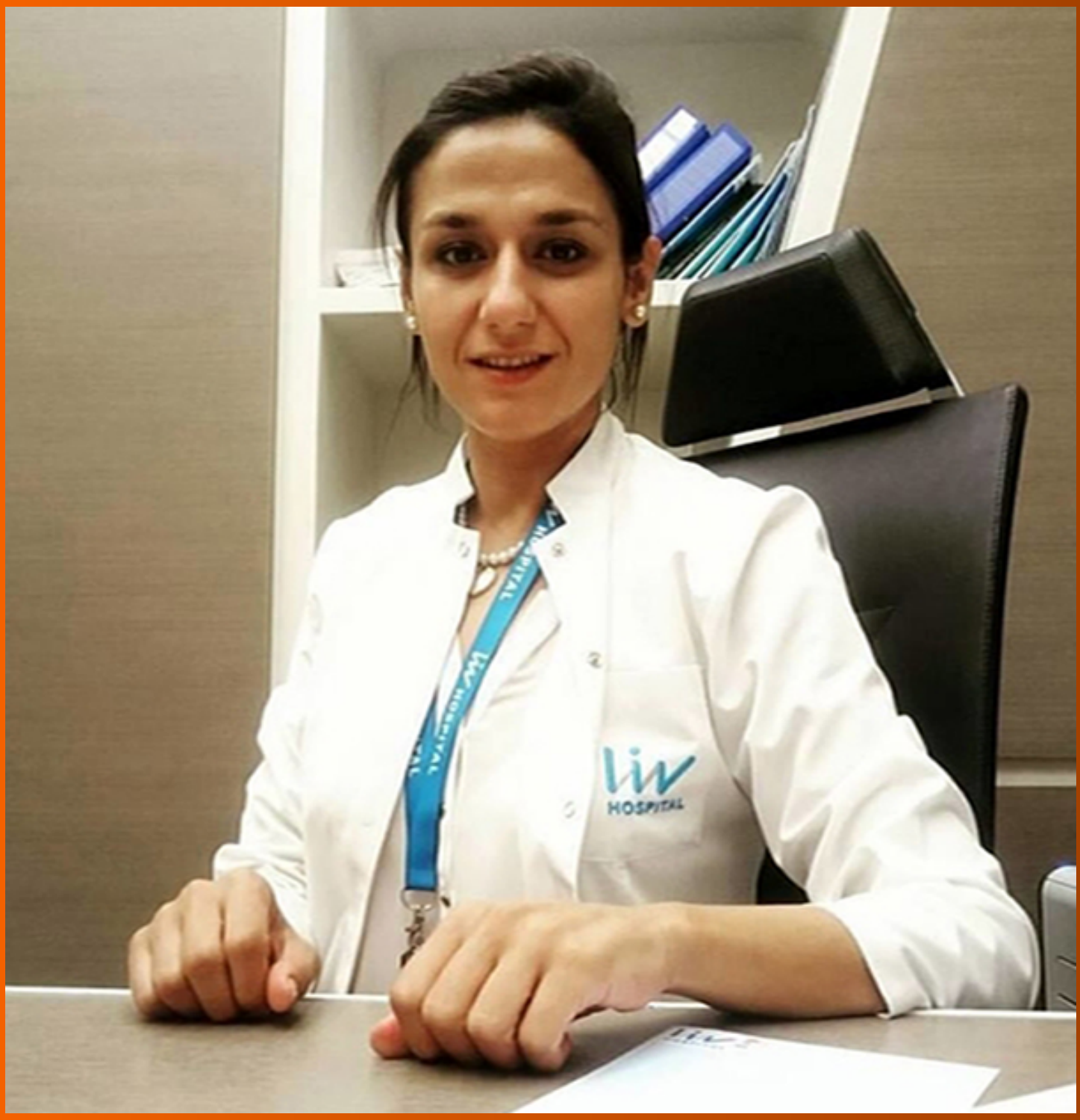

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Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Naciye Cigdem Arslan, MD, Assistant Professor, Surgeon, Surgical Oncologist, General Surgery, Istanbul Medipol University, Esenler 34320, Istanbul, Turkey

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EDITORIAL

Cancer-specific metabolism: Promising approaches for colorectal cancer treatment

Keun-Yeong Jeong

ORCID number: Keun-Yeong Jeong (0000-0002-4933-3493).

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Keun-Yeong Jeong, The Research Center, Metimedi Pharmaceuticals, Incheon 22006, South Korea

Corresponding author: Keun-Yeong Jeong, PhD, Executive Vice President, The Research Center, Metimedi Pharmaceuticals, 263, Central-ro, Yeonsu-Gu, Incheon 22006, South Korea.
alvirus@naver.com
Telephone: +82-32-2050541
Fax: +82-32-2050542

Abstract

Investigation of cancer-specific metabolism has made it possible to establish the principle that atypically reconstituted metabolism is considered a hallmark of cancer due to changes in physiological property. Recently, a variety of targets depending on the prompted aerobic glycolysis process, starting from the abnormal uptake of glucose, and cancer-specific metabolism due to impaired mitochondrial function and abnormal expression of drug-metabolizing enzymes have been investigated and discovered. Given that most solid cancers rely on cancer-specific metabolism to support their growth, it is necessary to examine closely the specific processes of cancer metabolism and have a detailed understanding of how cellular metabolism is altered in colorectal cancer (CRC) related to CRC survival and proliferation. The development of key methods to regulate efficiently cancer-specific metabolism in CRC is still in the initial stage. Therefore, targeting cancer-specific metabolism will yield treatable methods that are critical as a new area of development strategies for CRC treatment.

Key words: Colorectal cancer; Cancer metabolism; Warburg effect; Aerobic glycolysis; Mitochondria metabolism

Core tip: Studies of cancer-specific metabolism have been conducted for over half a century, and the importance of promoting aerobic glycolysis, cancer favorable metabolic changes in mitochondria, and abnormal expression of drug-metabolizing enzymes has been emphasized through the established theories to date. Cancer-specific metabolism is a major theoretical background that can explain the process of survival and proliferation of most solid cancers. Developing cancer-specific metabolism-target drugs provide a novel treatable method that will be critical in this new area of treatment strategies for colorectal cancer. They have not yet been conquered and have infinite growth potential.

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GENERAL VIEW ON CANCER METABOLISM

Cancer metabolism is classified as a classic but major research field in clinical and preclinical cancer biology. Studies on cancer-specific metabolism for over half a century have made it possible to establish the principle that abnormal metabolic changes are induced in normal cells[1]. This theory is primarily representative of the imbalance between the expression of oncogenes and the regulation of tumor suppressor genes, and these changes support the induction and maintenance of malignant characteristics in cancer cells[2]. Atypically reconstituted metabolism is considered a hallmark of cancer due to changes in a physiological property that are most commonly found in cancer cells[2]. Main issues while approaching the study of cancer metabolism are how abnormal functions in cancer-specific metabolism contribute to the survival of cancer cells and how to change these metabolisms using certain targets. The comprehensive principle of cancer metabolism is that altered metabolic activity improves the adaptability of cells to provide a selective benefit for tumorigenesis[3]. Well-known theories indicate that activities initiated by abnormal metabolic changes support cancer cell survival under stress conditions, such as hypoxic environment[3]. This is an important characteristic of malignant cancer metabolism and enables the abnormal proliferation of cancer cells[4].

Most solid cancers, including colorectal cancer (CRC), have inherent but similar metabolic characteristics. A well-known cancer-specific metabolism is the Warburg effect, and aerobic glycolysis has been well-established as a main metabolic feature of cancer cells[4,5]. This theory states that an increase in aerobic glycolysis is a physiological response to hypoxia and that cancer cells absorb a large quantity of glucose and produce lactate regardless of the oxygen supply, providing a secondary path that meets the metabolic needs of the cancer cells[1,4]. However, this well-established theory does not fully reflect cancer-specific metabolism. Although the Warburg effect has led to the widely held conception that cancer cells rely only on aerobic glycolysis to manage their major source of energy, the function of the mitochondria is not completely inactivated even under hypoxic environment[6]. In addition, the role of drug-metabolizing enzymes (DMEs) in anti-cancer drug resistance should also be noted. Therefore, when studying cancer-specific metabolism, not only should aerobic glycolysis be considered but also abnormal mitochondrial metabolism and DMEs in cancer cells.

TARGETABLE CANCER-SPECIFIC METABOLISM IN AEROBIC GLYCOLYSIS

Abnormal uptake of glucose is the most well-known metabolism in cancer[5]. Glucose transporter (GLUT) is a unique transporter and is considered responsible for a large amount of glucose uptake in cancer cells[5]. The expression of GLUT1 is increased in cancer cells among several subtypes of GLUT, and there is an alternative glucose uptake with passive GLUT, GLUT3, which is not expressed in most normal cells[7]. Following a large amount of glucose uptake, many enzymes involved in the process of producing pyruvate from glucose can be targeted. These include hexokinase 2, which produces glucose-6-phosphate from glucose, and phosphofructokinase 2, which produces fructose-6-phosphate from fructose-2, 6-bisphosphate[8]. The pyruvate kinase M2 isoform, which promotes aerobic glycolysis and produces pyruvate from phosphoenolpyruvate, is one of the important target proteins expressed by aerobic glycolysis[9]. Activation of the listed targets above led metabolism in cancer cells to produce a large amount of pyruvate from the excess glucose uptake. In normal cells, pyruvate is converted to acetyl-CoA *via* pyruvate dehydrogenase and generates energy through oxidative phosphorylation (OXPHOS)[6]. However, in cancer cells, pyruvate dehydrogenase kinase is activated, and pyruvate dehydrogenase is phosphorylated by pyruvate dehydrogenase kinase to suppress its activity. Excess pyruvate that cannot participate in OXPHOS is converted from pyruvate and NADH to lactate and NAD+ by lactate dehydrogenase A[6]. Then, the symbiotic relationship

between cancer cells following lactate production must be considered. There are cancer cells that release lactate through the monocarboxylate transporter and cancer cells that utilize the released lactate as an energy source. Therefore, to target lactate dehydrogenase A and monocarboxylate transporters involved in the producing and transferring lactate would be important[6,10].

TARGETABLE CANCER-SPECIFIC METABOLISM IN MITOCHONDRIA

Cancer cells accommodate to hypoxic condition by converting their metabolism to the oxygen-independent system by mitochondria[11]. Reductive carboxylation is induced in mitochondria under hypoxic condition, this feature is a driving force that maintains the viability by having tolerance about hypoxia[12,13]. These specific regulations in mitochondrial metabolism cause multiple changes in the composition of electron transport chain complexes, which could decrease O_2-dependent mitochondrial function, such as coupled metabolism with OXPHOS[14]. However, this metabolic change does not represent a complete loss of mitochondrial function. Instead of OXPHOS, which is referred to as the tricarboxylic acid cycle, mitochondria carry out cancer-specific metabolism adapting to the hypoxic condition for cancer survival[6]. Point mutations in isocitrate dehydrogenase 1 (IDH1) and IDH2 involve the production of d-2hydroxyglutarate (2HG), which is an inhibitor of α-ketoglutarate-dependent dioxygenase[15]. Since α-ketoglutarate-dependent dioxygenase is involved in the oxygen-sensing pathway that mediates the destabilization of hypoxia-inducible factor, the abnormal state of mitochondrial IDH and 2HG contributes to hypoxia-inducible factor stability and the transcriptional activation for expression of cancer favorable factors, such as vascular endothelial growth factor and GLUT[16].

Cancer favorable mitochondria can lead to lipid synthesis, amino acid synthesis, and nucleotide synthesis critical for cancer survival[17]. These diverse syntheses depend on the reverse metabolism of glutamine in cancer favorable mitochondria, and it replenishes the biosynthetic precursors[18]. Glutaminase catalyzes the conversion of glutamine to glutamate in a pathway involved in producing citrate[19]. Since glutaminase 1 is a source of 2HG production by mutated IDH1, it mediates the entry of glutamine into mitochondria and can thus enhance the proliferation of cancer cells[16,17,19]. Therefore, although mitochondria under hypoxic condition have a diminished function of energy metabolism, it should also be important to explore a variety of pathways that produce energy sources for cancer survival through changes in the cancer-specific metabolism in mitochondria by genetic variation or glutamine.

TARGETABLE CANCER-SPECIFIC METABOLISM IN DMES

Despite advances in medicine that lead to new drugs with specific molecular targets, major problems still remain with regard to anticancer drug resistance. This resistance is known to be caused by certain proteins that are attributed to DMEs in cancer, and DMEs can influence the susceptibility to therapeutic effects[20]. DMEs are classified in neoplastic tissues as phase I and phase II. Cytochrome P450-dependent monooxygenase (CYP) and dihydropyrimidine dehydrogenase (DPD), which are included in phase I enzymes, lead to the variations of efficacy or toxicity of the anticancer drugs[21]. Members of the subfamily of cytochrome P450 are represented to CYP family 1-3 (CYP1-3)[21]. Phase II enzymes mediate the conjugation of the products from phase I metabolism resulting in the subsequent elimination step of drug metabolism[22]. Glucuronide, glutathione system, beta-glucuronidase, aldehyde dehydrogenase, and nicotinamide adenine dinucleotide phosphate hydrogen quinone oxidoreductase-1 are members belonging to the phase II enzymes[22]. In CRC, it has been reported that CYP1B1, DPD, uridine diphospho-glucuronosyltransferase, and glutathione-transferase were highly expressed as compared to normal tissues[20,23]. Increase in such DMEs can induce resistance to various anticancer agents, in particular to cisplatin, paclitaxel, docetaxel, flutamide, and mitoxantrone, including 5-fluorouracil and irinotecan, which belong to the first or second line regimens for the CRC treatment[20]. The following mechanisms relating to DMEs expression have not been clearly elucidated. It can be explained either by metabolism of anticancer drugs and elimination of their action or by direct deactivation of drug molecules and mitogen-activated protein kinase pathways[20,23]. Further, several attempts have been made to develop potent inhibitors of DMEs, however many of these have been found to have a poor safety profile and to have many side effects[20,23]. Therefore, while focusing on molecular biological factors aimed at the intrinsic metabolism involved in growth and

metastasis, there is a continuing need to clarify the metabolism of DMEs, particularly by CYP1B1, DPD, uridine diphospho-glucuronosyltransferase, and glutathione-transferase, as a strategy overcoming cancer drug resistance.

THERAPEUTIC APPLICABILITY TARGETING CRC

Given that most solid cancers rely on cancer-specific metabolism to support their growth, survival, and multi-organ metastasis, targeting these metabolic activities may be main therapeutic strategies against CRC. In addition, the characteristic liver metastasis of CRC is also closely related to the metabolic abnormalities; therefore, the therapeutic and inhibitory effects on metastasis through targeting cancer-specific metabolism can be potentially anticipated. As described in the previous paragraphs, since various factors relating to cancer-specific aerobic glycolysis, mitochondrial metabolism, and DMEs have been identified in recent years, the stage has been reached where an optimal strategy to suppress effectively these metabolism-based targets should be established. A detailed understanding of how cellular metabolism is altered in CRC that leads to cancer progression and metastasis will provide insight into which proteins represent promising targets in CRC therapy, which will be raised from the analysis of cancer-specific metabolism. Building a theoretical context to understand metabolic regulation in CRC, however, remains a challenge for the successful construction of strategies. Therefore, the specific process of cancer metabolism related to the survival and differentiation of CRC must be closely examined, and work must focus to advance steadily the discovery of candidate proteins that can target it. It is important to point out that with the exception of 5-fluorouracil, Gemcitabine, or Pemetrexed, which were developed for inhibiting nucleic acid synthesis, other developing therapies are only in the early stages, where most pre-clinical studies have been completed[20]. Of course, it is not my purpose to raise concerns that there are few attempts to target cancer-specific metabolism for the treatment of CRC but to emphasize that the development of key methods to regulate efficiently cancer-specific metabolism is still under the initial stage. Thus, it is suggested that developing cancer-specific metabolism-target drugs provide a novel treatable method that will be critical in this new area of treatment strategies for CRC. They have not yet been conquered and have infinite growth potential.

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REVIEW

Race, the microbiome and colorectal cancer

Kendra J Royston, Babatunde Adedokun, Olufunmilayo I Olopade

ORCID number: Kendra J Royston (0000-0002-1575-7407); Babatunde Adedokun (0000-0002-6893-6468); Olufunmilayo I Olopade (0000-0002-9936-1599).

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Kendra J Royston, Olufunmilayo I Olopade, Division of Hematology Oncology, University of Chicago, Chicago, IL 60637, United States

Babatunde Adedokun, Center for Clinical Cancer Genetics and Global Health Department of Medicine, University of Chicago, Chicago, IL 60637, United States

Corresponding author: Kendra J Royston, PhD, Postdoctoral Fellow, Division of Hematology Oncology, University of Chicago, 900 E 57th Street, 8121B, Chicago, IL 60637, United States.
kendraroyston@gmail.com
Telephone: +1-773-8346289

Abstract

In the past decade, more cancer researchers have begun to understand the significance of cancer prevention, which has prompted a shift in the increasing body of scientific literature. An area of fascination and great potential is the human microbiome. Recent studies suggest that the gut microbiota has significant roles in an individual's ability to avoid cancer, with considerable focus on the gut microbiome and colorectal cancer. That in mind, racial disparities with regard to colorectal cancer treatment and prevention are generally understudied despite higher incidence and mortality rates among Non-Hispanic Blacks compared to other racial and ethnic groups in the United States. A comprehension of ethnic differences with relation to colorectal cancer, dietary habits and the microbiome is a meritorious area of investigation. This review highlights literature that identifies and bridges the gap in understanding the role of the human microbiome in racial disparities across colorectal cancer. Herein, we explore the differences in the gut microbiota, common short chain fatty acids produced in abundance by microbes, and their association with racial differences in cancer acquisition.

Key words: Diet; Epigenetics; Microbiome; Disparities; Colorectal; Colon; Cancer

Core tip: In this paper, we summarize the literature in relation to the gut microbiome and colorectal cancer. We provide unique perspectives and identify new areas of interest that will progress the field with relation to colorectal cancer disparities. This is significant because the comprehension of the microbiome is quickly becoming paramount for personalized medicine and combating disease progression.

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INTRODUCTION

It is safe to classify cancer as the disease of the 21st century. Ongoing investigations within the scientific community seek to identify novel approaches to remedy this debilitating disease, and much progress has been made in recent decades. From the discovery of immunologic drugs, to technological advancements in laser therapy, the wide array of research being done to resolve the ever-complicated ailment known as cancer continues to grow as researchers set out to find an effective treatment or cure[1-6]. Both cancer and chemo prevention are areas that have grown considerably as of late[7-10].The interest in these fields continues to thrive partially because of the high cost of cancer remediation in addition to the numerous side effects associated with chemotherapy drugs on the market[11-13]. In an effort to promote and conserve quality of life, in particular for African American and low income individuals, efforts to alter one's environment and increase the avoidance of carcinogenesis is a much needed area of investigation.

The microbiome is important in human health and disease, and while the microbiome is extremely complex, can change over time, and varies from person to person, we can still gain pertinent information that will help treat and cure disease. Researchers have undertaken the task of understanding the roles of the microbiome. It is common knowledge that a vast majority of commensal microbes have symbiotic relationships with their host organisms; we now seek to understand those relationships and the information we can gain about disease. Several studies have shown variance in microbial abundance and diversity among healthy and non-healthy individuals[10,14] (Figure 1). According to recent investigations, there are differences and trends of association in the gut microbiota based on race and ethnicity, sex, geography, diet, and other such demographics[15-17]. As a result of a study conducted by Chen *et al*[18], we now know that there are over 400 genes that can be used to distinguish the specific microbiomes of Asian, European, and American populations. Interestingly, Chen *et al*[19] at the Mayo Clinic in Rochester Minnesota report significant associations in microbial β-diversity according to body mass index, race, gender, and alcohol consumption. This study provides supporting evidence for the influence of environmental factors that affect the microbiome.

With respect to cancer and the roles of the microbes indigenous to the human gut, the need for further investigation and application is apparent. While we know there is an association with the microbiota and human health, we do not fully understand the significance of that association. It is believed that microbial transplantation could have some benefit in cancer prevention and or remediation[20,21]. If this is the case, trials need to be launched to assess this. We know that the consumption of certain foods can result in epigenetic modulation of oncogenes and tumor suppressors[22-25]. We also understand that metabolic processes are instrumental in the utilization of foods for nutrient absorption and energy production[26,27]. The question that arises from acknowledging the role microbes have in human health and disease becomes one of understanding how much of the metabolizing of nutrients is dependent on commensal microbes, and how these microbes drive epigenetic processes in the human organism. To begin unraveling these mysteries, we must first have a clear understanding of the typical microbial profile of a diseased and healthy individual. We (the scientific community) must also take on the daunting task of determining the functionality of the by-products of the microbes identified through our efforts.

Understanding the complexities of the microbiome gives rise to a better understanding of the complexities associated with the individuality of disease development and progression among different races and ethnicities. Diseases like cancer are difficult to appropriately address in part due to differences in chemotherapy effectiveness and adherence. Non-Hispanic Whites (NHW) tend to have overall better health outcomes when compared to Non-Hispanic Blacks (NHB) and other racial minorities in the United States[28-30]. A full comprehension of the differences among these groups at the microbiome level in concert with epigenetic and genetic differences will help decrease the health disparity in these populations.

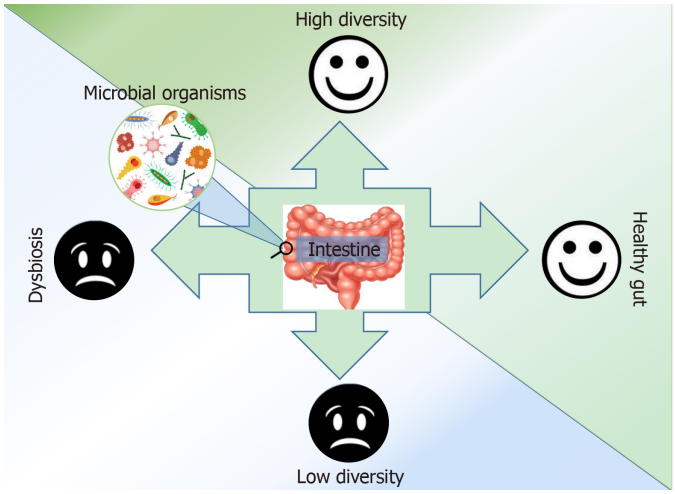

Figure 1 Gut health and the influence of gut microbes: Microbial diversity is associated with better health outcomes. A greater abundance of varied microbial species can be influenced by environmental and lifestyle factors. This diversity can be associated with better health outcomes. The above graphic summarizes the relationship between microbes human health.

RACE AND COLORECTAL CANCER IN THE UNITED STATES

Using incidence data from the Surveillance, Epidemiology and End Results Program, cancer registries and National Center for Health Statistics, incidence rate of colorectal cancer (CRC) was 49.2 per 100000 in NHB, higher than in NHW (40.2 per 100000) and Asian/Pacific Islander (API, 32.2 per 100000). Similarly, mortality rates from the disease using data from 2010 to 2014 were higher among NHB (20.5 per 100000) compared to NHW (14.6 per 100000) and APIs (10.3 per 100000). Among adults younger than 55 years, though mortality rates declined among blacks in the period 1970 (8.1 per 100000) to 2014 (to 6.1 per 100000), the rates were higher than for whites (3.6/100000 in 1970 to 4.1/100000 in 2014)[31]. Several factors could explain higher CRC rates in NHB. Blacks are usually diagnosed at more advanced stages of the disease and hence have higher mortality rates[32]. Socioeconomic disparities account for a significant proportion of the difference in CRC incidence between racial/ethnic groups, and these socioeconomic differences have been attributed to the higher prevalence of obesity, unhealthy diet and smoking[11,33]. Mortality differentials between racial groups can also be explained by inequalities in screening rates and access[34], and healthcare access and follow up care following abnormal findings on screening[35-37].

RACIAL DIFFERENCES IN DIET IN THE UNITED STATES

Several studies have reported a higher prevalence of unhealthy food intake among NHB compared to other racial groups. Dunford *et al*[38] examined trends in energy intake among United States adults aged 19 years and above from 1977 to 2012 using multiple national survey data. They found that NHB had a higher prevalence of snacking energy intake and salty snacks in the period reviewed. Using the Healthy Eating Index (HEI), Nowlin *et al*[39] analyzed NHANES data of United States adults aged 20 years and older between 2007 and 2012 and showed that among non-diabetics, NHB had significantly lower HEI scores compared to NHW. The authors also reported that the racial disparities were related to age, gender smoking status and time spent in the United States.

Another approach to assessing dietary quality was employed by Rehm *et al*[40]. The authors computed a composite variable to assess overall diet quality using 7 NHANES cycle from 1999 to 2012. They found that the estimated percentage of non-Hispanic white adults with a poor diet significantly declined (53.9% to 42.8%), while the corresponding figures were 64.7% to 57.7% among NHB and 66% to 58.9% among Mexican American adults. Furthermore, for specific food items, total vegetables, whole grains, unprocessed red meat, and milk consumption remained higher over time among non-Hispanic white adults compared to non-Hispanic black or Mexican American adults. Another noteworthy study conducted by Wang *et al*[41] used a composite index of dietary intake. The authors investigated trends in dietary quality

among adults from 1999 to 2010 using the NHANES dataset and found only minimal improvements in dietary quality over time among NHB. Additionally, in each survey cycle, they found a significantly lower mean Alternate Healthy Eating Index-2010 among NHB compared to Mexican Americans and NHW, though the difference between NHW and NHB disappeared after adjusting for socioeconomic factors such as income and education. The authors suggested that the observed differences between NHW and NHB were likely explained by socioeconomic variables. Among children and adolescents, using the NHANES data over a twenty-year period (1988 to 2008), Kant *et al*[42] found a greater increase in the number of eating occasions, the amount of energy from all beverages and non-nutritive beverages in NHB compared to other racial groups. Taken together, these studies indicate the poor dietary quality of NHB compared to other racial groups, and that could explain part of the racial disparities in different cancers, especially those cancers where type of diet is an established risk factor.

THE INTERPLAY BETWEEN DIET, THE MICROBIOME, AND CANCER

Cancer is an extremely complex and life altering disease which brings to rise the urgency to unravel its complexities. Focus areas in the cancer research community encompass a broad range of topics, and there has been a mass effort to minimize cancer's impact on quality of life and longevity. There are many approaches that can be taken; however, our interests lie in chemo adherence and prevention through modifiable factors. Knowledge about the disease and its causation associated with environmental exposures will help promote awareness and aversion to certain lifestyles that lead to mutations or epigenetic aberrancies, and cancer development. Since epigenetics is a field that focuses on changes in gene expression with no change in the underlying coding sequence[24], the regulation of epigenetic abnormalities is thought to have strong potential in the reversing cancer. One such Food and Drug Administration approved epigenetic therapy currently on the market treats cutaneous T cell lymphoma. Suberanilohydroxamic acid (SAHA), known commercially as Vorinostat, is a well-documented histone deacetylase (HDAC) inhibitor[43].

Researchers have suspected that lifestyle factors contribute to human health and disease for quite some time now. It was in 1964 that the surgeon general, Luther Terry and team, published the report that smoking had a direct impact on cancer development[44]. While this spurred changes in the regulations on tobacco, we believe that this report, and reports like it, placed a higher importance on environmental factors that lead to cancer. Takeshi Hirayama published one of the earlier documents on the impact of nutrition on cancer in 1979. Hirayama placed emphasis on the idea that lifestyle improvement (better diet, more exercise and smoking cessation) could be an effective community cancer plan[45]. Since then, many developments have been made with respect to cancer development and the environment over the decades. Fast-forwarding to 2003, Margaret Mason published a review paper that focused on the idea that diet could potentially modulate molecular signaling in cancer[8]; and to date, several studies have provided further evidence of this[7,9,23,24,46].

We now know with certainty that the foods we consume can have a direct impact on the state of human health and disease[39,47,48]. Several studies have begun to explore how these foods modulate gene expression of oncogenes and tumor suppressors[7,24,25,43]. Two recent papers detail how certain natural and dietary compounds promote cell death in breast cancer cell lines[7,25]. They demonstrate that sulforaphane, an isothiocyanate abundantly in cruciferous vegetables, and withaferin A a steroidal lactone from the Indian winter cherry, are efficient at downregulating epigenetic enzymes that are typically over expressed in breast cancer[25]. Further, this group also found that the prior-mentioned compounds increase the expression of tumor suppressor gene p21 through transcriptional activation at the promoter region through the up-regulation of a histone methylation marker associated with gene activation[7]. With respect to the epigenetic impact of nutritive compounds, other studies have found metabolic links among microbial organisms in the gut and choline consumption. Choline is a vital nutrient that acts as a source for the methyl groups needed for metabolic processes in humans. Apparently, choline utilizing bacteria can compete with the host for this nutrient, thereby impacting global DNA methylation patterns in mice[27]. In 2010, Moestue *et al*[49] noted that in breast cancer models, there were elevated levels of choline metabolites, and more recently it was found that while elevated choline metabolites were present in resistant breast cancers, chemotherapy induced an even greater increase in these metabolites[50]. Further study may reveal that choline-consuming bacteria like, Escherichia coli 536, are capable of altering

epigenetic aberrancies (*i.e*., hyper/hypo-methylation and tumor-suppressor silencing and oncogene activation respectively) that lead to cancer.

Interestingly, other studies have also reported anticancer effects at the gene regulatory level with respect to immune responsiveness. Recently, Rubio-Patino *et al*[51] published a study on the effects of a low-protein diet on inositol requiring enzyme 1α (IRE1α)-dependent anticancer immunosurvellience. IRE1α is partially responsible for the activation of the endoribonuclease domain that catalyzes splicing of X-box binding protein 1. In addition to this function, IRE1α RNAse activity has roles in IRE1-dependent decay of mRNA, rRNA, and microRNAs which in turn can lead to modulation of adaptive immunity[52]. Mediterranean, vegetable based, Japanese diets, and others all show promise at decreasing cancer risk and mortality[46] . Soldati *et al*[46] detail in their 2018 paper, that diet affects cancer progression through either systemic or local effects within the tumor microenvironment. Higher glucose levels are known to have an impact on immune activity, which in turn destabilized immune functionality. That being said, regulatory T cell function can be regulated by the metabolism, and the metabolism is regulated by patterns of dietary intake and physical activity[53].

The gut microbiome also has key roles in cancer risk reduction. In this modern era of research and medicine, the comprehension of the microbiome has expanded considerably. Causal mechanisms for cancer have been identified in microbes[54]. Some of these mechanisms are immunologic, and others appear to be epigenetic[55,56]. Iida *et al*[57] published one such immunologic study that details the influence of the microbiome in disease progression in 2013. They found that antibiotics actually decreased the efficacy of the tested immunotherapy leading to the conclusion that commensal bacteria have an effect on chemo adherence by modulating immunologic factors in the tumor microenvironment. More recently, studies have indicated that immune checkpoint inhibitors are regulated by gut microbes[58], with crosstalk occurring between intestinal epithelial and lymphatic cells. Interestingly, regulatory T cells appear to be inducible by intestinal microbial organisms of the clostridium genus which may suggest a therapeutic approach to immune response[59].

Maryann Kwa and co-authors published an article a couple years ago that suggests the bacterial species housed in the human intestine regulate estrogen metabolism. Interestingly, gut microbiota may affect the risk of acquiring estrogen-receptor-positive breast cancer due to certain microbes being capable of metabolizing estrogens in what they coin as the estrobolome[60]. In addition to this unique perspective, other leading minds have discovered that the bacterial organism *Clostridium perfringens* enterotoxin has the ability to suppress claudin-4 (a regulator of proliferation and cancer cell metastasis) protein expression, kill gastric cancer cells in-vitro, and inhibit tumor growth in mice xenografts[61]. Other studies have suggested that microbes can be used to biosynthesize nanoparticles designed to target and treat various forms of cancer[62]. Considering the broader implications of microbial composition and its utilization in cancer elimination, the field is subject to grow considerably.

Routine exercise and proper nutrition have proven to be effective in reducing the risk of carcinogenic related fatality in cancer patients. Individuals who consume more plant matter have overall better health outcomes according to a number of epidemiological studies. Clear patterns in dietary intake and cancer risk can be observed among different cultures, races, and ethnicities, which have led to the generation of hypotheses centered on the idea that racial and cultural differences are involved in dietary habits. Researchers have found that there are several variables apart from genetic factors that have been associated with increased cancer mortality in minority populations, including socioeconomic status, and the availability of healthy food options. That being said, it is important to recognize the significance these two modifiable factors have in cancer prevention in relation to the microbiome. Monda *et al*[26] state that exercise is capable of enhancing the amount of positive microbial organisms resulting in a richer diversity. According to another report, harmful changes with associated polychlorinated biphenyls, pollutants in air and water, are decreased in the gut microbiome of exercising mice[63]. This provides a bit of insight on lifestyle and environmental factors that regulate the abundance or lack of commensal bacteria in host organisms and how they impact health. It would be interesting to conduct an in depth study to determine if microbial diversity is regulated by diet and if microbial abundances attributable to certain diets are in turn responsible for regulation of carcinogenic processes. One study discusses fermentable carbs, or non-digestible carbohydrates that undergo fermentation by microbial organisms to produce short chain fatty acids (SCFAs) that can be utilized by the host[64]. It would be meritorious to observe the impact of SCFAs produced in this manner in cell metabolism. We may yet find that fermentable carbohydrates are capable of reducing the persistent activation of aerobic glycolysis in cancer.

CANCER RISK AND PREVENTION AMONG RACES AND THE INFLUENCE OF THE MICROBIOME

Several studies have indicated that microbial composition is strongly linked to dietary intake and differences among racial and ethnic groups unassociated with diet. We know that there are a number of events responsible for the progression of disease. As research progresses, the scientific community has found there to be trends and correlations with microbial composition, regulation of inflammatory processes, and immune response with microbes playing a significant role; at the very least serving as bio-indicators of some sort. It remains unknown as to whether or not these organisms and their by-products aid in the direct regulation of epigenetic mechanisms that help regulate the human organism's biological processes; however, it is plausible. With respect to African American susceptibility to cancer and a greater overall disease mortality, we may find a difference in the abundance of microbes in NHB when compared to racial groups with better disease outcomes. Pilot studies conducted by researchers confirm differences in microbial diversity in healthy individuals by race[18,65]. Alternatively, we may find that gut equilibrium conducive to individual survival differs from person to person.

Findley *et al*[14] published a comprehensive review in 2016 that noted the importance of not assuming race and ethnicity to be causal factors in microbial diversity. We recognize that there are numerous cultural differences that could potentially lead to the differences reported in the studies conducted to date. It remains that NHB and persons of low SES have a number of stressors and lifestyle factors that could directly influence the microbiome[66]. Despite these cautions (avoiding assumptions), one 2015 study shows, that Western diet consumed by individuals of African lineage can be damaging and creates an environment less capable of seamlessly carrying out biological processes (*i.e.*, cellular division and differentiation)[67]. With respect to choline, introduced in the previous section, O'Keefe *et al*[67] found that Africans on their original diet had a lower level of choline in their fecal water as compared to NHB on the Western diet suggesting the greater microbial diversity reported in the African's profile results in a greater metabolism of choline. In addition, epigenetic control of the expression or lack of expression of certain genes that are vital in the regulation of tumor suppression and oncogenesis is thought to be linked the gut flora. Histone modifying enzymes are sensitive to microbial metabolites, and they have been shown to mediate phenotype through programing gene expression thereby regulating cellular functionality[68]. This leads to the belief that NHB cancer susceptibility can somehow be linked to gut microbial composition, their metabolites, and their influence on the epigenetic regulation of processes that control oncogenes and tumor suppressors. Another paper that begins to establish the link between health disparities and the gut microbiome details key differences in the abundance or lack of certain SCFAs in the stool of African Americans, Caucasians and Asians[69].

We recognize the weakness in some of these studies stems from the fact that these data are self-reported questionnaires, which limit the accuracy of the similarities/differences of diet and environment. However, it is intriguing that preliminary results show significant differences in NHB SCFA production, fecal pH, and microbial patterns compared with other races[69]. It can also be noted that there is a clear difference in the microbial profiles of healthy and non-healthy individuals[70]. Interestingly, patients appear to be more susceptible to mortality from a disease when they have lower microbial diversity[71]. Since efficiency of certain immunotherapies for cancer treatment is thought to be influenced by the gut flora[56], it is becoming increasingly apparent how significant the interaction between the microbiome and cancer prevention and therapy is.

SHORT CHAIN FATTY ACIDS

There are a number by-products and metabolites resultant from the consumption of food products. The abundance and variety of these by-products, much like the abundance and variety of certain microbes, is determined by environmental and lifestyle factors (*i.e.*, diet and exercise). The main products absorbed as a result of digestion are long chain fatty acids, simple sugars like glucose, and monoacylglycerol, a class of glycerides[72]. In addition to these products are SCFAs, which are major nutrients resultant of bacterial fermentation. We briefly touched base on these fatty acids in the previous sections. The most studied SCFAs that have shown anti-cancer potential are acetate (much of which may be primarily derived from diet), butyrate and propionate, with acetate being the most abundant SCFA in the colon and propionate and butyrate being found at very low amounts in diseased individuals[73].

Interestingly, Krautkramer *et al*[74] conducted a study that demonstrated how the Western diet in mice limits SCFA production of microbes and negatively impacts the host's chromatin. They further display SCFA supplementation to be sufficient in mimicking epigenetic phenotypes resultant of gut microbiota, thereby demonstrating the importance of these microbial metabolites in host homeostasis[74]. Here on we will discuss the significance of these metabolites in their regulation of common cancers.

Acetate

Acetate is a SCFA found in abundance in the human colon. Over the years, this fatty acid has been reported to impact tumor cells as nutrient[75,76] and as an inducer of apoptosis[77-81]. Despite the contradictory reports, there is considerable evidence supporting acetate's importance in human physiological processes. Acetate is mandatory for histone acetylation[82,83], an epigenetic mechanism known for its roles regulating chromatin accessibility. Most notably, acetate is partially responsible for the appropriate binding of transcription factors for the activation of tumor suppressor genes. Conversely, inappropriate histone acetylation can allow for the activation of oncogenes. This subsection will delve into the roles of acetate in common cancers that affect the United Sates.

Colon cancer/CRC is one of the Western world's most common cancers in men and women with combined total of approximately 140250 new cases per year according to the American Cancer Society. There has been a considerable amount of research conducted over the years suggesting the significant roles of the microbiome in this particular cancer. Since acetate has been reported to be found in abundance in the colon and to be used in cancer cell metabolism, the regulation of this particular SCFA is of extreme intrigue within the scientific community. A number of studies have sought to determine the effect of acetate on CRC cells in-vitro. One in particular shows that this SCFA inhibits cell growth, decreases cell viability and induces apoptosis in two CRC cell lines[80]. Alternatively, in brain cancer, acetate competes with glucose for the generation of TCA cycle intermediates despite glucose being more abundant[84,85]. An interesting review from 2014 highlights the importance of acetate in fueling cancer cells[86]. Interestingly, this does not appear to be the case in CRC[77,80,87]. One paper that attempts to unravel the role of acetate in CRC cell death demonstrates the ability for this SCFA to increase the expression of MCT1 and MCT4, two plasma membrane lactate transporters and CD147, a multipurpose transmembrane protein associated with inflammation and tumor invasion[87]. The comprehension of the mechanisms involved in acetate's ability to influence CRC shows much therapeutic potential and promise for further scientific investigation.

Butyrate

A highly sighted review article published in 2011 by Berni Canani *et al*[22] highlights the numerous roles of butyrate (microbiome dependent) in modulating human health and disease. They detail how this particular SCFA has "potent regulatory effects on gene expression". Butyrate has been shown to regulate inflammation by inhibiting NFkB[88], it can improve memory function in Alzheimer's disease mouse models[89], and it has been shown to negatively impact cancer cell progression across a number of tumor types[23,90-93]. Butyrate also has significant roles in providing energy for colonocytes so that they may multiply and divide appropriately and avoid self-digestion. Interestingly, it accounts for approximately 70% of total colon cell energy utilization[94], but only comprises about 15% of the SCFA found in the colonic lumen[22]. There is considerable evidence in support of higher levels of butyrate being associated with better health outcomes, lower pH, and diets rich in fiber. Though butyrate has been shown to promote the growth and proliferation of colon cells, it is capable of inducing apoptosis in mutated cancer cells[91,92,95]. This SCFA has been studied quite a bit as an anticancer agent. A quick search of butyrate and cancer on PubMed will produce thousands of results with relevant material.

Several studies have shown butyrate to be an anti-cancerous agent resultant of bacterial fermentation of non-digestible carbohydrates[96]. Microbial organisms take up fibers that the host organism cannot digest in the colon and produce butyrate as one of three primary SCFAs. In 2017, Li *et al*[97] published an interesting study on butyrate in CRC cell lines. They report that this SCFA is capable of decreasing cancer cell migratory ability by inhibiting Akt/ERK signaling with dependency on HDAC3[97]. It is our opinion that the study could have been strengthened had the investigators chose to observe the effects of butyrate on HDAC3 gene and protein expression. There is quite a bit of evidence in support of butyrate's ability to inhibit HDAC activity across a number of cancer types[98-100]. A team recently published an article demonstrating butyrate's ability to act synergistically with the toxic chemo drug irinotecan[90].

The mechanisms by which butyrate has been demonstrated to promote CRC cell

senescence are varied. A number of reports reveal a complex network of oncogenes and tumor suppressors that have been modulated by this fatty acid. One study found that neuropilin-1, a receptor for vascular endothelial growth factor and a key player in apoptotic signaling, is down regulated by butyrate[101]. Other studies show butyrate to regulate CRC cell apoptosis through its impact on the pro-apoptotic pathway by up-regulating BAK and activating caspase-3[92]. There have been a number of studies that attribute butyrate's apoptotic ability to its regulation of epigenetic genes and microRNAs. In 2015, Hu *et al*[102] found that miRNA-92a, known for its role in promoting cancer cell proliferation, was inhibited by butyrate. It can be inferred that the inhibition of this oncogenic miRNA is in part due to butyrate's HDAC inhibitory abilities as they show similar results when comparing to the HDACi SAHA[102]. The fact that a naturally occurring compound can have such a beneficial impact is a significant discovery. Findings like these help usher in new discoveries that help improve the quality of life in cancer patients.

Propionate

Propionate is an important SCFA produced from the dicarboxylic acid pathway or the acrylate pathway and a substrate for hepatic gluconeogenesis[103]. It appears to play a key role in cholesterol metabolism principally as an inhibitory agent and potentially has a cholesterol lowering function[47,104]. Propionate is also known for its antifungal and antibacterial effects, anti-inflammatory and anti-carcinogenic properties[105]. Although butyrate has been consistently shown to be the most potent of the SCFA, propionate also demonstrated anticancer activity in many studies[9,106]. In addition, propionate enhances the anti-proliferative function of butyrate due to its inhibition of cell growth[107]. Concerning histone acetylation, Kiefer *et al*[108] showed that butyrate and propionate, but not acetate significantly enhanced histone acetylation in colon cancer cells after 24 h incubation. Furthermore, the additive effects of butyrate and propionate when combined in a mixture also amplified histone acetylation[108].

There is compelling evidence of the importance of SCFA including propionate in the cancer prevention and the potential of these chemicals in treatment. A study by Emenaker *et al*[106] treated fresh surgical colon cancer cells with SCFA and found all SCFA inhibited primary invasive human colon cancer invasion and significantly altered protein expression levels of established cancer genes: p53, Bax, Bcl2 and PCNA. Giardina *et al*[109] using HT-29 CRC cell lines showed that propionate and butyrate, but not acetate, could alter the metabolism of reactive oxygen species and increase cellular peroxide generation. This ability to produce peroxide enables the induction of apoptosis in CRC cell lines.

There is evidence that the anti-carcinogenic effects of propionate could differ from those of other SCFA. For example, Tedelind *et al*[105] compared the anti-inflammatory properties of butyrate, propionate and acetate and found that propionate and butyrate were equally effective in suppressing NF-KB reporter activity, immune related gene expression and cytokine release *in vitro*. The authors concluded the SCFA could be useful for treating inflammatory bowel disease. Another study using proteomic and cellomic analysis methods[95] showed that propionate had different mechanisms of action on cellular proteins compared to other SCFA such as butyrate and valerate. Specifically, propionate had less pronounced effects on keratins and intermediate filaments, and on b-tubulin isotypes expression and microtubules compared to butyrate and acetate. Concerning colitis associated CRC, propionate as part of a SCFAs mix prevented development of tumors and attenuated the colonic inflammation in a mouse model of colitis-associated CRC[110], and holds promise in the management of colitis associated CRC.

RACIAL DIFFERENCES IN THE PROFILE OF GUT MICROBIOTA AND SCFA

The bacterial composition of the gut and SCFA content has been shown to differ between racial/ethnic groups. Hester *et al*[69] compared bacteria and SCFA in the stools of a small pilot sample of 20 apparently healthy participants (five each were NHW, NHB, Hispanics and American Indians). The authors found lower acetate, butyrate and total SCFA content and a higher pH in NHB compared to the other racial groups. In addition, levels of *Firmicutes* bacteria were higher in NHB compared to Whites and Hispanics, and lower levels of *Lachnospiraceae*, known to be involved in the production of butyrate. Moreover, the ratio of *Firmicutes* compared to *Bacteriodes*, that has been associated with obesity was also higher among blacks and could partly explain the links between obesity and colon cancer.

Two recent studies have reported that *Bacteroides* were more commonly found

among NHB. Farhana *et al*[15] showed that *Bacteroides* were more abundant in colonic effluents of AAs compared to NHWs, especially *Fusobacterium nucleatuma* and *Enterobacter* species, whereas *Akkermansia muciniphilia* and *Bifidobacterium* were higher in NHW. The authors also showed that AA had decreased microbial diversity compared to NHW. *Bacteroides* were also shown to be more common among AA compared to NHW in a study of stress and microbiota among healthy individuals[66]. Higher stool counts of *Bacteroides* were also found in NHB in an older study that compared dietary habits and fecal microbiota of NHB and NHW[111]. In addition, NHB reported lower dietary calcium, magnesium and Vitamins A, C, D, and E.

A recent study compared colonic biopsies of healthy mucosa of CRC cases and tumor free controls and found a greater abundance of sulfidogenic bacteria among NHB compared to NHWs among cases and controls[65]. Additionally, *Bilophilia wadsworthia* (evidenced to be promoted by saturated fats from Western diets low in fiber[112,113]) and *Pyramidobacter spp* were significantly higher among AA cases compared to controls[65]. The study also showed a higher consumption of meat, fat and protein intake among NHB. David *et al*[113] also report that diet has the ability to alter the human gut microbiome. With regard to obesity, a known risk factor for various types of cancer, microbiota of the gut were shown to be instrumental in the regulation of diet induced obesity in lymphotoxin deficient mice[112]. This shows that this particular molecule key in gut immunity, is capable of modulating commensal responses enabling diet induced obesity, and we have a better understanding of the pathways involved in microbiota induced weight gain[112]. Comparison of the gut microbiota between populations with wide variations in dietary composition could shed light on the potential mechanisms involved in the role of diet and gut microbiota in carcinogenesis. A small study by Ou *et al*[114] studied the difference in gut microbiota between native Africans (NA) and NHB and showed that SFCA, total bacteria, microbial genes encoding for methanogenesis and hydrogen sulphide production were higher among NA while secondary bile acid was higher among NHB in fecal samples. The findings point to a higher saccharolytic fermentation and lower proteolytic fermentation among NA compared to NHB. Concerning specific bacteria, *Prevotella* was more common in NA while *Bacteriodes* were more abundant in NHB. The authors ascribed the observed differences in stool samples to NHB eating more dietary meat and fat and less complex carbohydrate and fiber. The same authors also showed in another study[115] comparing a small sample of high colon cancer risk in NA, NHB and NHW, that the levels of experimentally carcinogenic secondary bile acids (lithocholic and deoxycholic acids) was higher among NHB and NHW compared to NA. A related study[116] also showed that NA harbored a more diverse population of, methanogenic Archaea compared to NHB and NHW. The populations of other hydrogenotropic bacteria such as sulphate reducing bacteria was also more distinct among NA. Another study involving NA[117] examined total colonic evacuants for SCFA, vitamins, nitrogen and minerals and found total SCFA and butyrate were higher, but calcium, iron and zinc were lower among NA compared to NHB and NHW, supporting the mediatory role of these chemicals in the effects of microbiota in colon cancer development.

NA have been shown to have a propensity for methanogenic rather than sulfidogenic disposal of hydrogen generated from microbial fermentation in the human colon. In a South African study, NA excreted more methane in their breath compared to whites in the same country[118]. Furthermore, stool samples of European ancestry populations exhibited more of sulfate reduction activity. The differences observed in the bacterial composition of stool samples of NA and NHB is due to the dietary difference with the Africans consuming more coarse grains and vegetables.

Apart from intestinal microbiome, some other studies have examined the role of the oral microbiome in CRC risk using a multi-ethnic sample. For example, Li *et al*[16] showed unique differences in the patterns of saliva microbiome between individuals living in Alaska, Germany and Africa. In another study, Yang *et al*[17] using a nested case control study showed that *Treponema denticola* and *Prevotella intermedia* were associated with increased CRC risk among a sample made of predominantly EA and AA. Even though this study showed similar associations in AA and EA, they were more significant among AA, although AA were about three times more than EA in the sample. The authors however alluded to the fact that differences in the oral microbiome by race could explain the difference in association between the racial groups.

DISCUSSION AND CONCLUSION

We have reviewed the differences between race/ethnic groups concerning dietary

habits, how different diets affect the gut's environment, and the potential role of these differences in explaining disparities in CRC incidence in the United States and between native African populations. Our review reveals that unique opportunities exist in taking advantage of the racial differences in diet, other diet-related CRC risk factors such as obesity and diabetes, gut microbiome, and the genetic architecture of CRC for a greater understanding of the complexity of CRC etiology and carcinogenesis. Much of the literature we cite includes studies that show that environment, diet, and lifestyle can alter the microbiome and are associated with cancer risk, but whether these co-associations are linked is still unknown.

Several studies revealed that there is a direct link between dietary intake and the microbial profile of an individual, and this ultimately influences the risk of several diseases including cancer. A study we mention earlier in this review corroborates the link between dietary intake and microbiota. The authors switched regular diets between South African blacks and AA during a two-week period. Food exchanges of low fat, high fiber for AA and high fat low fiber for NA resulted in increased saccharolytic fermentation and suppressed secondary bile acid synthesis in AA[67]. Studies also consistently showed that AA consume less healthy diets and this could explain the higher rates of CRC among this population. Foods from an unbalanced diet deficient in fiber and high in meat, such as those consumed by AA, promotes proteolytic rather than saccharolytic fermentation and this results in branched chain SCFAs such as isobutyrate, isovaleric and 2-methylbutyroic acid. Moreover, proteolytic products including nitrogenous metabolites such as hipurate and ammonia have been shown to increase inflammation and carcinogenesis[48].

Evidence from studies demonstrating direct links between dietary habits, the gut microbiome and CRC risk indicates there is an urgent need for multiethnic studies of gut microbiome and SCFA and CRC. Moreover, such studies need to incorporate dietary intake, blood levels of nutrients and their metabolites, genes involved in the metabolism of various diets, and epigenetic factors. In addition, greater insights into colorectal etiology could be gained by exploring the large differences in diet, and several other environmental factors between native African populations and African Americans living in the United States. Recent estimates revealed greater than 10-fold difference in incidence rates of CRC between the United States and most of sub-Saharan Africa[119,120]. Multipronged approaches (such as integrative molecular epidemiology) to study the associations between dietary habits, profile of the gut microbiome and CRC across populations could provide great insights into the factors responsible for the widely varying CRC incidence rates across populations. Finally, most of the studies reviewed had small sample sizes that precluded adequate power to investigate CRC subtypes. Large sample multiethnic cohorts will afford greater understanding of CRC subtypes especially given the clear differences in the etiology, prognosis and approaches to the management of these subtypes.

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REVIEW

Targeted agents for second-line treatment of advanced hepatocellular carcinoma

Nicola Personeni, Tiziana Pressiani, Silvia Bozzarelli, Lorenza Rimassa

ORCID number: Nicola Personeni (0000-0002-7995-272X); Tiziana Pressiani (0000-0001-6919-1536); Silvia Bozzarelli (0000-0002-8118-7342); Lorenza Rimassa (0000-0001-9957-3615).

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Nicola Personeni, Tiziana Pressiani, Silvia Bozzarelli, Lorenza Rimassa, Medical Oncology and Hematology Unit, Humanitas Cancer Center, Humanitas Clinical and Research Center, IRCCS, Rozzano 20089, Milan, Italy

Nicola Personeni, Lorenza Rimassa, Department of Biomedical Sciences, Humanitas University, Pieve Emanuele 20090, Milan, Italy

Corresponding author: Lorenza Rimassa, MD, Associate Professor, Medical Oncology and Hematology Unit, Humanitas Cancer Center, Humanitas Clinical and Research Center, IRCCS, via Manzoni 56, Rozzano 20089, Department of Biomedical Sciences, Humanitas University, Pieve Emanuele 20090, Milan, Italy. lorenza.rimassa@hunimed.eu
Telephone: +39-2-82244573
Fax: +39-2-82244590

Abstract

Over the past ten years, sorafenib, a multikinase inhibitor, has been the standard of care for patients with unresectable hepatocellular carcinoma (HCC) and well-preserved liver function. Recently, lenvatinib, a different multikinase inhibitor, was shown to be non-inferior to sorafenib, in terms of survival, while all other agents previously tested failed to prove non-inferiority (or superiority) when compared to sorafenib. Similarly, in the second-line setting, most investigational drugs failed to provide better survival outcomes than placebo. However, in the last 2 years three positive phase III trials have been published in this setting. The RESORCE trial, a phase III study evaluating regorafenib in HCC patients who experienced disease progression after first-line treatment with sorafenib, showed better outcomes with regorafenib compared to placebo. More recently, the phase III CELESTIAL trial demonstrated the superiority of cabozantinib, a multikinase inhibitor targeting vascular endothelial growth factor receptor, MET, and AXL, *vs* placebo in the second- and third-line setting in patients progressing on or intolerant to sorafenib. The survival benefits of a sustained anti-angiogenic inhibition were demonstrated also with ramucirumab in the phase III REACH-2 trial in patients previously treated with sorafenib and who had high baseline alpha-fetoprotein levels. Overall, the adverse events reported in these trials were in line with the known safety profiles of the tested agents. After nearly a decade of a certain degree of stagnation, we are now witnessing a period of novel therapeutic advances with multikinase inhibitors and monoclonal antibodies that will likely change the treatment scenario of HCC.

Key words: Hepatocellular carcinoma; Advanced-metastatic; Second-line; Third-line;

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Regorafenib; Cabozantinib; Ramucirumab; Angiogenesis; Multikinase inhibitor; MET; AXL; Vascular endothelial growth factor receptor 2

Core tip: During the last decade, sorafenib, a multikinase inhibitor, has emerged as the only systemic agent available for the treatment of patients with unresectable hepatocellular carcinoma. However, in recent years, lenvatinib, which is a different multikinase inhibitor, was shown to be non-inferior compared to sorafenib. Despite several negative phase III trials, novel drugs with similar, but not overlapping, properties have been recently shown to improve patient outcomes, thereby confirming the role of sustained anti-angiogenic inhibition in further lines of treatment. Here, we will discuss the results of the positive phase III trials of regorafenib, cabozantinib, and ramucirumab in patients failing sorafenib.

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INTRODUCTION

Liver cancer ranks second among major causes of cancer-related deaths globally. In particular, hepatocellular carcinoma (HCC) roughly represents 90% of all primary liver cancers with 800000 new cases reported yearly[1]. In more than 80% of patients, cirrhosis is a predisposing condition[2], often related to prior infection with hepatitis B virus (HBV), hepatitis C virus (HCV), or alcohol abuse. Less frequently, HCC may also arise in a non-cirrhotic liver as a consequence of HBV genotoxic effects, nonalcoholic steatohepatitis (in patients with metabolic syndrome and diabetes), or malignant transformation of a hepatocellular adenoma.

Even under rigorous surveillance programs, a sizeable proportion of patients are often diagnosed at a stage not amenable to potentially curative approaches[3], thereby prompting the search for palliative treatments.

In recent years, transcriptome analyses have allowed to increase our understanding of HCC complexity with the identification of a proliferation class and a non-proliferation one[2,4]. Both classes display recurrent genetic alterations that affect deregulated pathways relevant to cellular homeostasis, senescence, proliferation, and differentiation. Although the ultimate goal of such advances is to inform future treatment strategies, none among driver mutations leading to oncogenic addiction in HCC is thus far considered as actionable[5].

On top of that, additional hurdles that hamper the development of personalized therapies lie within a substantial intra- and inter-tumor heterogeneity, that result from an admixture of mature hepatocytes and hepatic progenitor cells, both contributing to chronic inflammation, advanced fibrosis, and eventually cancer development[6].

Despite the obvious disappointment following the results of the first biomarker-driven phase III trial ever done in HCC, that reported negative results for tivantinib in patients with MET-high HCC in 2018[7], the quest for personalized approaches is still underway within newer studies that may finally provide a conceptual frame for precision medicine in this hard-to-treat malignancy. Similar approaches could also take advantage from next generation sequencing (NGS) platforms that were recently presented as a useful tool to individualize available targeted therapies in HCC patients[8]. Nevertheless, the clinical value of molecular profiling still needs to be demonstrated given that only few patients could receive targeted treatments matching with potentially actionable alterations identified by NGS[8].

Meanwhile, different strategies directed against relevant angiogenesis pathways have provided valuable therapeutic options in the management of advanced HCC. Indeed, a continuous dependence upon pro-angiogenic pathways is typical for HCC and it is reflected by an abnormal hypervascularity well known by the radiologist. This is mainly due to a hypoxic tumor microenvironment (TME) being a major determinant for hypoxia-inducible factor-1 transcription, that in turn leads to the over-production of vascular endothelial growth factor (VEGF). From a clinical

standpoint, these peculiar aspects render HCC rather unique in comparisons with other cancers[9] and have long been proven useful for either HCC diagnosis or embolization therapies.

More than a decade ago, the approval of the multikinase inhibitor sorafenib has paved the way of anti-angiogenic therapies targeting VEGF and the VEGF receptors (VEGFRs) in the treatment of advanced HCC patients, not amenable to curative treatments[10]. In addition to sorafenib, the therapeutic armamentarium for the frontline setting has been recently expanded with lenvatinib, which is a different multikinase inhibitor, still retaining anti-angiogenic properties. As reported in the overall survival analysis (OS) of the REFLECT trial, lenvatinib is non-inferior to sorafenib in untreated patients with advanced HCC[11].

However, intolerance or resistance to frontline sorafenib (or lenvatinib) may become major issues eventually leading to treatment failure. Whereas sorafenib targets encompass both drivers of cancer cell proliferation and the TME, such pharmacological complexity has greatly hampered the search for predictive markers and, arguably, the identification of resistance mechanisms. Nevertheless, after a decade with unsatisfactory results, three novel compounds sharing peculiar VEGFRs inhibition profiles have been recently reported superior when compared to placebo for OS in the second-line setting. These include regorafenib, cabozantinib (both belonging to the multikinase inhibitors class) and ramucirumab (a monoclonal antibody that targets VEGFR 2 signaling).

Aim of this review is to summarize current knowledge on the aforementioned agents and their role in the treatment of HCC patients who failed or are intolerant to sorafenib.

Regorafenib

Regorafenib is an orally administered tyrosine-kinase inhibitor that blocks the activity of several receptors such as VEGFR 1, 2, and 3, tyrosine-protein kinase receptor, and platelet-derived growth factor receptor β[12]. Based on the crucial role of angiogenesis in HCC development and progression, and on the results of a phase II study in patients with well-preserved liver function (Child-Pugh class A) progressing on sorafenib[13], regorafenib has been investigated in a large international phase III trial (ClinicalTrials.gov NCT01774344)[14].

The RESORCE trial was a multicenter, randomized, double-blind, placebo-controlled phase III trial assessing the role of regorafenib in patients affected by HCC progressing on sorafenib. Principal inclusion criteria were Barcelona clinic liver cancer (BCLC) stage B or C, preserved liver function (Child-Pugh class A), Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 or 1. Pathologic confirmation of diagnosis was not mandatory for patients with confirmed cirrhosis. Patients should have been treated with sorafenib at a minimum dose of 400 mg daily for at least 20 of the 28 d before discontinuation and should have stopped sorafenib no more than 10 wk before randomization. Reason for sorafenib discontinuation had to be documented radiologic progression, while patients intolerant to sorafenib or who had stopped sorafenib due to toxicity were not allowed to be included in the trial.

The trial randomized 573 patients from May 2013 to December 2015. Patients were randomized in a 2:1 ratio to receive regorafenib (n = 379) or placebo (n = 194) and were stratified by geographical origin (Asia *vs* rest of world), ECOG PS (0 *vs* 1), blood alpha-fetoprotein (AFP) levels (< 400 ng/mL *vs* ≥ 400 ng/mL), extrahepatic disease (yes *vs* no), and macrovascular invasion (yes *vs* no).

Patients in the two groups were well-balanced for baseline characteristics, including sex, race, geographical origin, stage of disease, stage of liver dysfunction, and etiology. The proportion of patients with Asiatic origin was 38%. The treatment consisted in four 40 mg tablets of regorafenib (160 mg) orally or matching placebo once daily for 21 consecutive days, followed by 7 d of rest in 4-wk cycles. Treatment could be interrupted for disease progression according to modified RECIST (mRECIST)[15], clinical progression, death, unacceptable toxicity, or decision by the investigator. Tumor assessments were performed every 6 wk for the first 8 cycles and every 12 wk thereafter. The primary end-point of the study was OS in the intent-to-treat population (ITT). Secondary endpoints were progression-free survival (PFS), time to treatment progression (TTP), objective response rate (ORR), and disease control rate (DCR) assessed by the investigators using mRECIST and RECIST v.1.1[16]. Further endpoints were safety, pharmacokinetics (PK), biomarker evaluation, and quality of life (QOL).

At the data cut-off for final analysis (February 29, 2016), among patients who started treatment (n = 567), 309 (83%) in the regorafenib arm and 183 (95%) in the placebo arm discontinued the study drug. The most frequent reason for treatment discontinuation was disease progression. Median treatment duration was 3.6 mo with regorafenib and 1.9 mo with placebo. With a median follow-up of 7 mo, median OS

was 10.6 mo in the regorafenib arm *vs* 7.8 mo in the placebo arm [hazard ratio (HR) = 0.63 95% confidence interval (CI): 0.50-0.79, $P < 0.0001$]. The same survival gain was confirmed in the updated survival analysis performed almost 1 year after the first one (10.7 mo *vs* 7.9 mo, HR = 0.61, $P < 0.0001$)[17]. Median PFS by mRECIST was 3.1 mo in regorafenib arm and 1.5 mo in the placebo arm. Regorafenib was superior to placebo in all the efficacy endpoints and similar results have been demonstrated by RECIST 1.1 assessment (Table 1)[18].

The safety population included 567 patients (99% of randomized patients), 374 in the regorafenib group and 193 in the placebo group. All patients in the regorafenib arm and 93% of patients in the placebo arm had at least one adverse event (AE), graded using NCI-CTCAE version 4.03. These AEs were deemed related to the study drug in 93% of patients on regorafenib and 52% of patients on placebo (Table 2). Most frequently observed grade 3-4 AEs were hypertension (15% of patients on regorafenib *vs* 5% of patients on placebo), hand-foot skin reaction (HFSR) (13% *vs* 1%), fatigue (9% *vs* 5%), and diarrhea (3% *vs* none). According to prior sorafenib dosing, grade ≥ 3 HFSR, fatigue, anorexia, and increased bilirubin were slightly higher in the group of patients that received < 800 mg compared with 800 mg, as last dose, while no difference was observed in rates of other treatment-emergent adverse events (TEAEs). Therefore, the last sorafenib dose may not predict the onset of TEAEs occurring with regorafenib[19]. Serious AEs (SAEs) and death rates were similar in the two groups; SAEs were attributed to the study drug in 10% of patients on regorafenib and 3% of patients on placebo. Grade 5 AEs occurring within 30 d after the last dose of treatment were observed in 13% of patients in regorafenib patients and 20% in placebo arm and were deemed related to the study drug in 7 patients on regorafenib and 2 patients on placebo (both liver failure). Regorafenib was interrupted or reduced in 68% of patients and discontinued in 25% of patients due to AEs, while 31% of patients on placebo interrupted or reduced treatment and 19% of patients on placebo discontinued due to AEs. The most common AEs responsible for regorafenib discontinuation were aspartate aminotransferase (AST) or alanine aminotransferase increase (2% and 1%) and HFSR (2%).

Quality of life during the study was assessed by several questionnaires (Functional Assessment of Cancer Therapy-General and Hepatobiliary, EQ-5D, EQ-VAS) and no statistically significant changes in QOL were detected between the two treatment arms.

Further exploratory data showed that the sequence of sorafenib followed by regorafenib achieved a median OS of 26 mo[19]. The efficacy of regorafenib was assessed according to the pattern of progression on prior sorafenib[20] and to the last sorafenib dose[19]. Regorafenib was shown to provide significant survival benefits regardless of the pattern of progression and the last sorafenib dose, although the development of new distant metastases or vascular invasion was confirmed to be a negative prognostic factor. Furthermore, a negative correlation between baseline AFP and circulating MET levels and prognosis was confirmed regardless of treatment[21]. Of note OS with regorafenib was significantly higher in patients suffering from HFSR[22], and this is in line with the correlation between skin toxicity and prognosis prospectively demonstrated with sorafenib[23].

In subsequent analyses regorafenib population PK (popPK) and exposure-response relationship were studied. PopPK analysis showed that most intrinsic factors had no statistically significant or clinically relevant impact on regorafenib exposure. Only age was found to be related to differences in exposure but the impact on efficacy was considered not significant[24]. No statistically significant correlations between exposure and outcomes were identified[25].

Preplanned, retrospective, optional biomarker analyses on archival tumor tissues and plasma samples collected at baseline were performed to identify potential biomarkers correlating with clinical outcome[26]. Baseline patient and disease characteristics were similar in the overall RESORCE population and in the plasma biomarker analysis cohorts, while several differences were reported between the overall study population and the tumor biomarker analysis cohorts due to the small sample size. Out of the 573 patients enrolled, only 68 archival tumor samples were collected while plasma samples were available for all the enrolled patients. For the NGS analysis, 23 tumor samples (all in the regorafenib arm) were selected, and 17 were of sufficient quality for analysis. For the immune profiling analysis, 62 samples had sufficient RNA, and 46 were of sufficient quality for analysis (32 in the regorafenib arm, 14 in the placebo arm). The NGS analysis showed mutations in *CTNNB1* in 3/10 progressors and 0/7 responders, and *VEGFA* amplification in 1/7 responders and 0/10 progressors. The immune profiling analysis defined immune gene expression signatures with 3 groups with low (46%), medium (37%), and high (17%) immune cell scores. However, the small sample size precluded any meaningful conclusions and these results can be considered only hypothesis generating. For the

Table 1 Efficacy results of the RESORCE phase III trial

Outcome based on assessment per mRECIST	Regorafenib *n* = 379 (%)	Placebo *n* = 194 (%)	HR (95%CI)	*P* value
Response				
Complete	2 (1)	0	-	NR
Partial	38 (10)	8 (4)	-	NR
Overall response rate	40 (11)	8 (4)	-	0.0047
Stable disease	206 (54)	62 (32)	-	NR
Disease control rate	247 (65)	70 (36)	-	< 0.0001
Overall survival in mo				
Median	10.6	7.8	0.63	< 0.0001
95%CI	9.1-12.1	6.3-8.8	(0.50-0.79)	
Progression-free survival in mo				
Median	3.1	1.5	0.46	< 0.0001
95%CI	2.8-4.2	1.4-1.6	(0.37-0.56)	
Time to progression in mo				
Median	3.2	1.5	0.44	< 0.0001
95%CI	(2.9-4.2)	(1.4-1.6)	(0.36-0.55)	
Outcome based on assessment per RECIST 1.1				
Response				
Complete	0	0	-	NR
Partial	25 (7)	5 (3)	-	NR
Overall response rate	25 (7)	5 (3)	-	0.02
Stable disease	223 (59)	62 (32)	-	NR
Disease control rate	249 (66)	67 (35)	-	< 0.0001
Progression-free survival in mo				
Median	3.4	1.5	0.43	< 0.0001
95%CI	2.9-4.2	1.4-1.5	(0.35-0.52)	
Time to progression in mo				
Median	3.9	1.5	0.41	< 0.0001
95%CI	(2.9-4.2)	(1.4-1.6)	(0.34-0.51)	

Adapted from: Bruix *et al*[14]; Bruix *et al*[18]. CI: Confidence interval; NR: Not reported; HR: Hazard ratio.

plasma analyses, 499 samples were of sufficient quality for protein analysis (332 in the regorafenib arm, 167 in the placebo arm), and 343 were of sufficient quality for RNA analysis (234 in the regorafenib arm, 109 in the placebo arm). The plasma analyses revealed multiple proteins and miRNAs possibly predictive for OS in patients treated with regorafenib. In particular, they identified 5 OS predictive biomarkers (angiopeietin-1, cystatin B, the latency-associated peptide of transforming growth factor beta1, oxidized low density lipoprotein receptor 1 C-C motif chemokine ligand 3), and 47 TTP predictive biomarkers[26]. Finally, an exploratory analysis on 328 whole blood DNA samples identified single nucleotide polymorphisms (SNPs) prognostic for TTP, one of which, in the *UGT1A1* gene, was also predictive of regorafenib TTP benefit. Also, two SNPs in the *VEGFA* gene were identified as having a prognostic or predictive treatment effect on grade ≥1 HFSR[27].

Based on the results of the phase III RESORCE trial, regorafenib has been approved by the United States Food and Drug Administration (FDA), the European Medicines Agency (EMA), and many other regulatory agencies for the treatment of patients with advanced HCC previously treated with sorafenib. The recommended dose and schedule for regorafenib in HCC is 160 mg administered orally once daily for 21 d every 28 d. As mentioned above, prior sorafenib tolerance and preserved liver function (Child-Pugh class A) remain crucial to determine the eligibility status for treatment with regorafenib.

Cabozantinib

Cabozantinib is an oral multikinase inhibitor of several receptors including MET, VEGFR 1, 2, 3, AXL (GAS6 receptor), and RET. Other known targets of cabozantinib include ROS1, TRKA, TRKB, TYRO3, MER, KIT, and FLT-3[28]. Based on preclinical studies in HCC models demonstrating the role of VEGFRs, MET, and AXL in tumor

Table 2 Adverse events in the RESORCE phase III trial occurring in ≥ 10% of patients–Safety population

	Adverse events, *n* (%)						Treatment-related adverse events, *n* (%)					
	Regorafenib *n* = 374			Placebo *n* = 193			Regorafenib *n* = 374			Placebo *n* = 193		
	Any G	G 3	G 4	Any G	G 3	G 4	Any G	G 3	G 4	Any G	G 3	G 4
Any AE	374 (100)	208 (56)	40 (11)	179 (93)	61 (32)	14 (7)	346 (93)	173 (46)	14 (4)	100 (52)	31 (16)	1 (1)
HFSR	198 (53)	47 (13)	NA	15 (8)	1 (1)	NA	196 (52)	47 (13)	NA	13 (7)	1 (1)	NA
Diarrhea	155 (41)	12 (3)	0	29 (15)	0	NA	125 (33)	9 (2)	0	18 (9)	0	0
Fatigue	151 (40)	34 (9)	NA	61 (32)	9 (5)	NA	110 (29)	24 (6)	NA	37 (19)	3 (2)	NA
Hypertension	116 (31)	56 (15)	1 (< 1)	12 (6)	9 (5)	0	87 (23)	48 (13)	1 (< 1)	9 (5)	6 (3)	0
Anorexia	116 (31)	10 (3)	0	28 (15)	4 (2)	0	88 (24)	10 (3)	0	12 (6)	0	0
Increased bilirubin	108 (29)	37 (10)	2 (1)	34 (18)	15 (8)	6 (3)	70 (19)	24 (6)	1 (< 1)	7 (4)	4 (2)	0
Increased AST	92 (25)	37 (10)	4 (1)	38 (20)	19 (10)	3 (2)	48 (13)	16 (4)	3 (1)	15 (8)	9 (5)	1 (1)
Fever	72 (19)	0	0	14 (7)	0	0	14 (4)	0	0	4 (2)	0	0
Nausea	64 (17)	2 (1)	NA	26 (13)	0	NA	40 (11)	1 (< 1)	NA	13 (7)	0	NA
Increased ALT	55 (15)	10 (3)	2 (1)	22 (11)	5 (3)	0	29 (8)	6 (2)	2 (1)	8 (4)	2 (1)	0
Weight loss	51 (14)	7 (2)	NA	9 (5)	0	NA	27 (7)	4 (1)	NA	3 (2)	0	NA
Oral mucositis	47 (13)	4 (1)	0	6 (3)	1 (1)	0	42 (11)	4 (1)	0	5 (3)	1 (1)	0
Vomiting	47 (13)	3 (1)	0	13 (7)	1 (1)	0	27 (7)	1 (< 1)	0	5 (3)	0	0
Cough	40 (11)	1 (< 1)	NA	14 (7)	0	NA	4 (1)	0	NA	2 (1)	0	NA
Hypophosphatemia	37 (10)	30 (8)	2 (1)	4 (2)	3 (2)	0	22 (6)	16 (4)	2 (1)	2 (1)	1(1)	0
Hoarseness	39 (10)	0	NA	1 (1)	0	NA	34 (9)	0	NA	0	0	NA

Adapted from: Bruix *et al*[14]. G: Grade; AE: Adverse event; HFSR: Hand-foot skin reaction; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NA: Not applicable.

progression[29], of MET in acquired resistance to antiangiogenic therapy including sorafenib[7,30-32], and on the results of a phase II randomized discontinuation trial in HCC[33], cabozantinib has been tested in the multicenter, randomized, double-blind, placebo-controlled phase III CELESTIAL trial (ClinicalTrials.gov NCT01908426)[34]. The CELESTIAL trial enrolled patients with pathologic diagnosis of HCC not amenable to curative treatment, preserved liver function (Child-Pugh class A), and good PS (ECOG 0 or 1). Enrolled patients had received previous treatment with sorafenib, they could have received up to two prior systemic regimens for advanced HCC and had progressed following at least one prior systemic treatment. From September 2013 to September 2017, 773 patients were randomized. At the time of the second interim analysis of OS (data cutoff of June 1, 2017) 707 patients were randomized (2:1 ratio) to receive cabozantinib (*n* = 470) or placebo (*n* = 237) and constitute the ITT population for efficacy analyses. Randomization was stratified by disease etiology (HBV with or without HCV *vs* HCV without HBV *vs* other), region (Asia *vs* other), macrovascular invasion and/or extrahepatic disease (yes *vs* no). Baseline patient characteristics were well-balanced between the two treatment arms. All patients had received prior treatment with sorafenib, and 192 patients (27%) had received two previous systemic therapies for advanced HCC. Patients received 60-mg cabozantinib tablets or matching placebo once per day continuously. Tumor assessment was performed every 8 wk according to RECIST 1.1[16]. Patients received treatment until loss of clinical benefit (treatment beyond radiographic progression was allowed) or unacceptable AEs. The primary endpoint was OS in the ITT population, secondary endpoints were PFS and ORR assessed by the investigators using RECIST 1.1[16]. At the time of data cutoff, 73 patients (16%) in the cabozantinib arm and 26 patients (11%) in the placebo arm were still on treatment. The most common reason for discontinuation was radiographic disease progression. One hundred and twenty-three patients (26%) in the cabozantinib arm and 78 patients (33%) in the placebo arm received post-study systemic or liver-directed therapy. Median OS was 10.2 mo (95%CI: 9.1-12.0) in the cabozantinib arm *vs* 8.0 mo (95%CI: 6.8-9.4) in the placebo arm, with a HR of 0.76 (95%CI: 0.63-0.92) and a *P* value of 0.005. This value met the criterion for statistical significance at the second interim analysis (stopping boundary *P* = 0.02), which included 484 deaths, corresponding to 78% of the 621 deaths planned for the prespecified final analysis. Cabozantinib performed better

than placebo in all the efficacy endpoints (Table 3)[34-35]. Preplanned and exploratory analyses confirmed the superiority of cabozantinib compared to placebo in all subgroups of patients. In the subgroup of patients who had received only sorafenib as previous systemic treatment, median OS was 11.3 mo in the cabozantinib group and 7.2 mo in the placebo group (HR = 0.70, 95%CI: 0.55-0.88), and median PFS was 5.5 mo in the cabozantinib group and 1.9 mo in the placebo group (HR = 0.40, 95%CI: 0.32-0.50). Cabozantinib improved clinical outcomes irrespective of prior sorafenib duration[36], age (cutoff 65 years)[37], baseline AFP values[38], prior transarterial chemoembolization (TACE)[39], tumor burden[40], and in patients with HBV etiology[41]. Furthermore, 47% of patients on cabozantinib compared to 11% of patients on placebo had any reduction in target lesions, and among patients with elevated baseline AFP levels, 23% of patients on cabozantinib compared to 5% of patients on placebo achieved ≥ 50% reduction in AFP levels[35]. AFP response rate, defined as ≥ 20% decrease in AFP level from baseline at week 8, was higher with cabozantinib *vs* placebo and was associated with longer OS and PFS with cabozantinib[42]. Although different cutoffs were adopted, these findings are in line with previous reports suggesting a benefit from systemic treatments in patients achieving an AFP response[43].

The safety population included 704 patients who started treatment, 467 in the cabozantinib group and 237 in the placebo group. Median duration of treatment was 3.8 mo with cabozantinib and 2 mo with placebo. Ninety-nine percent of patients who received cabozantinib and 92% of patients who received placebo had ≥ 1 AE (graded according to NCI-CTCAE version 4.0), and 68% of patients on cabozantinib and 32% of patients on placebo had ≥ 1 grade 3-4 AE. Most common grade 3-4 AEs were palmar-plantar erythrodysesthesia (PPE) (17% of patients on cabozantinib *vs* 0% of patients on placebo), hypertension (16% *vs* 2%), increased AST level (12% *vs* 7%), fatigue (10% *vs* 4%), and diarrhea (10% *vs* 2%) (Table 4). SAEs were reported in 50% of patients in the cabozantinib arm and in 37% of patients in the placebo arm. Grade 5 AEs occurring within 30 d after the last dose of treatment, mostly disease progression, were observed in 12% of patients in both arms and were deemed related to the study drug in 6 patients on cabozantinib and in 1 patient on placebo. Dose reductions (to 40 mg and then to 20 mg daily) and discontinuations due to AEs occurred in 62% and 16% of patients on cabozantinib and in 13% and 3% of patients on placebo, respectively. AEs leading to treatment discontinuation in > 1.0% of patients in the cabozantinib group were PPE, fatigue, decreased appetite, diarrhea, and nausea. Median average daily dose was 35.8 mg for cabozantinib and 58.9 mg for placebo, and median time to first dose reduction was 38 d in the cabozantinib arm[34]. The safety results for cabozantinib reported in the exploratory analyses were consistent with the results in the overall study population[36-41]. Of note, albeit patients ≥ 65 years more frequently discontinued treatment due to AEs, rate of dose reductions and median average daily dose were similar irrespective of age[37]. Grade 3-4 AEs were similar for HBV-positive patients and for patients with prior TACE compared to the overall study population and to patients without prior TACE, respectively [39-41]. Also, a post hoc QOL analysis estimated the incremental quality-adjusted life-years (QALYs) accrued with cabozantinib. Although cabozantinib was associated with an initial, small reduction in health utility compared to placebo, the difference reduced with dose adjustments and considering the overall within-trial health utility experience, cabozantinib was associated with a clinically and statistically significant benefit in mean QALYs[44]. Finally, a popPK analysis showed that PK of cabozantinib in HCC patients was similar to that observed for other cancer types and healthy volunteers, and that HCC patients with mild and moderate hepatic dysfunction had consistent exposure with the patients of normal liver function[45].

Based on the results of the phase III CELESTIAL trial, cabozantinib has been approved by the EMA and the FDA for the treatment of patients with HCC who have been previously treated with sorafenib. The recommended dose and schedule for cabozantinib in HCC is 60 mg, administered orally once daily (tablet formulation).

Given a strong preclinical rationale showing the effect of cabozantinib on immune-mediated killing of tumor cells and immune TME permissiveness[46], ongoing studies are testing cabozantinib in combination with immune checkpoints inhibitors. Notably, the multicenter, randomized, open-label, controlled phase III COSMIC-312 trial (ClinicalTrials.gov NCT03755791) is evaluating the efficacy and safety of cabozantinib in combination with atezolizumab *vs* the standard of care sorafenib in patients with advanced HCC who have not received previous systemic therapy.

Ramucirumab

Ramucirumab is a recombinant human IgG1 monoclonal antibody that interferes with high affinity with the extracellular domain of VEGFR 2, blocking the binding of its ligands VEGF-A, VEGF-C, and VEGF-D, that play an important role in tumor

Table 3 Efficacy results of the CELESTIAL phase III trial

Outcome	Cabozantinib	Placebo	HR	*P* value
Intent to treat population	***n* = 470 (%)**	***n* = 237 (%)**	**(95%CI)**	
Overall response rate				0.009
Partial response	18 (4)	1 (< 1)	-	
95%CI	(2.3-6.0)	(0.0-2.3)		
Stable disease	282 (60)	78 (33)	-	NR
Disease control rate	300 (64)	79 (33)	-	NR
Overall survival in mo				
Median	10.2	8.0	0.76	0.005
95%CI	9.1-12.0	6.8-9.4	(0.63-0.92)	
Progression-free survival in mo				
Median	5.2	1.9	0.44	< 0.001
95%CI	4.0-5.5	1.9-1.9	(0.36-0.52)	
Time to progression in mo				NR
Median	5.4	1.9	0.41	
95%CI	(4.0-5.6)	(1.9-1.9)	(0.34-0.49)	
Patients who have only received sorafenib as prior therapy	*n* = 335 (%)	*n* = 174 (%)	HR (95%CI)	*P* value
Overall survival in mo				
Median	11.3	7.2	0.70	NR
95%CI	9.5-13.9	5.8-9.3	(0.55-0.88)	
Progression-free survival in mo				
Median	5.5	1.9	0.40	NR
95%CI	4.6-5.7	1.9-1.9	(0.32-0.50)	

Adapted from: Abou-Alfa *et al*[34]; Merle *et al*[35]; Kelley *et al*[36]. CI: Confidence interval; NR: Not reported; HR: Hazard ratio.

angiogenesis and tumor growth[47]. Two phase I trials evaluated ramucirumab in order to define the maximum-tolerated dose with doses ranging from 2 mg/kg per week to 20 mg/kg per 3 wk intravenously and two patients with advanced HCC experienced disease control longer than 6 mo[47-48]. These results provided the rationale for a phase II study that confirmed the antitumor activity with an acceptable safety profile of ramucirumab 8 mg/kg per 2 wk in first-line HCC[49]. The phase III REACH trial (ClinicalTrials.gov NCT01140347) evaluated ramucirumab 8 mg/kg per 2 wk *vs* placebo in 565 patients with advanced HCC as second-line treatment following sorafenib. With a median OS of 9.2 mo in the ramucirumab arm and of 7.6 mo in the placebo arm (HR = 0.87, 95%CI: 0.72–1.05, P = 0.14), this trial did not meet its primary endpoint. However, in the prespecified analysis of the subgroup of patients with baseline AFP levels ≥ 400 ng/mL (n = 250), ramucirumab showed a significant survival benefit, with a median OS of 7.8 mo *vs* 4.2 mo (HR=0.67, P =0.006), with a good safety profile. In addition, the REACH trial confirmed in the overall study population the negative prognostic role of baseline elevated AFP levels[50].

In HCC the AFP value is included in several prognostic scoring systems[51-53] and a concentration > 400 ng/mL has been associated with worse prognosis[50,54]. Also, increased VEGFR expression and angiogenesis have been demonstrated in patients with HCC and elevated AFP concentration[2,55,56].

Based on these data and on the results achieved in the REACH trial in patients with high baseline AFP levels, ramucirumab was further tested in the phase III multicenter, randomized, double-blind, placebo-controlled REACH-2 trial (ClinicalTrials.gov NCT02435433)[57].

The REACH-2 trial enrolled patients with histologic or cytologic diagnosis of HCC or, in the absence of histologic confirmation, with cirrhosis and HCC, BCLC stage B, or C disease not suitable for locoregional therapy, preserved liver function (Child-Pugh class A), and good PS (ECOG 0-1), AFP levels ≥ 400 ng/mL, progressing on or intolerant to first-line treatment with sorafenib. From July 26, 2015, to August 30, 2017, 292 patients were randomly assigned (2:1 ratio), 197 to the ramucirumab group and 95 to the placebo group. Randomization was stratified by geographical region [region 1 (Americas, Europe, Australia, Israel) *vs* region 2 (Asia, excluding Japan) *vs* region 3 (Japan)], macrovascular invasion (yes *vs* no), and ECOG PS (0 *vs* 1). Patients

Table 4 Adverse events in the CELESTIAL phase III trial occurring in ≥ 10% of patients regardless of causality–Safety population

	Adverse events, *n* (%)			
	Cabozantinib *n* = 467		Placebo *n* = 237	
	Any G	G 3-4[1]	Any G	G 3-4[1]
Any AE	460 (99)	316 (68)	219 (92)	86 (37)
Diarrhea	251 (54)	46 (10)	44 (19)	4 (2)
Decreased appetite	225 (48)	27 (6)	43 (18)	1 (< 1)
PPE	217 (46)	79 (17)	12 (5)	0
Fatigue	212 (45)	49 (10)	70 (30)	10 (4)
Nausea	147 (31)	10 (2)	42 (18)	4 (2)
Hypertension	137 (29)	74 (16)	14 (6)	4 (2)
Vomiting	121 (26)	2 (< 1)	28 (12)	6 (3)
Increased AST	105 (22)	55 (12)	27 (11)	16 (6)
Asthenia	102 (22)	32 (7)	18 (8)	4 (2)
Dysphonia	90 (19)	3 (1)	5 (2)	0
Constipation	87 (19)	2 (< 1)	45 (19)	0
Abdominal pain	83 (18)	8 (1)	60 (25)	10 (4)
Weight loss	81 (17)	5 (1)	14 (6)	0
Increased ALT	80 (17)	23 (5)	13 (5)	5 (2)
Mucosal inflammation	65 (14)	8 (2)	5 (2)	1 (< 1)
Fever	64 (14)	0	24 (10)	1 (< 1)
Upper abdominal pain	63 (13)	3 (1)	31 (13)	0
Cough	63 (13)	1 (< 1)	26 (11)	0
Peripheral edema	63 (13)	4 (1)	32 (14)	2 (1)
Stomatitis	63 (13)	8 (2)	5 (2)	0
Dyspnea	58 (12)	15 (3)	24 (10)	1 (< 1)
Rash	58 (12)	2 (< 1)	146 (6)	1 (< 1)
Ascites	57 (12)	18 (4)	30 (13)	11 (5)
Dysgeusia	56 (12)	0	5 (2)	0
Hypoalbuminemia	55 (12)	2 (< 1)	12 (5)	0
Headache	52 (11)	1 (< 1)	16 (7)	1 (< 1)
Thrombocytopenia	52 (11)	16 (3)	1 (< 1)	0

[1]Mostly grade 3; Adapted from: Abou-Alfa *et al*[34]. G: Grade; AE: Adverse event; PPE: Palmar-plantar erythrodysesthesia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

received ramucirumab 8 mg/kg or placebo intravenously every 14 d until disease progression, unacceptable toxicity, or withdrawal of consent. Tumor assessment was performed by the investigators every 6 wk according to RECIST 1.1 during the first 6 mo of treatment, and every 9 wk thereafter. Patient-reported outcomes were assessed at baseline, every 6 wk, and at treatment discontinuation with the Functional Assessment of Cancer Therapy Hepatobiliary Symptom Index 8 (FHSI-8), which specifically evaluates the most frequently observed symptoms of patients with hepatobiliary malignancies: lack of energy, nausea, pain, weight loss, back pain, fatigue, jaundice, and stomach pain or discomfort[58]. Serum AFP levels were measured at baseline, every 6 wk during the treatment period, and at the end of the treatment period.

The primary endpoint of REACH-2 trial was OS, secondary endpoints were PFS, ORR, TTP, safety, time to deterioration in scores on the FHSI-8, and time to deterioration in ECOG PS. Efficacy analyses were conducted by ITT, safety analyses were done in all patients who received at least one dose of study drug. Baseline patient characteristics were well-balanced between the two treatment arms, except for baseline AFP levels that were higher in the ramucirumab group [2741 ng/mL (IQR 1178–11861) in the placebo group *vs* 3920 ng/mL (IQR 1175–20000) in the

ramucirumab group]. Median duration of prior sorafenib was 4.1 mo in both groups, and 50 patients (17%) discontinued sorafenib due to intolerance. At the time of data cutoff, March 15, 2018, 281 patients were off treatment, and 11 patients in the ramucirumab group were still receiving therapy; 206 patients (71%) had disease progression, and 221 (76%) had died. Median OS was 8.5 mo (95%CI: 7.0–10.6) in the ramucirumab arm *vs* 7.3 mo (95%CI: 5.4–9.1) in the placebo arm, with a HR of 0.71 (95%CI: 0.53-0.95) and a *P* value of 0.0199. Ramucirumab also significantly improved PFS (2.8 *vs* 1.6 mo, HR = 0.45, 95%CI: 0.339–0.603, *P* < 0.0001), and DCR (59.9% *vs* 38.9%, *P* = 0.0006). These results were confirmed in almost all predefined subgroups (Table 5).

Median duration of treatment was 12 wk with ramucirumab and 8 wk with placebo. Treatment discontinuations due to any AEs (graded according to NCI-CTCAE version 4.0) (18% *vs* 11%) and to treatment-related AEs (11% *vs* 3%) of any grade were more frequent in the ramucirumab group compared to the placebo group. Dose reductions (5% *vs* 2%), delays (6% *vs* 3%), and omissions (29% *vs* 11%) due to AEs were more common in the ramucirumab arm than in the placebo arm. Most common AEs of any grade in the ramucirumab group were fatigue (27%), peripheral edema (25%), hypertension (25%), and decreased appetite (23%). Hypertension (12% *vs* 4%) and hyponatremia (5% *vs* 2%) were the only grade ≥ 3 AEs reported in ≥ 5% of patients (Table 6). SAEs occurred in 35% of patients in the ramucirumab group and 29% in the placebo group. Grade 5 AEs occurring within 30 d after the last dose of treatment were observed in 20% of patients in the ramucirumab arm and 17% of patients in the placebo arm, and were deemed related to the study drug in 4 patients on ramucirumab (3 liver failure and 1 arterial thromboembolic event) and in no patient on placebo. FHSI-8 was completed in 99% of patients at baseline and 67% at the end of treatment in both groups. Median time to deterioration of FHSI-8 scores (3.7 mo *vs* 2.8 mo, *P* = 0.238) and ECOG PS (*P* = 0.77) were not different between the ramucirumab and placebo arms, although the number of events did not allow a meaningful statistical assessment of ECOG PS deterioration[58].

An exploratory analysis investigated the potential relationship between changes in AFP during treatment and efficacy in terms of survival, considering AFP response defined as ≥ 20% decrease from baseline. Ramucirumab was shown to prolong time to AFP progression and radiographic TTP and to slow the rate of AFP increase compared to placebo. AFP response was significantly higher in the ramucirumab arm compared to the placebo arm (42% *vs* 11%, *P* < 0.0001). Also, regardless of treatment OS was longer in patients with AFP response (13.5 mo in AFP responders *vs* 6.7 mo in non-responders, HR = 0.47, *P* < 0.0001), and changes in AFP levels were associated with radiographic TTP (3 mo in AFP responders *vs* 1.6 mo in non-responders, HR = 0.43, *P* < 0.0001)[59].

A preplanned pooled meta-analysis of individual data of patients (*n* = 542) enrolled in the REACH-2 trial and patients with AFP ≥ 400 ng/mL enrolled in the REACH trial (ramucirumab, *n* = 316; placebo, *n* = 226) confirmed significant improvements in OS (median 8.1 *vs* 5.0mo with placebo, HR = 0.69, 95%CI: 0.57–0.84, *P* = 0.0002), PFS (median 2.8 *vs* 1.5mo with placebo, HR = 0.57, 95%CI: 0.47–0.69, *P* < 0.0001), and DCR (56.3% *vs* 37.2% with placebo, *P* < 0.0001). Of note, median baseline AFP levels were lower in the REACH-2 trial compared to those in the above mentioned cohort of the REACH trial [3394 ng/mL (IQR 1177–16812) *vs* 5736 ng/mL (IQR 1322–291000)][58]. The same pooled analysis showed a reduction in disease-related symptoms with ramucirumab compared to placebo, with a significantly delay in FHSI-8 time to deterioration (3 mo with ramucirumab *vs* 1.9 mo with placebo)[60]. Also, the pooled analysis confirmed the efficacy and safety results regardless of etiology, including HCV, HBV, and other[61], and in Japanese patients[62]. Finally, an exploratory analysis evaluated the prognostic utility of Child-Pugh score *vs* albumin-bilirubin (ALBI) grade, showing a similar prognostic utility of the two scoring system and a higher incidence of liver AEs in patients with a high score in either system, and confirming the efficacy of ramucirumab in patients with ALBI score 1 or 2 or Child-Pugh score 5 or 6[63].

Based on these results ramucirumab, pending approval, will be a new treatment option for patients previously treated with sorafenib and with baseline elevated AFP levels. The recommended dose and schedule for ramucirumab is 8 mg/kg intravenously every 14 d.

CONCLUSION

Despite numerous negative trial results in the second-line setting, the current clinical scenario is quickly expanding with the anticipated availability of three anti-angiogenic agents-regorafenib, cabozantinib, and ramucirumab-shown to prolong OS in recent phase III second-line trials. In contrast, preliminary data from a phase III trial of pembrolizumab[64] suggest that single-agent immune checkpoint inhibitors might

Table 5 Efficacy results of the REACH-2 phase III trial

Outcome	Ramucirumab *n* = 197 (%)	Placebo *n* = 95 (%)	HR (95%CI)	*P* value
Response				
Complete	0	0	-	NR
Partial	9 (4.6)	1 (1.1)	-	NR
Overall response rate	9 (4.6)	1 (1.1)	-	0.1697
Stable disease	109 (55.3)	36 (37.9)	-	NR
Disease control rate	118 (59.9)	37 (38.9)	-	0.0006
Overall survival in mo			0.71 (0.53–0.94)	0.0199
Median	8.5	7.3		
95%CI	7.0-10.6	5.4-9.1		
Progression-free survival in mo			0.45 (0.33–0.60)	< 0.0001
Median	2.8	1.6		
95%CI	2.8–4.1	1.5–2.7		
Time to progression in mo			0.42 (0.31–0.58)	< 0.0001
Median	3.0	1.6		
95%CI	(2.8–4.2)	(1.5–2.7)		

Adapted from: Zhu *et al*[57]. CI: Confidence interval; NR: Not reported; HR: Hazard ratio.

not be superior to placebo in patients who had received prior sorafenib. In fact, despite numerically longer OS and PFS in the pembrolizumab arm of the KEYNOTE-240 study, the statistical significance per pre-specified statistical plan was not met. Pending additional details from KEYNOTE-240 that need to be considered, these disappointing results do not necessarily imply a dead end for immunotherapy studies in HCC.

Rather, combinations of immune checkpoint inhibitors and anti-angiogenics may represent a sound evolution of current treatment options. In this respect, it is predicted that some agents may also move from the second-line to a frontline setting, as it is the case for current phase I trials of regorafenib plus pembrolizumab (ClinicalTrials.gov NCT03347292), or cabozantinib plus nivolumab as neoadjuvant treatment (ClinicalTrials.gov NCT03299946). In principle, robust preclinical data do support similar strategies aiming to improve the effectiveness of immunotherapy converting an immunosuppressive milieu into an immunosupportive one[65]. However, most clinical studies are still in their very early stages of development, while other studies are already making their way into more advanced phase III contexts (ClinicalTrials.gov NCT03755791).

For the time being, in the absence of additional agents looming in the spotlight of placebo-controlled studies, an anti-VEGFR strategy is overall regarded as the only one increasing survival, and thereby establishing a standard of care after prior sorafenib treatment. Still, when it comes to specific treatment choices, the debate remains open as no direct comparative studies testing regorafenib, cabozantinib, and ramucirumab are available. With the notable exception of ramucirumab (whose efficacy could not be proven in patients with low AFP levels), there is no approved biomarker that can aid patient selection, and this observation clearly speaks to the huge translational efforts needed. As in other oncology settings, clinical factors informing treatment selection should include first-line therapy, tolerance, and duration of response to prior treatment. In fact, inclusion and exclusion criteria provided by each clinical study protocol should be an additional aid for the selection of the most adequate second-line agent. For instance, poor tolerability of prior sorafenib excludes an individual patient from treatment with regorafenib. Similarly, low AFP levels clearly contraindicate ramucirumab. In view of a treatment sequencing that includes up to three lines, consistent with CELESTIAL study[34], one may consider cabozantinib as a third-line treatment. Even patients' clinical conditions by the time of disease progression, liver function, and the adverse events profiles are variables that need to be considered in the decision-making process.

Further, will the results of well-conducted clinical trials fulfill the expectations of the real-world setting? This is not a trivial point, and this will undoubtedly pose additional questions, especially in light of a limited benefit of sorafenib previously reported in a cohort of Medicare beneficiaries[66].

The scientific community is witnessing a turning point for our knowledge of the

Table 6 Adverse events in the REACH-2 phase III trial occurring in ≥ 10% of patients–Safety population

	Adverse events, *n* (%)						Treatment-related adverse events, *n* (%)					
	Ramucirumab *n* = 197			Placebo *n* = 95			Ramucirumab *n* = 197			Placebo *n* = 95		
	Any G	G 3	G 4	Any G	G 3	G 4	Any G	G 3	G 4	Any G	G3	G 4
Fatigue	54 (27)	7 (4)	NA	16 (17)	3 (3)	NA	28 (14)	2 (1)	NA	5 (5)	0	NA
Peripheral edema	50 (25)	3 (2)	0	13 (14)	0	0	15 (8)	2 (1)	0	5 (5)	0	0
Decreased appetite	46 (23)	3 (2)	0	19 (20)	1 (1)	0	21 (11)	0	NA	4 (4)	0	0
Abdominal pain	39 (20)	3 (2)	NA	12 (13)	2 (2)	NA	8 (4)	1 (1)	1 (< 1)	3 (3)	0	NA
Nausea	37 (19)	0	NA	11 (12)	0	NA	23 (12)	0	0	2 (2)	0	NA
Diarrhea	32 (16)	0	0	13 (14)	1 (1)	0	14 (7)	0	1 (< 1)	5 (5)	1 (1)	0
Headache	28 (14)	0	NA	5 (5)	1 (1)	NA	12 (6)	0	3 (1)	0	0	NA
Constipation	27 (14)	1 (1)	0	19 (20)	1 (1)	0	3 (2)	1 (1)	0	3 (3)	0	0
Insomnia	21 (11)	0	NA	6 (6)	1 (1)	NA	1 (1)	0	NA	0	0	NA
Pyrexia	20 (10)	0	0	3 (3)	0	0	4 (2)	0	2 (1)	1 (1)	0	0
Vomiting	20 (10)	0	0	7 (7)	0	0	5 (3)	0	NA	1 (1)	0	0
Bleeding or hemorrhage events	48 (24)	9 (5)	1 (1)	12 (13)	2 (2)	1 (1)	21 (11)	1 (< 1)	0	5 (5)	0	1 (1)
Epistaxis	27 (14)	1 (1)	0	3 (3)	0	0	14 (7)	0	0	2 (2)	0	0
GI hemorrhage events	12 (6)	7 (4)	0	5 (5)	2 (2)	0	1 (1)	1 (1)	0	0	0	0
Hepatic hemorrhage events	1 (1)	0	1 (1)	0	0	0	0	0	0	0	0	0
Pulmonary hemorrhage events	5 (2)	1 (1)	0	0	0	0	0	0	0	0	0	0
Hypertension	49 (25)	25 (13)	0	12 (13)	5 (5)	0	32 (16)	15 (8)	0	6 (6)	2 (2)	0
Proteinuria	40 (20)	4 (2)	0	4 (4)	0	0	27 (14)	4 (2)	0	3 (3)	0	0
Arterial TE events	5 (3)	0	1 (1)	1 (1)	0	0	4 (2)	0	1 (1)	0	0	0
Venous TE events	2 (1)	0	0	2 (2)	1 (1)	0	1 (1)	0	0	1 (1)	0	0
GI perforation	2 (1)	2 (1)	0	2 (2)	2 (2)	0	1 (1)	1 (1)	0	0	0	0
Congestive heart failure	1 (1)	0	0	1 (1)	1 (1)	0	0	0	0	0	0	0
Fistula	1 (1)	0	0	0	0	0	0	0	0	0	0	0
Liver injury or failure	78 (40)	28 (14)	4 (2)	28 (29)	14 (15)	1 (1)	17 (9)	3 (2)	0	2 (2)	0	0
Ascites	35 (18)	7 (4)	0	7 (7)	2 (2)	0	4 (2)	1 (1)	0	1 (1)	0	0
Hepatic encephalopathy	8 (4)	5 (3)	1 (1)	0	0	0	2 (1)	1 (1)	0	0	0	0
Infusion related reactions	17 (9)	28 (14)	0	3 (3)	0	0	13 (7)	0	0	2 (2)	0	0

Adapted from: Zhu *et al*[57]. G: Grade; AE: Adverse event; NA: Not applicable; GI: Gastrointestinal; TE: Thromboembolic.

genetic and immunologic landscape of HCC. Encouraging efficacy signals are now emerging from the use of second-line anti-angiogenic agents after sorafenib. Arguably, from a clinical perspective, the next challenge will be the implementation of well-designed studies that include sound correlative translational investigations. This is a great opportunity to bridge the enormous gap between clinical practice and basic science still existing in the field of HCC research.

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REVIEW

Precision medicine in gastric cancer

Patrizia Bonelli, Antonella Borrelli, Franca Maria Tuccillo, Lucrezia Silvestro, Raffaele Palaia, Franco Maria Buonaguro

ORCID number: Patrizia Bonelli (0000-0002-0916-4015); Antonella Borrelli (0000-0002-4439-6882); Franca Maria Tuccillo (0000-0003-4167-2535); Lucrezia Silvestro (0000-0002-4209-7694); Raffaele Palaia (0000-0002-4299-3448); Franco Maria Buonaguro (0000-0002-7491-7220).

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Patrizia Bonelli, Antonella Borrelli, Franca Maria Tuccillo, Franco Maria Buonaguro, Molecular Biology and Viral Oncology, Istituto Nazionale Tumori - IRCCS - Fondazione G Pascale, Napoli 80131, Italy

Lucrezia Silvestro, Abdominal Medical Oncology, Istituto Nazionale Tumori - IRCCS - Fondazione G Pascale, Napoli 80131, Italy

Raffaele Palaia, Gastro-pancreatic Surgery Division, Istituto Nazionale Tumori - IRCCS - Fondazione G Pascale, Napoli 80131, Italy

Corresponding author: Patrizia Bonelli, BSc, Research Scientist, Molecular Biology and Viral Oncology, Istituto Nazionale Tumori - IRCCS - Fondazione G Pascale, via Mariano Semmola, Napoli 80131, Italy. p.bonelli@istitutotumori.na.it
Telephone: +39-81-5903270

Abstract

Gastric cancer (GC) is a complex disease linked to a series of environmental factors and unhealthy lifestyle habits, and especially to genetic alterations. GC represents the second leading cause of cancer-related deaths worldwide. Its onset is subtle, and the majority of patients are diagnosed once the cancer is already advanced. In recent years, there have been innovations in the management of advanced GC including the introduction of new classifications based on its molecular characteristics. Thanks to new technologies such as next-generation sequencing and microarray, the Cancer Genome Atlas and Asian Cancer Research Group classifications have also paved the way for precision medicine in GC, making it possible to integrate diagnostic and therapeutic methods. Among the objectives of the subdivision of GC into subtypes is to select patients in whom molecular targeted drugs can achieve the best results; many lines of research have been initiated to this end. After phase III clinical trials, trastuzumab, anti-Erb-B2 receptor tyrosine kinase 2 (commonly known as ERBB2) and ramucirumab, anti-vascular endothelial growth factor receptor 2 (commonly known as VEGFR2) monoclonal antibodies, were approved and introduced into first- and second-line therapies for patients with advanced/metastatic GC. However, the heterogeneity of this neoplasia makes the practical application of such approaches difficult. Unfortunately, scientific progress has not been matched by progress in clinical practice in terms of significant improvements in prognosis. Survival continues to be low in contrast to the reduction in deaths from many common cancers such as colorectal, lung, breast, and prostate cancers. Although several target molecules have been identified on which targeted drugs can act and novel products have been introduced into experimental therapeutic

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protocols, the overall approach to treating advanced stage GC has not substantially changed. Currently, surgical resection with adjuvant or neoadjuvant radiotherapy and chemotherapy are the most effective treatments for this disease. Future research should not underestimate the heterogeneity of GC when developing diagnostic and therapeutic strategies aimed toward improving patient survival.

Key words: Gastric cancer; Molecular characterization; Biomarkers; Precision medicine; Targeted therapy

Core tip: The onset of gastric cancer is linked to genetic alterations, and environmental and lifestyle factors. Recent classifications (TCGA, ACRG) based on genetic alterations have shown significant neoplasia heterogeneity, which makes the practical application of such approaches more difficult. Numerous studies have been conducted on new specific targeted therapies in advanced gastric cancer in the field of precision medicine. The results have not been satisfactory in terms of survival, so elective therapy remains surgery associated with adjuvant and neoadjuvant chemotherapy. Future research should not underestimate the heterogeneity of gastric cancer when developing diagnostic and therapeutic strategies aimed toward improving patient survival.

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INTRODUCTION

Gastric cancer (GC) is a complex disease whose onset is linked to a series of genetic and environmental factors such as smoking and a high salt diet. *Helicobacter pylori* (*H. pylori*) is considered one of the most significant risk factors of GC. It is present in more than 70% of non-cardia GC cases and 90% of chronic gastritis cases[1], and its presence increases the risk of cancer (as compared to uninfected individuals)[2,3]. Recent research has shown that there is a correlation between the risk of GC and the characteristics of specific strains of *H. pylori*[4]. Moreover, *H. pylori* infection has been demonstrated to be essential for promoting chronic inflammation of the gastric epithelium and histological changes that sequentially lead to GC[5]. In this process, genetic and epigenetic alterations occur such as hypermethylation of DNA or mutations in genes including APC, WNT signaling pathway regulator (*APC*), tumor protein p53 (*TP53*), and KRAS proto-oncogene, GTPase (*KRAS*)[6].

Regarding treatment options, surgical resection with adjuvant or neoadjuvant radiotherapy and chemotherapy with cisplatin, 5-fluorouracil (5-FU), taxane, or irinotecan are the most effective treatments for GC. However, despite the increasing knowledge and progress in drug development, this disease has a very poor prognosis due to late diagnosis and extreme intra- and inter-tumor heterogeneity. The heterogeneity makes the choice of therapy difficult, emphasizing the need for both new indicators for patient classification and novel therapies capable of addressing genetic, molecular, and cellular heterogeneity within tumors. This review highlights the progress achieved in the molecular characterization of GC and how it has impacted diagnosis, prognosis, and therapy in clinical practice.

EPIDEMIOLOGY OF GC

GC is the fifth most malignant tumor in worldwide and the third leading cause of cancer-related deaths[7]. Unfortunately, the disease becomes symptomatic in the advanced stage; thus, the 5-year survival rate is only high (90%) in Japan where diagnosis and early tumor resection are done[8]. In European countries, however, the survival rate is low, varying between 10% and 30%[9]. The incidence of GC has geographical variation, with more than 50% of new cases occurring in developing

countries. The areas most at risk are represented by China and Japan, Eastern Europe, Central, and South America, while the areas with lowest risk are South Asia, North America, New Zealand, Australia, and North and East Africa[10]. In recent decades, a decrease in the incidence rate has been observed, especially in young patients with non-cardia, sporadic, and intestinal GC[11,12]. The decreased incidence of GC can be attributed to the better preservation of foods, higher hygienic standards, higher intake of fruits and vegetables, and the eradication of *H. pylori*[13]. Figure 1 summarizes the epidemiology of GC.

PATHOLOGICAL CLASSIFICATION OF GC

According to World Health Organization (commonly known as WHO) guidelines, GC can be classified as adenocarcinoma, ring-cell carcinoma, and undifferentiated carcinoma[14]. The Lauren's classification, which is widely used, classifies GC into intestinal, diffuse, and mixed/unclassified types based on macroscopic and microscopic differences[15]. It has been hypothesized that intestinal GC is associated with chronic atrophic gastritis and intestinal metaplasia, whereas the diffuse type originates from normal gastric mucosa. In European countries, the intestinal type is currently the most common GC[16-20]. It tends to occur more often in the distal part of the stomach, in high-risk areas and is often preceded by long-standing precancerous lesions[17]. On the other hand, the diffuse type is predominant among young patients. However, Lauren's classification has a couple of key flaws. First, a large group of carcinomas do not fall into the two main types of carcinomas, intestinal or diffuse. This group of "unclassified" or "undetermined" gastric carcinomas include undifferentiated carcinomas and carcinomas that have dual differentiation (mixed intestinal and diffuse carcinomas). Second, there has been confusion regarding the "intestinal" term. Therefore, a change to Lauren's classification has been proposed in which GCs are classified into four subtypes: Glandular, solid, isolated cell type, and mixed carcinoma[21].

MOLECULAR CHARACTERIZATION OF GC

Advances in next-generation sequencing (NGS) and microarray technologies and a better understanding of cancer biology have provided opportunities to characterize the genome of tumors including GC. The molecular profile of the GC has enabled The Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG) to classify GC into subtypes. The new molecular classification of GC is complementary to the subtyping classification based on histopathological characteristics. It is important to note that the molecular classification of GC helps to identify the molecular alterations that may be targeted by therapy. Furthermore, the molecular profiles of GCs obtained from individual patients have offered new opportunities to identify biomarkers that can be predictive of the tumor response to treatment[22-24] and to guide the selection of cytotoxic drugs and targeted therapies. TCGA and ACRG classifications of GC should facilitate the development of personalized prognosis and treatment, as well as better patient stratification for the design of clinical trials. The molecular characterization of GC from TCGA has used different platforms, including exome sequencing, DNA copy number analysis, DNA methylation, mRNA and microRNA (miRNA) expression. It divides GC into four subtypes: Epstein-Barr virus (EBV)-positive, microsatellite instable (MSI), chromosomal instability (CIN), and genomically stable (GS) (Figure 2). Each of these GC subtypes is characterized by distinct features that provide prognostic information and suggest the potential benefits of targeted therapy.

EBV-positive tumors have mainly been found in the fundus and gastric body[25,26], and represent about 9% of GC cases. High DNA hypermethylation has been demonstrated in EBV-positive tumors, particularly of the cyclin-dependent kinase inhibitor 2A (commonly known as *CDKN2A*) promoter[27]. An estimated 80% of EBV-positive subtype tumors contain mutations in phosphatidylinositol 3-kinase CA (*PIK3CA*)[28] and amplification of Janus Kinase 2 (*JAK2*), CD274 molecule, and programmed cell death 1 ligand 2 (*PDCD1LG2*), which encode for respectively tyrosine kinase receptors, PD-L1 and PD-L2[29]. Based on these results, JAK2 inhibitors and PD-L1/2 antagonists should be explored as treatment options for EBV-positive tumors. Promising initial results have been reported with pembrolizumab, a humanized monoclonal antibody against programmed cell death 1 (PD-1)[30,31]. In addition to *PIK3CA* mutations, EBV-positive tumors have more recurrent AT-rich interaction domain 1A (*ARID1A*) (55%) and BCL6 corepressor (commonly known as

Figure 1 Epidemiology of gastric cancer. Frequency of diagnosis, leading cause of cancer death, and risk areas worldwide of gastric cancer.

BCOR) (23%) mutations[29,32], whereas only rare *TP53* mutations have been observed.

Patients with MSI subtype generally have intestinal tumors, which are diagnosed in old age. MSI tumors (21.7% of GC cases) are characterized by genomic instability due to methylation of DNA mismatch repair genes including MutL homolog 1 (*MHL1*) and to a high incidence of mutations in *PIK3CA*[33], Erb-B2 receptor tyrosine kinase 3 (*ERBB3*)[34], ring finger protein 43 (*RNF43*)[35], phosphatase and tensin homolog (*PTEN*)[36], *TP53*[37], *KRAS*[38], and *ARID1A*[32] genes. Increased expression of components of the mitotic pathway such as the E2F transcription factor (*E2F*), aurora kinase A (*AURKA*), polo-like kinase 1 (*PLK1*), and forkhead box M1 (*FOXM1*) has been described in MSI tumors[29].

GS tumors (19.7%) are mainly diffuse and are diagnosed in younger patients[39]. GS tumors, which lack chromosomal alteration or MSI, exhibit the high expression of molecules involved in cell adhesion and pathways related to angiogenesis. They also have low mutation rates and *ARID1A*, ras homolog family member A (*RHOA*), and cadherin 1 (*CDH1*) are the most frequently mutated genes[40]. Previous studies have shown the loss of *CDH1*, which encodes the E-cadherin cell adhesion molecule, in hereditary diffuse GC[41]. TCGA data have also revealed the fusion of claudin 18-Rho GTPase activating protein 6 (*CLDN18-ARHGAP6*) or claudin 18-Rho GTPase activating protein 26 (*CLDN18-ARHGAP26*) and recurrent mutations in *RHOA*. CLDN18 and ARHGAP6 are respectively involved in the intercellular structure of the tight junction and the activation of Rho signaling (a signaling pathway in which intracellular and extracellular stimuli activate GTPase Rho), whereas RHOA modulates programmed cell death and contractility and motility of actomyosin-dependent cells[42-44]. Therefore, alterations in RHOA or CLDN18-ARHGAP6 could contribute to the lack of cell cohesion, dispersed growth, and programmed cell death resistance.

CIN tumors represent almost half of GC cases (49.8%), are mainly intestinal, and are most frequent in the cardia-gastro-esophageal junction. Chromosomal deletions affecting *CDH1*, catenin alpha 1 (*CTNNA1*) and RB transcriptional corepressor 1 (*RB1*) and mutations in *TP53* (71%) are frequent in these tumors. CIN tumors present with amplification of genes encoding tyrosine kinase receptors such as epidermal growth factor receptor (*EGFR*), *ERBB2*, *ERBB3*, fibroblast growth factor receptor 2 (*FGFR2*), and MET proto-oncogene, receptor tyrosine kinase (*MET*); some transcription factors including the MYC proto-oncogene, basic helix-loop-helix transcription factor (*MYC*) and GATA binding protein 4 (*GATA4*); cell cycle regulators such as cyclin-dependent kinase 6 (*CDK6*), cyclin E1 (*CCNE1*), and cyclin D1 (*CCND1*) and other genes such as *PDCD1LG2* and *PIK3CA*[29]. Alterations of these genes have been observed in advanced/metastatic GC[24]. By contrast, the ACRG analyzed samples from 300 Korean patients, classifying GC based on particular genetic signatures such as the activation status of *TP53* and the MSI condition[45]. Four molecular subtypes have been

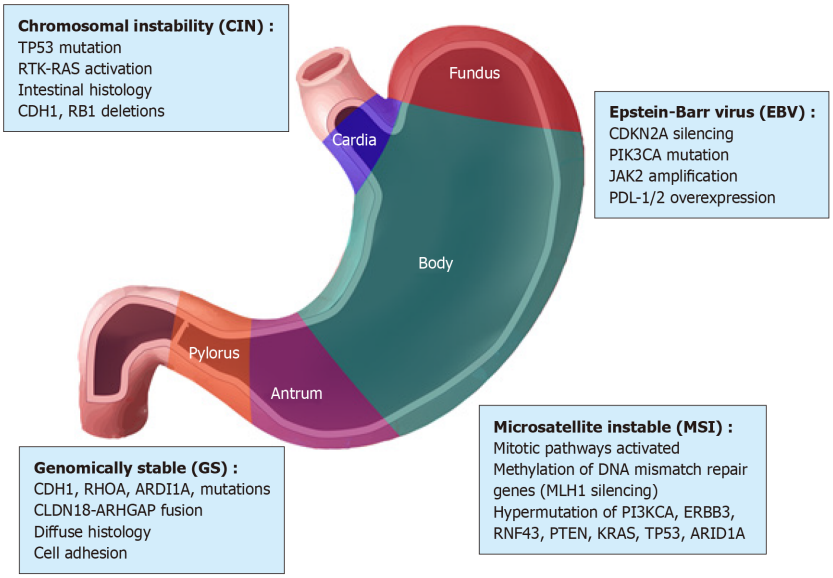

Figure 2 The Cancer Genome Atlas gastric tumor classification. TCGA study divides GC into four molecular subtypes: CIN (chromosomal instability); EBV (Epstein-Barr virus); GS (genomically stable); and MSI (microsatellite instable). GC: Gastric cancer; TCGA: The Cancer Genome Atlas.

identified: MSI, microsatellite stable (MSS) with active *TP53* (MSS/TP53+), MSS with inactive *TP53* (MSS/TP53-), and MSS with epithelial-mesenchymal transition (EMT) signature (MSS/EMT) (Figure 3).

These subtypes are associated with survival and recurrence. The MSI subtype has a better prognosis and a lower tendency to relapse. The MSS/TP53+ and MSS/TP53- subtypes have an intermediate prognosis, whereas the MSS/EMT subtype is associated with a high rate of recurrence and a lower survival rate. Moreover, MSI tumors are diagnosed at an early stage (I/II), and about 60% are intestinal and show a high frequency of mutations of *PIK3CA*, *KRAS*, *ARID1A*, and ALK receptor tyrosine kinase (*ALK*) genes; they also show loss of *MLH1*. Tumors of the MSS/TP53+ subtype include many EBV-positive cases compared to the other subtypes, and have a high prevalence of mutations in the *APC*, *KRAS*, *PIK3CA*, *ARID1A*, and SMAD family member 4 (*SMAD4*) genes[46] compared to the MSS/TP53- subtype. They also present amplification of the *CCNE1* gene. The MSS/TP53- subtype is mainly Lauren intestinal and has *TP53* mutations, with a low frequency of mutations affecting the other genes. This subtype also has amplification of *EGFR*, *MYC*, *ERBB2*, and *CCNE1* genes. The MSS/EMT subtype predominantly consists of Lauren diffuse tumors, and tend to be diagnosed at a younger age. This subtype has low cell adhesion due to loss of *CDH1* and has the least number of mutations. *ARID1A* is among the most frequently mutated gene. The ACRG classification is also applicable to other large independent cohorts[45]. The differences between the two classifications (TGCA and ACRG) reflect the different approaches and platforms used, and the ethnicity of the samples. In the ACRG cohort, GCs of the diffuse type are more represented. However, both identified the MSI subtype with hypermethylation of *MHL1*, high mutation frequency and a better prognosis. The EBV and MSS/TP53 + subtypes are similar in that many cases belonging to the MSS/TP53+ subtype is EBV+ and present mutations in *PIK3CA* and *ARID1A*. The GS and MSS/EMT subtypes, which include younger patients, are mostly diffuse and show low intercellular adhesion. The CIN and MSS/TP53- subtypes present with mutations in *TP53* and amplification of members of the *EGFR* family, and are mostly intestinal.

APPLICATION OF THE GC MOLECULAR PROFILE IN CLINICAL PRACTICE: PRECISION MEDICINE

Due to new technologies, such as NGS and microarray, recent discoveries have made possible to integrate diagnostic and therapeutic method, based on genotype and phenotype, and to apply them to individual patients with GC in the age of precision medicine.

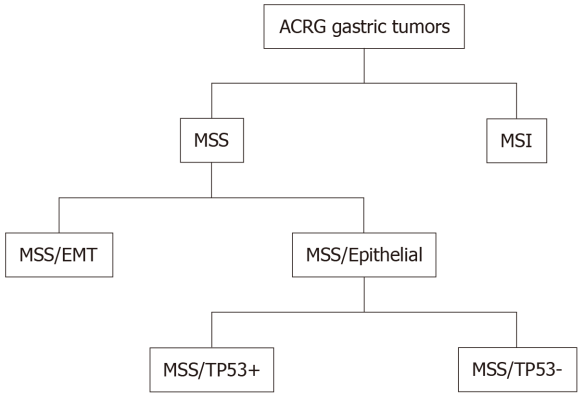

Figure 3 Asian Cancer Research Group gastric tumor classification. Gastric cancer was classified into four subtypes: MSI (microsatellite instable); MSS (stable microsatellite); MSS/TP53+ (MSS with active TP53); MSS/TP53- (MSS with inactive TP53); MSS/EMT (MSS with epithelial-mesenchymal transition). ACRG: Asian Cancer Research Group.

Biomarkers for diagnosis and prediction

Tumor markers are used to determine the clinical stage, assess the treatment response, and predict the risk of recurrence after treatment. Currently, markers such as α-fetoprotein (AFP), carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA-125), and carbohydrate antigen 19-9 (CA19-9) are frequently used in clinical practice. CEA is a risk factor for liver metastases[47], and increased CEA levels have been observed in all advanced GCs. The sensitivity and specificity of CEA for predicting GC recurrence is < 60% and < 80%, respectively[48]. CA19-9 is a marker commonly used in GC, although it is also present in other neoplastic pathologies. In combination with other tumor markers, CA19-9 can provide more information to predict GC recurrence[49]. Other markers such as AFP and CA-125 are widely used in the diagnosis of GC. AFP is an indicator of a high stage and presence of hepatic metastases[50], and CA-125 is associated with peritoneal diffusion[47].

Among the new biomarkers, human epidermal growth factor receptor 2 (encoded by *ERBB2* commonly referred to as *HER2*) represents the first biomarker available in clinical practice for patients with GC. HER2 belongs to the EGFR family and has tyrosine kinase activity[51]. An estimated 6%-23% of GCs have overexpression and/or amplification of *HER2*[52,53], and it is mainly found in intestinal tumors[54,55]. HER2 is used in clinical practice for targeted therapy. EGFR or ERBB1 is expressed in about 30% of GCs[56]. The overexpression of EGFR in the pathogenesis of GC is associated with a poorly differentiated histology, vascular invasion, and shorter survival[57]. Tyrosine kinase inhibitors, particularly gefitinib and erlotinib, have shown efficacy in *EGFR*-amplified tumors. Mutations of *EGFR* confer resistance to these drugs[58,59]. In addition to anti-HER2 monoclonal antibodies, anti-EGFR therapy also includes gefitinib and erlotinib, tyrosine kinase inhibitors, as well as monoclonal antibodies such as cetuximab and panitumumab.

Other markers have attracted substantial attention as useful therapeutic candidates for targeted anti-cancer agents. For example, a high frequency (30%) of FGFR2 overexpression has been observed in GC. The amplification of *FGFR2* is related to poor overall survival (OS)[60]. Furthermore, a study found that FGFR2 could be a biomarker for predicting the long-term failure of adjuvant chemotherapy for advanced GC[61]. Thus, FGFR2 may be a candidate for targeted anticancer agents. E-cadherin, a molecule involved in calcium-mediated cell adhesion, is a tumor suppressor whose deactivation is correlated with invasion and metastasis[62]. The deactivation of *CDH1* may occur due to mutations, hypermethylation, loss of heterozygosity, and *H. pylori* infection[62]. Patients with *CDH1* changes have generally worse survival than negative patients. It could be a useful marker for the diagnosis of preoperative biopsies[63]. Genetic deregulation of the PI3K/AKT/mTOR pathway has been frequently identified in GC[64,65], and mechanistic target of rapamycin kinase (mTOR) is activated in 60% of GCs[66].

Mutations of *PI3KCA*, which encodes the p110α catalytic isoform of PI3K, have been identified in up to 25% of patients with GC[67]. These mutations are involved in resistance to antitumor drugs and the acquisition of a metastatic phenotype; moreover, they are found mainly in the EBV-positive subtype of GC[68]. It has been reported that the amplification and/or overexpression of *MET* is involved in carcinogenesis, the efficacy of therapy, and the outcome of GC[69]. MET expression is

associated with invasion and overall poor survival[70]. Vascular endothelial growth factor (*VEGF*) encodes for a growth factor that promotes the formation of new blood vessels. VEGF and vascular endothelial growth factor receptor (VEGFR) are upregulated in about 40% of GC cases[71], and their inhibition results in decreased cell proliferation and invasion. VEGF andsVEGFR2 are also used in clinical practice in targeted therapy[72,73]. *TP53* is a tumor suppressor whose incidence of mutation in GC is about 3%-65%[74]. In the EBV-positive subtype, the incidence of *TP53* mutation is lower[28]; moreover, an increased incidence has been observed in the intestinal type[75]. In many human tumors, *TP53* mutations are associated with a poor prognosis[76]. For GC, there is no well-established clinical significance between the *TP53* status and the outcome of patients. Recent studies, however, have integrated the mutational status of *TP53* and other genetic alterations to define subpopulations of GCs in order to define the clinical relevance[77]. *TP53* mutations appear to be a cofactor that supports the expression of genes involved in various signaling pathways; and whose aberrant activation leads to high proliferation, increased metastatic potential, and resistance to treatment. *AURKA* and MDM2 proto-oncogene (*MDM2*) encode negative regulators of *TP53*. *AURKA* is amplified and overexpressed in GC[78]. By regulating the ubiquitination of TP53 through MDM2, AURKA promotes tumor growth and cell survival[79].

DNA damage is repaired by a series of mechanisms, including basic excision repair, mismatch repair, nucleotide excision repair, single-strand annealing, homologous recombination, and non-homologous end joining. The poly (ADP-ribose) polymerases, known as PARPs, are proteins involved in the basic excision repair pathway and catalyze the transfer of ADP-ribose to target proteins[80,81]. PARP1 and PARP2 are the best known of these proteins. Numerous studies have highlighted an upregulation of PARPs in different tumors, including GC[82,83]. A high expression of PARP1 in GC is associated with tumor invasion and a poor prognosis[84].

Proteins in the matrix metalloproteinase (MMP) family are involved in breakdown of the extracellular matrix in normal physiologic processes and can promote cancer cell invasion and metastasis by degrading the extracellular matrix. Increased matrix metallopeptidase 15 (MMP15) expression is associated with poor prognosis in GC[85]. In addition, overexpression of matrix metallopeptidase 9 (MMP9) is a poor prognostic factor in patients with GC[86].

Fibrinogen C domain containing 1 (FIBCD1) is an acetyl group-binding receptor, which shows high affinity and calcium-dependent binding to acetylated structures such as chitin, some N-acetylated carbohydrates, and amino acids but not to their non-acetylated counterparts. The expression of *FIBCD1* is significantly increased in GC tissues compared with normal tissues, and its overexpression is related to a poor prognosis[87]. FIBCD1 may be a novel prognostic marker in gastric GC; however, the mechanisms underlying its function require further studies.

PD-1 and PD-2 are the immune checkpoint receptors expressed on T and B lymphocytes, natural killer T cells, and monocytes[88]. After binding with PD-L1 and PD-L2 on activated T cells, they downregulate the activity of cytotoxic T cells and thus induce immunotolerance to the tumor. In 15%-70% of patients with GC, PD-L1 expression has been observed and this expression correlates with poor outcome[89]. Upregulation of *PD-L1*/*PD-L2* expression in the EBV-positive subtype has been observed[29].

Circulating tumor cells (CTCs), single or in clusters, originate from primary tumor or metastases[90]. Clinically, they are related to the progression and metastatic processes, and therefore can be used as surveillance markers. CTCs can identify early stages of metastasis and thus identify patients who may benefit from treatment after primary tumor surgery[90,91]. The presence of CTCs, which have the characteristics of stem cell-like or EMT cells, allows evaluation of the tumor stage and the prediction of recurrence. Circulating cell-free DNA is more sensitive than CTCs, originates from normal and cancerous cells, and is present in the blood[92]. Circulating tumor DNA (ctDNA) originates from the primary tumor or metastases and can be used for the specificity of the diagnosis, even if the sensitivity is lower than the common markers used[93]. The ctDNA shows the presence of EBV DNA, and is useful for identifying EBV-positive subtypes[94]. The response to therapy can also be assessed with ctDNA.

MiRNAs are small, non-coding RNAs that, by regulating gene expression, play a role in the processes of proliferation, differentiation, and cell invasion[95]. They can increase the expression of oncogenes or reduce the expression of oncosuppressor genes[96]. Numerous miRNAs have been identified and play a role in GC[97,98]. Circulating cell-free miRNAs can be used as non-invasive biomarkers for the diagnosis and relapse of GC[99-101]. Approximately 135 long non-coding RNAs (lncRNAs), non-transcribed RNA sequences longer than 200 nucleotides, are dysregulated and strongly correlated with tumorigenesis, metastasis, and prognosis of GC[102-103]. Some lncRNAs are overexpressed in GC compared to healthy control

tissue and may be prognostic markers[104,105]. However, further studies are needed to determine their possible clinical use.

Biomarkers for targeted therapy

Surgery is the elective treatment for many stages of GC. In a patient with GC at stage 0, I, II, or III, surgery (often together with other treatments) is currently the only treatment. Depending on the type and stage of GC, it is possible with surgery to remove all or part of the stomach including the nearby lymph nodes (the principles). Even when the tumor is too widespread to be removed entirely, patients can be helped by surgery because it can help prevent bleeding from the tumor or remove stomach obstruction due to tumor growth. This is termed palliative surgery because it allows the reduction or prevention of symptoms, but is not indicated for the treatment of GC[106].

Minimally invasive surgery, including laparoscopic gastrectomy and robotic gastrectomy, is receiving much attention in GC management[107,108]. The laparoscopic gastrectomy has the advantage of leading to a faster recovery with shorter hospital stays compared to the traditional surgery[109]. However, it has the disadvantage of limited movements. The robotic gastrectomy has overcome these limitations and its use is spreading rapidly[110-112]. Some studies have been carried out in order to compare both the advantages and disadvantages of two technologies[113-115]. The disadvantages of robotic gastrectomy concern its cost, duration of the procedure, and training needs[116]. Unfortunately, the lack of controlled and randomized studies has precluded the ability to establish a clear indication of robotic gastrectomy in the treatment of GC[116].

Surgical resection with pre- and post-operative chemotherapy and/or radiotherapy is the primary curative treatment of early-stage GC with a 5-year survival of about 30%[117-119]. Systemic chemotherapy is used to treat patients with localized and advanced GC. Palliative systemic therapy and chemo/radiotherapy are standard treatment options for patients with unresectable or metastatic advanced GC. Neoadjuvant chemotherapy with surgery is associated with the improved survival of patients with metastatic disease[120]. Perioperative chemotherapy with docetaxel, oxaliplatin, fluorouracil, and leucovorin (FLOT) significantly improves progression-free survival (referred to herein as PFS) and OS among patients with resectable GC compared with epirubicin, cisplatin, and fluorouracil or capecitabine (ECF/ECX)[121]. A Bayesian network meta-analysis obtained an estimate of the efficacy of perioperative FLOT and neoadjuvant treatments for resectable GC. Compared with surgery alone, perioperative cisplatin with fluorouracil (CF), perioperative ECF/ECX, and perioperative FLOT significantly improved survival. The most effective neoadjuvant treatment for the disease is likely to be perioperative FLOT[122]. Targeted therapy, a new therapeutic strategy, may improve the survival of patients with advanced GC. Clinical trials with targeted therapies have been performed in patients with GC. Table 1 shows some clinical trials, completed or ongoing, classified by specific molecular target.

EGFR signaling pathway

The EGFR signaling pathway is activated in the GC[56,123]. Overexpression of EGFR has been associated with reduced OS[56,71]. This behavior may depend on the observation that EGFR targeting molecules may be potential agents for target therapy. Trastuzumab is the first molecular targeted agent approved as standard therapy for GC. It is a monoclonal antibody against HER2, which binds to the extracellular domain of the receptor. A phase III clinical trial (ToGA) (NCT01041404) enrolled 594 patients with GC who had high HER2 expression. These patients were randomized to chemotherapy alone or combined with trastuzumab. Treatment with trastuzumab led to an increase in OS of 2.7 mo and the PFS was heightened compared to that of patients treated with chemotherapy alone[124]. The benefits observed in patients treated with the combination of trastuzumab and chemotherapy were even more evident in patients who expressed high levels of HER2 compared to those with low HER2 expression. The 2015 National Comprehensive Cancer Network guidelines recommended the first-line treatment of trastuzumab combined with chemotherapy in patients overexpressing HER2. To date, trastuzumab is the only targeted therapy allowed for the treatment of advanced GC[124]. A clinical trial (NCT01736410), which evaluated the efficacy of trastuzumab with tegafur, gimeracil, oteracil (TS-1) and cisplatin as first-line treatment for advanced HER2-positive GC, has been completed. The combination of trastuzumab with TS-1 and cisplatin demonstrated good activity, was well tolerated, and is a first-line treatment that can be used for advanced HER2-positive GC[125]. In the GATSBY multicenter phase II/III study (NCT01641939), the efficacy of trastuzumab emtansine was evaluated in patients with advanced HER2-positive GC who had already received previous treatment. The results obtained were

Table 1 Clinical trials classified on molecular targets

Signaling	Molecular target	Therapeutic agents	Clinical trial (Identifier)	Type of trial	Line of treatment	Phase	Patient's stage	Status	Ref.
EGFR	HER2	Trastuzumab ± 5-FU/cisplatin/capecitabine	ToGA (NCT01041404)	Multicenter, randomized, open-label	First	III	Advanced gastric cancer, HER2-positive	Completed	[124]
	HER2	Trastuzumab /TS-1/cisplatin	NCT01736410	Multicenter, non-randomized, open-label	First	II	Advanced gastric cancer, HER2-positive	Completed	[125]
	HER2	Trastuzumab emtansine *vs* docetaxel-paclitaxel	GATSBY (NCT01644193 9)	Multicenter, randomized, open-label	Second	II/III	Advanced gastric cancer, HER2-positive	Completed	[126]
	HER2	Pertuzumab ± trastuzumab/5-FU/cisplatin/capecitabine	JACOB (NCT01774786)	Multicenter, randomized, double	First	III	Metastatic gastric cancer, HER2-positive	Active	[127]
	HER2	Lapatinib ± oxaliplatin/capecitabine	TRIO-013/LOGiC (NCT00680901)	Multicenter, randomized, quadruple	First	III	Advanced/metastatic gastric cancer, HER2-positive	Active	[128]
	HER2	IMU-131 vaccine (HER-Vaxx) + 5-FU/cisplatin or capecitabine	NCT02795988	Non-randomized, open-label	First/second	Ib/II	Advanced/metastatic gastric cancer, HER2-positive	Recruiting	[130]
	HER2	Pyrotinib	(NCT02500199)	Open-label	Second	I	Advanced gastric cancer, HER2-positive	Recruiting	[131]
	HER2	Pyrotinib ± docetaxel	(NCT02378389)	Open-label	Second	I	Advanced gastric cancer, HER2-positive	Recruiting	[132]
	EGFR	Cetuximab ± cisplatin/capecitabine	EXPAND (NCT00678535)	Multicenter, randomized, open-label	First	III	Advanced gastric cancer	Completed	[134]
	EGFR	Panitumumab + 5-FU/cisplatin/docetaxel	NCT01716546	Multicenter, open-label	First	I/II	Locally advanced/metastatic gastric cancer	Terminated	[135]
	EGFR	Nimotuzumab + irinotecan *vs* irinotecan	ENRICH (NCT01813253)	Randomized, open-label	Second	III	Advanced/recurrent gastric cancer, EFGR overexpressed	Terminated	[136]
	EGFR	Nimotuzumab ± S-1/cisplatin	NCT02370849	Randomized, open-label	First	II	Locally advanced/metastatic gastric cancer	Completed	[138]
mTOR/PI3K/AKT	mTOR	Everolimus	NCT00519324	Multicenter, open-label	Second/third	II	Advanced/metastatic, refractory gastric cancer	Completed	[142]
	mTOR	Everolimus	NCT00729482	Open-label	Second	II	Advanced, refractory gastric cancer	Completed	[143]
	mTOR	Everolimus + BSC *vs* placebo + BSC	GRANITE-1 (NCT00879333)	Multicenter, randomized, quadruple	Second/third	III	Advanced gastric cancer	Completed	[144]

	PI3KCA	Alpelisib + AUY922	NCT01613950	Multicenter, open-label	Second/third	Ib	Advanced/metastatic gastric cancer, PIK3CA mutations and/or HER2 amplification	Completed	[145]
	AKT	Ipatasertib ± 5FU/oxaliplatin/leucovorin	NCT01896531	Randomized, double	Second	II	Advanced/metastatic gastric cancer	Active	[146]
HGF/MET	HGF	Rilotumumab *vs* rilotumumab ± epirubicin/cisplatin/capecitabine	NCT00719550	Multicenter, randomized	First	Ib/II	Locally advanced/metastatic gastric cancer	Completed	[149]
	HGF	Rilotumumab ± epirubicin/cisplatin/capecitabine	RILOMET-1 (NCT01697072)	Multicenter, randomized, triple	First	III	Locally advanced/metastatic gastric cancer, MET-positive	Terminated	[150]
	HGF	Rilotumumab ± /cisplatin/capecitabine	RILOMET-2 (NCT02137343)	Multicenter, randomized, triple	First	III	Advanced gastric cancer	Terminated	[151]
	MET	Onartuzumab ± 5-FU/leucovorin/oxaliplatin	NCT01662869	Multicenter, randomized, double	First	III	Metastatic gastric cancer, HER2 negative, MET-positive	Completed	[152]
VEGF/VEGFR	VEGFR2	Ramucirumab + BSC *vs* placebo + BSC	REGARD (NCT00917384)	Randomized, quadruple	First	III	Metastatic/locally recurrent gastric cancer	Completed	[72]
	VEGFR2	Ramucirumab ± paclitaxel	RAINBOW (NCT01170663)	Multicenter, randomized, double	Second	III	Metastatic, refractory gastric cancer	Completed	[73]
	VEGFR2	Apatinib *vs* placebo	NCT01512745	Randomized, quadruple	Third	III	Advanced/metastatic refractory gastric cancer	Completed	[156]
TP53	TP53	Polymorphisms of xenobiotic metabolism, DNA repair, and TP53 genes	NCT01470404				Gastric cancer treated with adjuvant chemotherapy	Completed	[161]
	TP53	APR-246 + 5-FU/cisplatin	NCT02999893	Open-label	Second	I/II	Advanced/metastatic platinum resistant gastroesophageal cancer, TP53 mutated	Recruiting	[167]
	TP53	AZD1775 (WEE inhibitor) + paclitaxel	NCT02448329	Single center	Second	II	Advanced gastric cancer, TP53 mutated	Recruiting	[169]
	TP53	HDM201 (inhibitor TP53/MDM2 interaction)	NCT02143635	Multicenter, non-randomized, open-label	Second	I	Advanced/metastatic gastric cancer, TP53 wild-type	Active	[170]
PARP	PARP	Olaparib + paclitaxel *vs* paclitaxel	Study 39 (NCT01063517)	Multicenter, randomized, double	Second	II	Metastatic/recurrent gastric cancer, low ATM expression	Active	[174]
	PARP	Olaparib + paclitaxel *vs* placebo + paclitaxel	GOLD (NCT01924533)	Multicenter, randomized, double	Second	III	Advanced gastric cancer	Active	[175]

	PARP	Olaparib + ramucirumab	NCT03008278	Open-label	Second	I/II	Metastatic/recurrent gastric cancer	Recruiting	[178]
	PARP	Veliparib + FOLFIRI	NCT01123876	Open-label	First/second	I	Advanced gastric cancer	Completed	[179]
Immune Checkpoint	PD-1	Pembrolizumab *vs* pembrolizumab ± 5FU/cisplatin or capecitabine	KEYNOTE-059 (NCT02335411)	Multicenter, non-randomized, open-label	Second/third	II	Metastatic/recurrent gastric cancer	Active	[181]
	PD-1	Pembrolizumab *vs* paclitaxel	KEYNOTE-061 (NCT02370498)	Randomized, open-label	Second	III	Advanced gastric cancer	Active	[183]
	PD-1	Pembrolizumab *vs* pembrolizumab ± 5FU/cisplatin or capecitabine	KEYNOTE-062 (NCT02494583)	Randomized, quadruple	First	III	Advanced gastric cancer	Active	[184]
	PD-1	Pembrolizumab ± 5-FU/cisplatin or oxaliplatin/capecitabine	KEYNOTE-0859 (NCT03675737)	Randomized, double	First	III	Advanced/metastatic gastric cancer	Recruiting	[185]
	PD-1	Pembrolizumab/trastuzumab ± cisplatin/capecitabine/oxaliplatin	NCT02954536	Single group, open-label	First	II	Advanced/metastatic gastric cancer, HER2-positive	Recruiting	[186]
	PD-1	Nivolumab *vs* placebo	ATTRACTION-2 (NCT02267343)	Multicenter, randomized, quadruple	Second	III	Advanced/recurrent gastric cancer	Active	[187]
	PD-1	Nivolumab ± capecitabine/oxaliplatin	ATTRACTION-4 (NCT02746796)	Multicenter, randomized, quadruple	First	II/III	Advanced/recurrent refractory gastric cancer	Active	[188]
	PD-1	Nivolumab ± ipilimumab	CHECKMATE-032 (NCT01928394)	Multicenter, randomized, open label	First/second	I/II	Advanced/metastatic, refractory gastric cancer	Active	[189]
	PD-1	Nivolumab ± ipilimumab *vs* nivolumab + chemotherapy *vs* chemotherapy	CHECKMATE-649 (NCT02872116)	Multicenter, randomized, open-label	First	III	Advanced/metastatic gastric cancer	Recruiting	[190]
	PD-1	Tislelizumab ± oxaliplatin/capecitabine or 5-FU/cisplatin	NCT03777657	Randomized, triple	First	III	Locally advanced/metastatic gastric cancer	Recruiting	[191]

EGFR: Epidermal growth factor receptor; mTOR: Mechanistic target of rapamycin kinase; HGF: Hepatocyte growth factor; VEGF: Vascular endothelial growth factor; TP53: Tumor protein p53; PARP: Poly (ADP-ribose) polymerase; PD-1: Programmed cell death 1.

not encouraging since treatment with trastuzumab emtansine did not yield increases in OS compared to standard treatment with a taxane (docetaxel, paclitaxel)[126].

Other agents that target HER2, such as pertuzumab and lapatinib, have been used in clinical trials in patients with advanced GC and HER2 overexpression. One study (JACOB, NCT01774786) was performed with pertuzumab, trastuzumab, and chemotherapy in patients with untreated HER2-positive metastatic GC. This trial was the first to investigate the dual antibody blockade of HER2. Unfortunately, no significant improvement in OS was observed in the dual blockade group[127]. The

clinical trial (TRIO-013/LOGiC, NCT00680901) performed with lapatinib in combination with oxaliplatin and capecitabine did not produce significant results in terms of OS[128]. A parallel study of biomarkers was conducted using immunohistochemistry and NGS. The most common alteration found in HER2-positive patients was amplification of *CCNE1*, which correlated with a lack of response to therapy. Patients with high levels of *ERBB2* amplification were more responsive to therapy. The analysis of cell-free DNA showed that the amplification of *ERBB2*, detectable in the plasma of patients, was a predictive response. During disease progression, genetic changes were detected such as amplification of *MYC*, *EGFR*, *FGFR2*, and *MET*[129]. A phase I clinical trial (NCT02795988) evaluated the safety, tolerability, and immunogenicity of IMU-131, a peptide composed of three epitopes selected from the protein structure of HER2. In the phase II portion of the same trial, IMU-131 was used in combination with chemotherapy in patients overexpressing HER2. The study is ongoing, as only phase I has been completed, and no conclusions have been drawn[130]. Pyrotinib is an irreversible inhibitor of both HER2 and EGFR. The phase I studies (NCT02500199, NCT02378389) with pyrotinib and pyrotinib plus docetaxel in patients with HER2-positive GC are recruiting[131,132].

Studies have also been performed to identify markers to be used in monitoring the efficacy of trastuzumab alone or in combination with chemotherapy. Resistance has occurred in patients treated with trastuzumab. One of the main mechanisms that lead to this resistance are mutations in *PI3KCA* and *PTEN*[64,65,67]. The combination of trastuzumab with PI3K inhibitors may bring substantial benefits to patients with HER2-positive GC. One of the markers of resistance to trastuzumab is *CCNE1*, whose amplification is negatively correlated with the response to therapy directed against HER2[133]. Other monoclonal antibodies used to target EGFR include cetuximab and panitumumab. The results showed that anti-EGFR antibodies did not provide further benefits for patients with advanced GC receiving chemotherapy as first-line treatment (EXPAND) (NCT00678535)[134]. Panitumumab was used as first-line treatment in a clinical phase I/II trial (NCT01716546) in association with 5-FU, cisplatin, and docetaxel for locally advanced or metastatic GC. However, this study did not reach its primary endpoint because in an intermediate analysis, the number of responses obtained was lower than the prefixed limit[135]. Nimotuzumab is the first EGFR humanized monoclonal antibody that binds with high specificity to the extracellular region of EGFR. Two clinical trials have been concluded. The phase III study (NCT01813253) was performed to evaluate the OS in advanced GC patients with EGFR overexpression who were treated with nimotuzumab in combination with irinotecan and compared to a group of patients who received only irinotecan. This study, completed in 2018, has not yet had its results reported[136]. The second study (NCT02370849) evaluated the efficacy of cisplatin and S-1 with and without nimotuzumab in patients with advanced GC who were not previously treated[137]. The combination of nimotuzumab and S-1-cisplatin provided no additional benefit compared to chemotherapy alone in the first-line treatment of unresectable or metastatic GC[138]

mTOR/PI3K/AKT signaling pathway

Everolimus (RAD001) is an mTOR inhibitor with antitumor activity. In a phase I clinical trial, RAD001 was used in combination with capecitabine in patients with refractory GC; the clinical benefits were modest[139]. In phase I clinical trials (NCT01049620) and (NCT01042782), RAD001 was used in combination with capecitabine and oxaliplatin and with mitomycin C, respectively, in patients with advanced GC; the results of these trials are unknown[140,141]. In a multicenter phase II study (NCT00519324), RAD001 was used in patients with metastatic GC with previous chemotherapy failure; particularly, 10.1 mo was the median OS for which the monotherapy with everolimus, in patients in which the previous chemotherapy had failed, showed a satisfying disease control rate[142]. Another clinical trial (NCT00729482) evaluated the efficacy of RAD001 as a monotherapy in patients with advanced GC in whom standard first-line treatment had failed. In addition to the efficacy of RAD001, the expression of markers was evaluated in order to identify biomarkers of response to therapy. Tumors that did not have mTOR pathway activation did not benefit from treatment with RAD001[143]. The median OS was lower than that reported in the study conducted by Doi *et al*[142]. The results of this study showed that the efficacy of RAD001 was unsatisfactory compared to conventional treatment for advanced GC[143]. In the phase III GRANITE-1 study (NCT00879333), the median OS in patients treated with RAD001 *vs* placebo was 5.4 *vs* 4.3 mo. Compared to best supportive therapy (referred to as BSC in Table 1), RAD001 did not significantly improve OS in patients with advanced GC who were previously administered one or two lines of systemic chemotherapy[144]. A clinical trial (NCT01613950) was performed to investigate the efficacy of the combination of

alpelisib (BYL719), a potent and selective inhibitor of mutated *PI3KCA* and AUY922, an inhibitor of heat shock protein 90 (HSP90), in patients with advanced GC with *PIK3CA* mutations and/or amplification of *HER2*, respectively. The results are not yet known[145]. Ipatasertib (GDC-0068), an inhibitor of serine/threonine kinase (AKT), has been used in combination with 5-FU, folinic acid, and oxaliplatin (mFOLFOX6) in advanced or metastatic GC in a multicenter placebo-controlled clinical trial (NCT01896531). The trial is ongoing[146].

Hepatocyte growth factor/MET signaling pathway

High MET expression has been observed in intestinal GC rather than in the diffuse type and in advanced stage disease[147]. MET positivity is a prognostic factor for OS in GC[148]. Patients with GC and MET expression can benefit from anti-MET drugs. Rilotumumab is a hepatocyte growth factor (HGF) monoclonal antibody that blocks binding between HGF and its receptor MET. The efficacy of first-line rilotumumab in patients with GC in combination with ECX was demonstrated in a phase Ib/II clinical study (NCT00719550). The group of patients who received ECX plus rilotumumab showed a better prognosis than placebo[149]. The RILOMET-1 clinical trial (NCT01697072) evaluated the efficacy of rilotumumab in combination with epirubicin, cisplatin, and capecitabine. Regarding OS, the addition of rilotumumab to chemotherapy did not bring about benefits compared to chemotherapy alone in MET-positive patients[150], unlike the phase II study in which OS was 10.6 *vs* 5.7 mo in MET-positive patients who received rilotumumab compared to the placebo group[149]. The multicenter phase III clinical trial, RILOMET-2 (NCT02137343), in which patients with advanced GC were treated first-line with rilotumumab plus cisplatin and capecitabine, was closed for a review of the safety of the study[151]. The randomized, multicenter study (NCT01662869) evaluated the efficacy of onartuzumab (monoclonal anti-MET antibody) in combination with mFOLFOX6 in patients with metastatic HER2-negative and MET-positive GC. Onartuzumab did not yield satisfactory results in combination with FOLFOX[152].

VEGF/VEGFR signaling pathway

Antibodies against VEGF and VEGFR have shown anti-tumor effects in combination with chemotherapy as first and second-line treatments for GC. Bevacizumab, a humanized monoclonal antibody against VEGF, inhibits the VEGF/VEGFR signaling pathway[153]. A phase II study (NCT00447330) was performed in patients with metastatic GC in combination with capecitabine and oxaliplatin; an OS of 7.2 and 10.8 mo was demonstrated in the two groups of patients treated with chemotherapy alone and with the combination with bevacizumab, respectively[154]. Ramucirumab is a humanized monoclonal antibody specific for VEGFR2. By blocking downstream VEGFR2 signaling, ramucirumab provides antitumor effects both as a single agent (REGARD trial, NCT00917384)[72] and in combination with paclitaxel (RAINBOW trial, NCT01170663)[73] in patients with metastatic refractory GC. The median OS was significantly longer in the group of patients treated with ramucirumab plus paclitaxel (9.6 mo) compared to those treated with paclitaxel plus placebo (7.4 mo). Thus, ramucirumab may be a new second-line treatment for patients with metastatic GC. The addition of ramucirumab to FOLFOX (leucovorin, 5-FU, oxaliplatin) did not improve OS in patients with advanced GC[155]. In a phase III study (NCT01512745), apatinib *vs* placebo was used in patients with advanced/metastatic GC who failed two lines of chemotherapy. The median OS was 6.5 *vs* 4.7 mo[156]. Other studies with apatinib have been started but have not yet been completed regarding the use of apatinib alone as first-line maintenance treatment in patients with advanced GC (NCT03255811)[157] and as maintenance treatment with capecitabine (NCT03598348) after first-line chemotherapy[158]. The clinical trial (NCT03104283) assessed the efficacy and safety of apatinib as monotherapy in elderly advanced GC patients, and determined the relationship between VEGFR2 expression and efficacy of apatinib treatment[159]. In a retrospective study, the efficacy of the association of apatinib with docetaxel *vs* apatinib as monotherapy as a second- or third-line treatment in advanced GC was evaluated. The median OS was 3.3 *vs* 6.0 mo in patients with apatinib monotherapy and those with apatinib and docetaxel combination, respectively. Patients with advanced GC benefited more with the combination of apatinib and docetaxel than with apatinib monotherapy[160].

TP53 signaling pathway

A pharmacogenomic study (NCT01470404) was performed to evaluate the effects of germline polymorphisms in xenobiotic metabolism genes on the toxicity profile, and the role of germline polymorphisms of genes involved in DNA repair and the *TP53* tumor suppressor to predict disease recurrence and survival in GC patients treated with adjuvant chemotherapy[161]. *TP53* mutations represent a very attractive target for

cancer therapy. One of the objectives being pursued is to identify molecules that can restore the function of wild-type TP53. Among these, APR-246 was identified[162], and has already been tested in mouse models of cancer[163-165] and in phase I/II clinical trials on hematological and prostate malignancies[166]. A phase I/II study (NCT02999893) was prepared for the treatment of gastroesophageal tumors with mutated *TP53*[167]. Because *TP53* mutations are still somewhat difficult to address adequately, identifying TP53-dependent targets may provide new opportunities for alternative targeted therapies. For example, targeting WEE1 G2 checkpoint kinase (WEE1), a protein kinase that plays a role in the G2-M cell cycle checkpoint, prevents cells from entering mitosis in response to DNA damage[168]. AZD1775, an inhibitor of WEE1, was used in the clinical trial (NCT02448329) as second-line therapy in combination with paclitaxel in GC harboring *TP53* mutations[169]. Overexpression of AURKA improves the stabilization of MDM2 and promotes the degradation of TP53, inhibiting its proapoptotic function in response to chemotherapy[79]. This result justifies the use of AURKA inhibitors in the treatment of GC. The TP53/MDM2 interaction inhibitor (HDM201) was used in the clinical trial (NCT02143635) in patients with advanced GC characterized by wild-type TP53; this study is ongoing[170]. No clinical trial has been performed on the use of MMP inhibitors in GC.

PARP signaling pathway

In response to DNA damage, sensors and effectors are activated that induce cell cycle arrest, damage repair, and eventually cell apoptosis. PARP inhibitors act by preventing breakage of the single DNA strand and induce tumor cell death[171]. *In vitro*, gastric carcinoma cell lines, particularly those in which the ATM serine/threonine kinase expression levels are low, were sensitive to the action of olaparib (PARP inhibitor)[172]. In a phase II study, the efficacy of olaparib (AZD-221) plus paclitaxel was evaluated *vs* paclitaxel in patients with recurrent or metastatic GC whose ATM expression levels were low or undetectable (Study 39; NCT01063517)[173]. The combination of olaparib plus paclitaxel significantly improved OS compared to placebo/paclitaxel, both in the general population and in the population with low ATM levels (13.1 *vs* 8.3 mo)[174]. A multicenter phase III trial has evaluated the efficacy of olaparib in combination with paclitaxel *vs* placebo plus paclitaxel in patients with advanced GC who are progressing after first-line treatment (GOLD, NCT01924533). The OS did not differ between treatment groups in the overall population (median OS 8.8 mo in the olaparib group *vs* 6.9 mo in the placebo group or the negative ATM population (12.0 mo *vs* 10.0 mo)[173].

The GOLD study did not achieve its primary objective to show a significant improvement in OS with olaparib in the overall or ATM-negative population of patients with advanced GC. However, the study provided data on efficacy and safety related to the use of olaparib in combination with a chemotherapy drug and the study itself is foundational for other studies in this type of patients[175]. The GOLD trial has been negative for its endpoints of improved OS, both in overall patient population and the ATM-negative population. The differences between the GOLD trial and Study 39 are the enriched population of ATM-negative patients in Study 39 (51% *vs* 19%) with respect to the GOLD study. PARP inhibitors are effective in tumors with a definite molecular signature, so it may not be realistic to expect efficacy from olaparib in an unselected marker population[176].

Furthermore, the time of exposure to olaparib was shorter in the GOLD trial than in Study 39[177]. A phase I/II pilot study was prepared to analyze the efficacy of olaparib in combination with ramucirumab in patients with metastatic, recurrent or unresectable GC (NCT03008278). The study is currently recruiting[178]. Another phase I clinical trial (NCT01123876) studied the combination of veliparib (PARP inhibitor) with FOLFIRI in patients with advanced solid tumors, including GC[179]. The antitumor activity of veliparib in combination with FOLFIRI and the acceptable safety profile lay the foundation for further studies.

Immune checkpoint signaling pathway

Pembrolizumab is the first immune checkpoint inhibitor approved by the United States Food and Drug Administration (FDA) for the treatment of advanced or metastatic GC[180]. In a multicenter phase II trial (KEYNOTE-059, NCT02335411), the efficacy of pembrolizumab alone was demonstrated in patients with advanced GC who had previously been treated[181]. Treatment with pembrolizumab showed a higher overall response rate in PD-L1-positive patients than in PD-L1-negative patients. Furthermore, in MSI-high patients, the response was higher than that in non-MSI high patients. These results suggest that PD-L1 and MSI levels may be predictive biomarkers of pembrolizumab efficacy[182]. In the trial KEYNOTE-061 (NCT02370498), which was performed in patients with advanced PD-L1-positive GC, the efficacy of second-line treatment of pembrolizumab *vs* paclitaxel was compared. Pembrolizumab

did not significantly improve OS compared to paclitaxel but had a better safety profile than paclitaxel[183]. Pembrolizumab was used as first-line monotherapy or in combination with cisplatin, 5-FU, or capecitabine in patients with advanced PD-L1-positive GC (KEYNOTE-062, NCT02494583); the results of this study are not yet known[184]. In the clinical trial KEYNOTE-859 (NCT03675737), the efficacy of pembrolizumab in association with chemotherapy with cisplatin and 5-FU or oxaliplatin and capecitabine, in advanced/metastatic HER2-negative GC expressing PD-L1, will be evaluated[185]. Another clinical trial (NCT02954536) is evaluating the first-line efficacy of the combination of pembrolizumab and trastuzumab in combination with chemotherapy in patients with HER2-positive metastatic GC. Preliminary results have been obtained on the safety and efficacy of the treatment. Resistance phenomena have occurred because mutations of *TP53* (63%) and *KRAS* (16%) and loss of *ERBB2* amplification in disease progression have been observed[186]. Nivolumab is a monoclonal antibody that targets PD-1 and has received FDA approval for neoplastic pathologies. In a clinical trial (NCT02267343) performed in patients with locally advanced or metastatic GC refractory to chemotherapy, nivolumab was effective with improvement of the median OS[187]. In another clinical trial (NCT02746796), the efficacy of nivolumab in combination with chemotherapy as first-line treatment was tested in patients with advanced or recurrent non-resectable GC[188]. In the clinical trial CheckMate-032 (NCT01928394), performed on solid tumors including GC, the efficacy of nivolumab in combination with ipilimumab, an anti-cytotoxic T-lymphocyte associated protein 4 antibody (*CTLA4*), was evaluated. Nivolumab with ipilimumab demonstrated encouraging long-term OS in patients with GC refractory to chemotherapy[189]. In the clinical trial CheckMate-649 (NCT02872116), the efficacy of nivolumab as first-line treatment in combination with ipilimumab *vs* nivolumab plus chemotherapy *vs* chemotherapy alone, is being evaluated in patients with advanced or metastatic GC[190]. The clinical trial (NCT03777657) is evaluating the efficacy of tislelizumab, a humanized anti-PD1, in combination with oxaliplatin and capecitabine or 5-FU and cisplatin[191].

Other targets

Clinical trials have been performed to study the significance of CTCs in advanced/metastatic GC. Some trials (NCT03156777[192], NCT01625702)[193] have been designed to evaluate CTCs as markers of prognosis and response to chemotherapy. In a clinical trial (NCT01625702) in HER2-positive patients, an increased HER2 extracellular domain was a predictor of a better prognosis. The elevated levels of HER2 after therapy were correlated with a negative therapeutic response[194]. Other trials have been designed to evaluate CTCs and cell-free DNA as clinical prognosis markers (NCT01299688)[195] and response to HER2 (NCT02610218)[196] or VEGFR (NCT02048540)[197] targeting. Only one clinical trial (NCT01848015) was designed to establish the predictive value of CTCs in the recurrence of advanced GC after radical resection[198]. Some of these trials have been completed, but the results are not yet known. A study conducted in patients with HER2-positive metastatic GC revealed that the ctDNA of these patients provided useful information for monitoring the response to trastuzumab, for the purpose of developing therapeutic strategies for HER2-positive but trastuzumab-resistant patients[199]. A phase III clinical study (NCT01178944) was performed to determine if miR-215-5p levels could be predictive of the response to pralatrexate (a folate analog metabolic inhibitor) in association with oxaliplatin in patients with non-resectable GC[200]. Another study (NCT03253107) was conducted to determine if miRNAs levels may be predictive biological markers for the response to chemotherapy[201]. The results of these trials are not yet known. A study (NCT03057171) is ongoing on the control of *H. pylori* on the expression of lncRNAs in gastrointestinal diseases including GC[202].

CONCLUSION

GC is the fifth most malignant tumor worldwide and the third leading cause of cancer-related deaths[7]. Unfortunately, the disease becomes symptomatic in the advanced stage. GC is a complex disease whose onset is linked to a series of environmental and genetic factors[1-6]. Despite the increasing knowledge and progress in drug development, due to late diagnosis and extreme intra- and inter-tumor heterogeneity, the prognosis of GC patients is poor. The heterogeneity of GC is mainly linked to genetic and epigenetic alterations, but also interactions with the microenvironment and the presence of intratumoral cellular clones. Hence, there are variations between patients and within the same tumor. The new classifications, TCGA and ACRG, based on molecular profiles and complementary to those based on

pathological characteristics[15], have highlighted four GC subtypes, each characterized by specific genetic alterations[29]. The molecular classification of GC has helped to identify molecular alterations that may be targeted by the therapy. Furthermore, the molecular profiles of GCs obtained from individual patients has provided new opportunities to identify biomarkers that may predict the tumor response to treatment[22-24]. Unfortunately, even today, the molecular characteristics of tumors are not taken into significant consideration in the management of patients.

H. pylori is responsible for the onset of peptic ulcers and 80% of GC cases. Eradication of the *H. pylori* infection treats gastritis and peptic ulcers and is a mean to prevent GC. Obviously, for the treatment of the eradication of the *H. pylori*, guidelines have been issued by three separate authoritative groups[203-205] but none overcome the problem of resistance. Fallone *et al*[206] recently revised the guidelines to arrive at the best treatment options; however, GC still develops after the eradication. Many Japanese investigators have reported that the presence of severe atrophy after eradication represents a risk factor for the development of GC[207]. Hence, there is a need for specific endoscopic surveillance programs for this type of patients.

Endoscopy plays an important role in the diagnosis of GC[208]. More than 90% of GC cases are reportedly revealed by biopsy-associated endoscopy. The increased use of endoscopy, thanks also to the revolutionary developments that have occurred recently and that have produced new, more sophisticated systems, has allowed highlighting of the "early" GC[209,210]. Ultrasonographic endoscopy is useful for TNM staging of GC patients, having a high diagnostic value. This technique allows the patient to be managed for the most appropriate treatment, limiting the occurrence of unnecessary exploratory surgical procedures[211]. Endoscopy can also be curative for early GC or used as palliative care for more advanced cases. In early GC, the endoscopic mucosal resection provides similar effects as traditional surgical resection[212,213].

Surgical resection with adjuvant or neoadjuvant radiotherapy and chemotherapy with cisplatin, 5-FU, taxane, or irinotecan, remains the most effective treatment for advanced GC. The recent MRC MAGIC/UK study (ISRCTN93793971) showed that perioperative ECF/ECX chemotherapy led to an improvement in OS and PFS in patients with resectable GC[214]. Perioperative chemotherapy is the standard of care in most of Europe for localized GC with accepted ECF or ECX regimens[215]. However, objective response rates to chemotherapy range from 20% to 40%, indicating variable clinical responses that are mostly likely caused by the biologic heterogeneity of the tumor. As with chemotherapy, therapeutic regimens based on targeted therapy have recently been introduced, which makes use of small molecules or monoclonal antibodies that can act on specific molecules capable of modifying molecular pathways involved in proliferation, differentiation, and cell invasion.

Based on phase III clinical trials in patients with advanced/metastatic GC, trastuzumab (anti-HER2) and ramucirumab (anti-VEGFR2) have been approved as first- and second-line therapies in these patients[72-74,124]. However, the survival of patients receiving these therapies is not very high, and with the exception of HER2, there are no markers that can be used to evaluate the response to therapy. Data from preclinical studies have shown a relationship between HER2 overexpression and activation of angiogenesis in breast cancer cells[216]. A retrospective study showed significant efficacy of the combination of a biological therapy with ramucirumab with a chemotherapeutic (paclitaxel) in patients in whom trastuzumab therapy had failed[217]. This study has demonstrated the crosstalk between HER2 signaling and angiogenesis in GC, which can explain tumor survival. Therefore, trastuzumab resistance could be overcome by inhibiting the angiogenic pathway. An analysis of subgroups extrapolated from the RAINBOW study showed that patients who had already been treated with trastuzumab benefited from treatment with ramucirumab in combination with paclitaxel[218]. New studies will be needed to evaluate the efficacy of sequential blockades of both pathways to improve the survival of patients with GC.

Other monoclonal antibodies, such as cetuximab and panitumumab (anti-EGFR), have also been tested in advanced/metastatic GC but the results on survival rates have not been encouraging[134-135]. Unacceptable results were obtained with RAD001, an mTOR inhibitor. The efficacy of RAD001 is unsatisfactory compared to conventional treatment for advanced GCs[139,143]. Anti-MET monoclonal antibodies, such as rilotumumab and onartuzumab, in combination with chemotherapy, did not bring benefits compared to chemotherapy alone[149-152]. A study was prepared to more effectively target MET with a mixture of two humanized monoclonal antibodies that target two non-overlapping MET epitopes. The results represent efficacy data demonstrated on preclinical models and are part of a clinical trial (NCT02648724)[219] carried out on patients with NSLC and MET amplification[220]. The advantage of antibody mixtures is their ability to orchestrate the internalization of the receptor and its degradation more effectively than a single monoclonal antibody, as previously

shown for the EGFR family[221].

PARP inhibitors are very effective in the treatment of ovarian and breast tumors in which DNA repair systems are altered and BRCA1/2 mutations are present, which makes them more sensitive to these inhibitors[222]. New biomarkers are being explored, which go beyond BRCA1/2 mutations and DNA repair mechanism deficits to stratify sensitive patients, new combinations of PARP inhibitors, and/or combinations with checkpoint inhibitors to determine who will be eligible for this treatment for other solid tumors, including GC[223]. It has been hypothesized that the inhibition of PARP may trigger mechanisms based on the recognition of new tumor cell antigens by the immune system, making the PARP inhibitors potential partners for combination with immune checkpoint inhibitors.

Recently, patients with GC have also been studied from an immunotherapy viewpoint. Pembrolizumab and nivolumab received FDA approval for GC[180]. Anti-PD1 antibodies have been used in phase II and phase III clinical trials and appear to be promising, especially in patients overexpressing PD-L1. Further clinical trials are underway to evaluate the efficacy of these antibodies in association with chemotherapy. At the same time, other pathways such as the TP53 signaling pathway, are being studied to identify inhibitory molecules[162-166]. Strategic opportunities can also be provided by studying the potential of biomarkers such as CTCs, ctDNA, miRNAs, and lncRNAs to predict response to therapy and resistance phenomena.

There is no doubt that targeted therapies allow patients to live longer, whether they are administered alone or in combination with chemotherapy. Today the probability of observing patients who survive several years after the diagnosis of cancer is much higher, thanks to the targeted therapies. The targeted therapies must be provided to groups of patients who can benefit from them, screened on the molecular profiles to which the therapy is effective. Molecular profiling regarding the overexpression and/or mutation of the targets must be carried out on tissue biopsies, both in resectable and unresectable patients, to establish the correct targeted therapy to be used alone or associated with chemotherapy. It is necessary to continue to study the heterogeneity of GC. The fact that GC has genetic variations between different patients and/or in the same patient during its progression and/or during or after therapy (conventional or targeted) should drive investigations into the molecular characteristics present in tumor tissue, and the use of circulating biomarkers to predict and monitor disease progression and response to therapy. Furthermore, the association of several markers should be considered in order to appropriately classify the tumor and to establish therapeutic strategies that increase survival rates.

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MINIREVIEWS

Endoscopic management of esophageal cancer

Osman Ahmed, Jaffer A Ajani, Jeffrey H Lee

ORCID number: Osman Ahmed (0000-0002-0422-9932); Jaffer A Ajani (0000-0001-9946-0629); Jeffrey H Lee (0000-0001-6740-3670).

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Osman Ahmed, Jeffrey H Lee, Department of Gastroenterology, Hepatology and Nutrition, University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

Jaffer A Ajani, Department of Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX 77030, United States

Corresponding author: Jeffrey H Lee, MD, Professor, Department of Gastroenterology, Hepatology and Nutrition, University of Texas MD Anderson Cancer Center, 1515 Holcombe Avenue, Houston, TX 77030, United States. jefflee@mdanderson.org
Telephone: +1-713-5638906
Fax: +1-713-5634408

Abstract

Esophageal cancer (EC) generally consists of squamous cell carcinoma (which arise from squamous epithelium) and adenocarcinoma (which arise from columnar epithelium). Due to the increased recognition of risk factors associated with EC and the development of screening programs, there has been an increase in the diagnosis of early EC. Early EC is amenable to curative therapy by endoscopy, which can be performed by either endoscopic resection or endoscopic ablation. Endoscopic resection consists of either endoscopic mucosal resection (preferred in cases of adenocarcinoma) or endoscopic submucosal dissection (preferred in cases of squamous cell carcinoma). Endoscopic ablation can be performed by either radiofrequency ablation, cryotherapy, argon plasma coagulation or photodynamic therapy, amongst others. Endoscopy can also assist in the management of complications post-esophageal surgery, such as anastomotic leaks and perforations. Finally, there is a growing role for endoscopy to manage end-of-life palliative symptoms, especially dysphagia. The growing use of esophageal stents, debulking therapy and dilation can assist in improving a patient's quality of life. In this review, we examine the multiple roles of endoscopy in the management of patients with EC.

Key words: Esophageal cancer; Endoscopy; Resection; Ablation; Stent; Barrett's esophagus

Core tip: The endoscopic management of esophageal cancer is continuously evolving. Although, endoscopy was generally reserved for diagnosis, but due to the growing evidence around screening, early cancers are now being detected. Therefore, endoscopy has now grown to include an increasing therapeutic role in esophageal cancer. This

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includes resection by either endoscopic mucosal resection or endoscopic mucosal dissection. Ablative therapies by endoscopy including the use of radiofrequency ablation and photodynamic therapies are also growing. Finally, the role of endoscopy entails palliative management, such as the use of esophageal stent placements.

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INTRODUCTION

Esophageal cancer (EC) is an overarching term generally used to describe two separate malignancies, esophageal squamous cell carcinoma (SCC) and esophageal adenocarcinoma. Esophageal SCCs arise in the squamous epithelium (generally in the mid-proximal esophagus but can occur throughout the esophagus) whereas esophageal adenocarcinomas arise in the columnar epithelium and are generally found in the distal esophagus.

The epidemiology of EC is evolving. Although ECs only make up one percent of all new cancer cases in the United States, they make up 2.6% of all cancer deaths. The overall incidence of all types of EC has remained steady over the past two decades with an estimated 17000 new cases annually in the United States. The 5-year survival rate of EC varies according to how advanced the tumor is at diagnosis. Those with localized disease have a 5-year survival rate of 45.2%, while those with distant metastases have a 5-year survival rate of only 4.8%[1,2].

Due to the aggressive nature of the disease and the high mortality rate, it is imperative to identify patients early in the course of the disease. Currently, only 19% of cases are staged as localized disease at diagnosis. The benefit of localized disease is that it opens up a whole array of treatment options including the use of endoscopic therapy. The increasing recognition of risk factors associated with metaplasia and dysplasia has led to an increased interest in screening and surveillance programs. For example, the role of gender, obesity and gastroesophageal reflux disease in the development of Barrett's esophagus (BE) has allowed for the development of screening and surveillance guidelines, which has then lead to treatment guidelines for pre-cancerous and early cancerous lesions[3,4].

Although endoscopy was initially limited to the diagnosis of EC, recent advancements have allowed the modality to play a growing role in the management of the tumor. The development of advance camera technology has allowed better recognition of the disease, while simultaneously the introduction of novel endoscopic techniques and instruments has allowed endoscopists to treat pre-cancerous lesions and even early ECs.

In this review, the current indications for endoscopy in the management of EC are reviewed. The pre-endoscopic management work-up, endoscopic options for curative therapy, the role of endoscopy in managing complications of surgery as well as how endoscopy can play an essential part in the palliative management of EC are described.

PRE-ENDOSCOPIC MANAGEMENT INVESTIGATIONS

The first step in performing endoscopic management in patients with EC is to recognize the setting where it is appropriate. Multiple studies have demonstrated improved outcomes and less complications in high-volume centers, and therefore consideration should be given to referring patients to centers with experience when endoscopic curative management is an option[5,6]. Certain guidelines recommend that endoscopic resection (ER) of early EC only be done in high-volume centers. Similarly, a multi-disciplinary approach, with involvement of surgery, oncology and pathology is critical as the diagnosis of dysplasia can be controversial with poor intra- and inter-observer agreement[8]. A second opinion, ideally from a gastrointestinal pathologist, should be sought if there are doubts about the presence of dysplasia. Additionally, a multi-disciplinary approach will allow for more flexibility and options for the patients and assist in managing any potential complications.

Once a patient has been referred for endoscopic management of pre-cancerous lesions or early EC, it is vital to establish the stage and characteristics of the tumor. This is done through a combination of endoscopic investigations, as well as potentially other modalities to ensure the tumor has not progressed. In terms of endoscopy, careful examination of the lesion is essential prior to any decision regarding endoscopic therapy. After washing the esophagus to remove any food, liquid or debris, careful examination of affected areas with white-light endoscopy should be performed. Recent studies have demonstrated that high-definition endoscopy is superior to standard definition in assessing mucosal changes in patients with BE (Figure 1)[9].

In addition, although there has been an increase in the use of adjuncts to white-light imaging, their evidence in the diagnosis of EC is still controversial with the exception of narrow-band imaging (NBI). NBI is a technique that allows increased highlighting of mucosa and the mucosal vasculature (Figure 2). A meta-analysis on the use of NBI to identify high-grade dysplasia (HGD) in patients with BE demonstrated a pooled sensitivity of 0.96 (95% confidence interval: 0.93-0.99) and a pooled specificity of 0.94 (95% confidence interval: 0.84-1.0). The meta-analysis included eight studies with 446 patients and a total of 2194 lesions. Based on these studies, there has been an increasing use of NBI to identify high-risk lesions[].

Similar to NBI, there has been extensive investigation into the use of chromoendoscopy. Chromoendoscopy is the use of selective dyes to highlight specific features on the mucosa and potentially increase the contrast between normal mucosa and abnormal mucosa. The most commonly used dye in chromoendoscopy is methylene blue, which is thought to selectively stain intestinal metaplasia. A previous randomized control trial on the use of methylene blue as compared to random 4-quadrant biopsies showed that although there was no increased detection of dysplasia, the use of methylene blue led to a smaller requirement for the number of biopsies[11]. On the other hand, a separate randomized control trial showed that methylene blue detected less dysplasia compared to random 4-quadrant biopsies[]. Finally, a systematic review and meta-analysis was performed in 2009 and included nine studies with a total of 450 patients. The study demonstrated no incremental yield in the use of chromoendoscopy as compared to standard 4-quadrant biopsies[]. Subsequently, current guidelines do not recommend the routine use of chromoendoscopy when assessing esophageal lesions for advanced or high-risk features.

When inspecting a lesion with white-light endoscopy or NBI, there are certain features that should be carefully sought for in the mucosa as they will likely change therapy. When examining BE, it is important to document landmarks including any potential hiatal hernia, the location of the gastroesophageal junction, the top of the gastric folds, the location of the squamo-columnar junction and the length of columnar mucosa both circumferentially and the maximal longitudinal length. One commonly used classification for reporting BE is the Prague classification, which documents circumferential and maximal longitudinal length and has been found to have high validity and inter-observer agreement[14,15].

In addition, it is critical to document any nodularity found and the location of the nodularity as it will likely require separate management from the remainder of the BE. Nodules are also suggestive of advanced lesions requiring therapy. In addition to nodules, other high-risk features that portend to malignancy include the presence of ulceration or structuring[16]. Careful examination should be done in the 12 o'clock to 6 o'clock (or the right hemisphere) as these have higher rates of EC in BE[17].

Although a careful examination of a lesion using white-light endoscopy is the gold standard, there have been previous studies looking into adjunctive methods to determine resectability. One potential option was the use of endoscopic ultrasound (EUS). EUS would allow the clinician to determine the depth of the lesion as well as any potential locoregional lymph nodes. Initially, the thought was that EUS could provide the ability to determine whether any invasive cancer was present and therefore assist in determining which lesions endoscopic therapy should be avoided. Although initial results were promising, they have not been followed up by similar outcomes in subsequent studies[18].

A systematic review and meta-analysis examining the role of EUS found that EUS only had a 65% concordance for T-staging when compared to surgical or endoscopic mucosal resection (EMR) based pathology[19]. A follow-up meta-analysis found better results but was limited due to the heterogeneity between studies[]. A more recent study examined the same utility of EUS in pre-malignant lesions and found poor correlation with a sensitivity of 50% and a specificity of 93%[21,22]. Interestingly, previous studies have found that EUS-guided mini-probe based examinations have better sensitivity than radial echoendoscopes[]. Due to all the previous studies, the use of EUS to determine resectability is limited and should not be done to guide

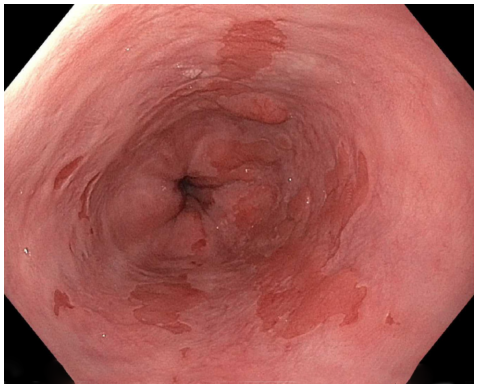

Figure 1 Barrett's esophagus with nodularity.

management decisions in patients with pre-malignant lesions.

Nevertheless, EUS can provide a helpful role in patients with early EC. Although EUS has difficulty staging cancers, it can be a useful tool in both identifying and sampling lymph nodes (Figure 3). EUS generally has been found to over-stage T2 malignancies and therefore caution should be taken before labelling a lesion as unresectable[24]. When it comes to lymph nodes, EUS was found to have fairly high sensitivity and specificity as compared to positive electron-transmission scans and has the added benefit of being able to sample nodes through a fine needle aspiration or biopsy[25,26].

In general, when approaching a patient for potential endoscopic management, it is important to ensure that care is provided in a center with expertise not only in endoscopy but also in surgery, pathology and radiology. The most important investigation is a careful examination during upper endoscopy both with white light endoscopy and NBI. Although adjunctive investigations have so far not yielded fruit, consideration can be given to performing EUS if there is concern for locoregional invasion.

When it comes to the endoscopic management of EC, it can generally be divided into two categories, curative and palliative therapy. Curative therapy is generally reserved for early ECs limited to the mucosa with no lymph node involvement. In this section, we will review the common methods for endoscopic management, as well as upcoming frontiers.

ER

ER is the mainstay of endoscopic management of early ECs. ER can be performed in two ways, by EMR or by endoscopic submucosal dissection (ESD). ER can be performed for both adenocarcinomas and SCCs. In adenocarcinoma patients, the spectrum of disease where ER can be performed generally includes pre-malignant low-grade dysplasia in a patient with BE up to in some cases stage T1b adenocarcinoma (as per the TNM staging of tumors). For SCCs, ER can be performed in patients with early EC that is staged as T1 or intramucosal.

EMR is generally performed by two distinct methods: the cap-assisted method and the ligation-assisted method. The cap-assisted method, also known as the "suck and cut" method involves suctioning the mucosa into a cap-fitted endoscope and then using a snare to cut the mucosa. The snare is pre-opened prior to suctioning and generally comes as part of a pre-assembled ensemble kit. In the ligation-assisted method, or multi-band ligator method, the upper endoscope is fitted with an apparatus similar to a variceal band ligator, and the mucosa is suctioned and has a band placed around it. Subsequently, a snare is passed, and the mucosa upheld by the band is resected (Figure 4).

The evidence comparing the two methods of EMR showed that they are generally comparable. In a randomized control trial comparing the techniques, the ligation-assisted method was shown to be quicker with smaller resection specimens compared to the cap-assisted method. However, both techniques had similar maximal thickness in their resection specimens and similar adverse event rates (Figure 5)[27]. Previous studies that compared the two techniques in a non-randomized manner also demonstrated similar results[28,29]. The use of the lifting and then direct snare technique that is commonly used in the colon is discouraged in the esophagus due to an increased risk of perforation[30].

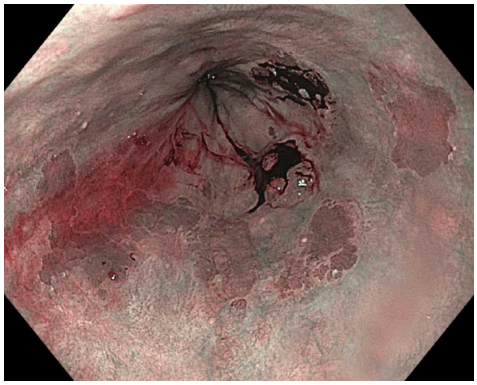

Figure 2 Narrow-band imaging of Barrett's esophagus.

ESD is a more recent technique that involves careful dissection of the submucosa of the lesion in systematic fashion followed by en bloc removal of the desired tissue. Although the benefit is that it provides en bloc specimen and can give information about the margins of resection, the disadvantage is that it is time consuming and requires a deeper resection potentially leading to increase adverse events. Indeed, in a systematic review and meta-analysis comprising of 15 non-randomized trials comparing ESD to EMR, they found that although ESD had higher curative resection rates and lesser local recurrence rates, it was balanced by more time-consuming procedures and higher rates of bleeding and perforation[]. Another meta-analysis looking specifically at esophageal neoplasms found no difference between EMR and ESD in terms of margins, lymph node positivity or metachronous cancers but found less recurrence with ESD though balanced by an increased risk of strictures[32].

The one situation where ESD has had positive results (as compared to EMR) is in the setting of SCCs. A previous study examining resection techniques found less recurrence when en bloc resection was performed by ESD in patients with SCC as compared to patients that had piecemeal resection[33]. Based on this study, EMR is generally considered sufficient for small lesions (less than 10 mm) if the diagnosis is SCC, but patients with larger lesions should ideally undergo ESD. Overall, current guidelines recommend EMR for resection of BE or early esophageal adenocarcinomas unless the lesions are larger than 15 mm, are poorly lifting or are at risk for submucosal invasion in which case ESD should be performed. For patients with SCC, current guidelines generally recommend ESD though EMR is acceptable in smaller lesions[34].

ENDOSCOPIC ABLATION

Ablative therapy is generally reserved for flat lesions or treatment of BE after ER. There are many ways to perform ablative therapy with the most common being radiofrequency ablation (RFA) (Figure 6). Other methods that are less commonly used include photodynamic therapy (PDT) and cryoablation. The main purpose of ablative therapy is to destroy the remaining residual malignant or pre-malignant tissue to prevent recurrence.

RFA is the application of thermal energy that is generated by radiofrequency waves to destroy tissue. It involves contact ablation and can be done in localized areas or in a circumferential manner. The seminal study examining the effects of RFA was published in 2009. It was a multi-center randomized control trial that compared RFA to sham therapy in patients with dysplastic BE. The primary outcome (complete eradication) was followed until 12 mo post-therapy. In the RFA group, when using intention-to-treat analysis, 90.5% of patients had complete eradication whereas in the sham group only 22.7% had eradication. The main adverse event related to RFA was the development of chest pain post-treatment[35]. Similar results have been shown in the other multi-center studies including European and Asian populations[36,37].

The role of endoscopic therapy in patients with low-grade dysplasia has been controversial, and there has been debate on whether to pursue endoscopic management or only perform careful observation. A previous study examining patients with BE with only low-grade dysplasia found a decrease in the progression of the dysplasia and the development of cancer with the use of RFA[38]. Finally, there have been studies on whether RFA should be applied to patients with BE but no evidence of dysplasia. A study looking at the cost-effectiveness of RFA therapy found

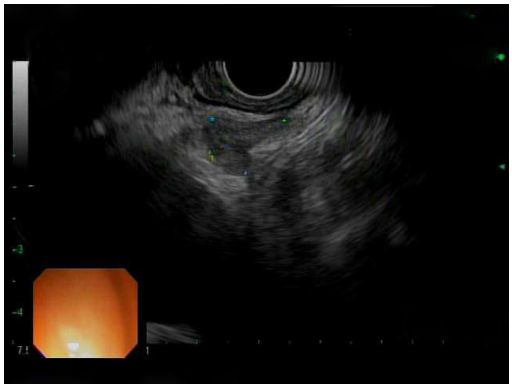

Figure 3 Endoscopic ultrasound image of subcarinal lymph node.

that treatment of patients with BE without dysplasia did not provide cost-effective therapy[39].

Current guidelines generally recommend RFA in patients with dysplasia with non-nodular lesions or intra-mucosal cancer. RFA should also be performed to treat residual BE in patients who have undergone ER. Additionally, although RFA has become well-established in the management of patients with BE or adenocarcinoma, its role in the management of SCC is still developing. Recent studies have showed promise in early SCC with high complete eradication rates and low recurrence rates[40,41].

Other types of ablative therapy include argon plasma coagulation (APC), cryoablation and PDT. APC is widely available, generally due to its use in multiple conditions and diseases and has been widely investigated in the management of BE. In one study examining the role of APC in patients with non-dysplastic BE, complete eradication was successful in 77% of the patients (37/48). The mean number of sessions required was 2.8 (range 1-5) though 9.8% (5/51) had major complications including perforation, hemorrhage and stricture formation[42]. Nevertheless, other studies have showed similar positive results with APC[43,44].

Cryoablation of the esophagus has also been studied in the management of pre-malignant and malignant conditions of the esophagus. The most widely used method is the application of liquid nitrogen therapy. Previous studies have shown high eradication rates in patients with intestinal metaplasia and HGD with minimal adverse events[45]. There have also been long-term retrospective studies to determine the sustained ability of cryotherapy. A 5-year follow-up of patients who received cryotherapy revealed complete eradication rates of 93% in HGD and 75% in intestinal metaplasia. The rate of progression to HGD or adenocarcinoma was 1.4% per patient-year in those treated with cryotherapy[46]. Cryotherapy has also been studied as rescue or salvage therapy in patients who have had recurrence after initial RFA therapy. The complete eradication of dysplasia rate was 75% in those subsequently treated with cryotherapy, including two patients who initially had intramucosal adenocarcinoma and were both successfully treated[47].

PDT is an ablative process in which a photosensitizer drug is activated by the use of laser light, which leads to mucosal destruction. PDT has evidence in the management of both SCC and esophageal adenocarcinomas. Treatment of either cancer staged as either T1 or T2 showed a complete response rate of 87% with the majority of the complications being either cutaneous photosensitization or esophageal strictures[48]. Long-term follow-up has shown sustained response and low rates of recurrence as well[49,50]. Comparisons between PDT and APC in the eradication of both BE and dysplasia have showed similar effectiveness though higher costs associated with PDT[51,52]. A study comparing RFA to PDT in patients with BE with dysplasia found that RFA had higher complete response rates and was significantly less costly. Though caution must be taken in interpreting these results as the study was non-randomized with major differences in the baseline characteristics of the two groups[53].

In summary, there are many methods that have evolved to treat flat mucosal lesions with pre-malignant or malignant findings. RFA is generally the most widespread method with increasing evidence of its utility backed by a strong safety record. The development of circumferential balloons as well as through-the-scope segmental pads has made it more user-friendly. In patients who have failed RFA after multiple attempts, consideration should be given to alternative modalities including APC, cryoablation and potentially PDT based on local expertise.

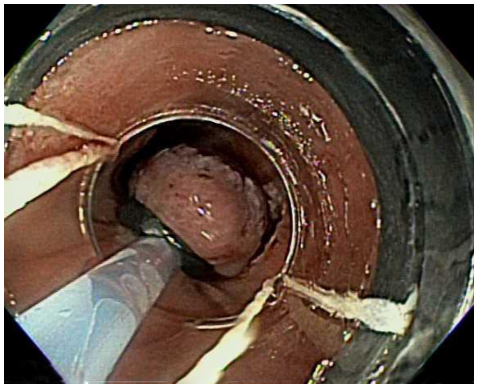

Figure 4 Band-ligation method of endoscopic mucosal resection.

ENDOSCOPIC MANAGEMENT OF POST-OPERATIVE COMPLICATIONS

Although an increasing number of patients are being diagnosed with early EC that is amenable to curative resection by endoscopy, a large proportion still progress to surgery. Depending on the features of the tumor and its aggressiveness, the algorithm of neo-adjuvant therapy followed by surgery is generally followed. Nevertheless, endoscopy can play a central role in patients who develop post-operative complications after surgery for EC. The most common complication is the development of a post-operative leak generally at the anastomosis (Figure 7). The incidence of post-operative complications can be as high as 22.9% of post-esophageal resection cases[54]. The rates of esophageal leaks have been shown to be as high as 7.9% of all esophageal surgeries[55]. Prompt recognition and management of esophageal leaks is imperative as the mortality rate associated with leaks can be as high as 35%[56].

Esophageal stent placement is an alternative to a re-operation for an anastomotic leak. Most commonly, a self-expanding metal stent (SEMS) is placed to overlap the site of the leak and allow it to heal. Although SEMS generally come in varying sizes, consideration should be given to place the largest tolerable diameter to prevent migration of the stent as there likely is no narrowing to hold the stent in place. In our practice, we generally use SEMS with a diameter of 23 mm to treat esophageal anastomotic leaks. The securing of the esophageal stent can be done by a variety of methods, including placing a hemostatic clip between the stent and the mucosa or possibly using an endoscopic suturing device to secure the stent in place. The evidence for the role of esophageal stents in the post-operative setting is variable with studies ranging from a technical success (ability to place the stent) rate between 80% to 100% to a clinical success rate (resolution of the leak and removal of the stent) that can be as low as 45%[57,58]. The most common complications post-stent placement is pain, stent migration and bleeding[59].

Other methods can be considered for anastomotic leaks including endoscopic clip placement to close the defect. The development of over-the-scope clips have allowed larger defects to be closed endoscopically. Multiple trials on the use of endoscopic clip placement have demonstrated high rates of clinical success and closure. A recent large study examined the role of over-the-scope clips in closure of luminal defects. A total of 188 patients were included of which 108 had fistulas, 48 had perforations and 32 had leaks. Successful closure occurred in 90% of patients with perforations, 73% with leaks but only 42.9% of patients with fistulas[60].

PALLIATIVE ENDOSCOPIC MANAGEMENT

Once a patient has advanced disease not amenable to curative therapy, the shift of care turns towards palliative management. The role of endoscopy in palliative care is generally the improvement of symptoms especially dysphagia. As patients focus more on end of life care, the need to ensure the ability to take oral contents becomes a matter of quality of life. The main components of endoscopic management in palliative care are dilation, debulking and esophageal stent placement.

In regard to dilation, the reducing caliber of the esophagus secondary to tumor is the main reason for dysphagia and intermittent periodic dilations are an option to treat the disease (Figure 8). Unfortunately, dilation alone rarely provides long-lasting

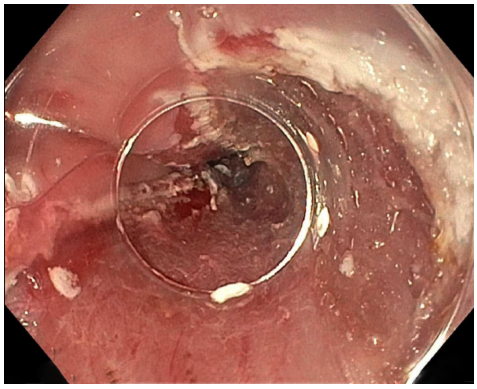

Figure 5 Post-endoscopic mucosal resection.

efficacy, and this is compounded by high rates of complications especially perforations[61]. Endoscopic debulking therapy can be achieved by the use of laser therapy, PDT or chemical therapy. Chemical therapy, including the use of absolute alcohol, generally only provides transient relief and requires multiple ongoing sessions[]. PDT has generally been found to be better than laser therapy as shown in randomized comparison trials that have showed similar efficacy between laser therapy (*e.g*., Nd : YAG) and PDT but less perforations associated with PDT[63].

Finally, the mainstay of esophageal palliation is the placement of esophageal stents. The most common form of esophageal stents are SEMS, and they can come in covered, partially covered and uncovered forms (Figure 9). The evidence for the role of esophageal stents is controversial. Although they have been shown to have durable effectiveness towards dysphagia and lower rates of perforation as compared to dilation alone, they are limited due to patient intolerance of chest pain as well as the risk of stent migration[64].

CONCLUSION

As the epidemiology and presentation of EC evolves, so does the role of endoscopy in its care. No longer relegated to diagnosis only, endoscopy can provide curative therapy in early EC as well as provide therapy for pre-malignant changes. It can also be used to manage complications related to the management of EC specifically post-operative complications. Finally, there is a growing role for endoscopy in the palliative management of EC with an increasing use of debulking therapy as well as the ongoing relief of dysphagia with esophageal stent placements.

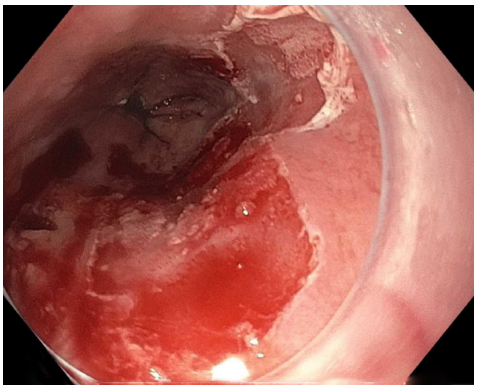

Figure 6 Post-radiofrequency ablation.

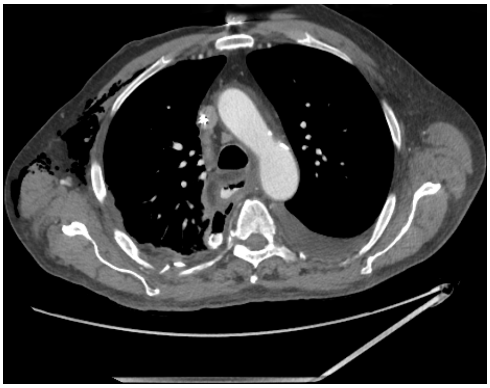

Figure 7 Post-operative anastomotic leak.

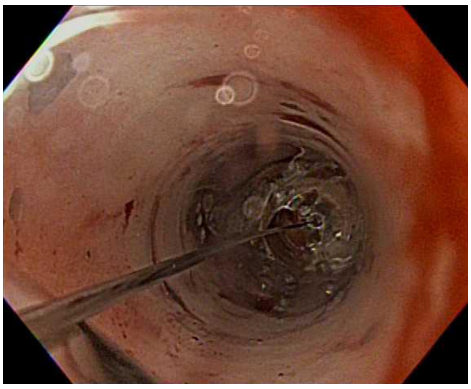

Figure 8 Esophageal balloon dilation.

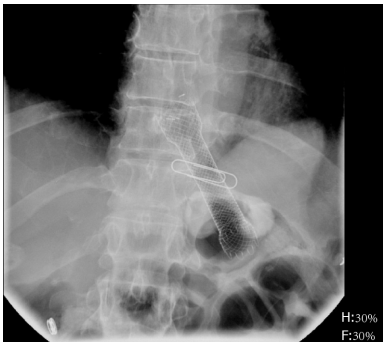

Figure 9 Self-expanding metal stent esophageal stent.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-320a suppresses tumor progression by targeting PBX3 in gastric cancer and is downregulated by DNA methylation

Yong-Shuang Li, Ying Zou, Dong-Qiu Dai

ORCID number: Yong-Shuang Li (0000-0002-0474-853X); Ying Zou (0000-0003-2067-0995); Dong-Qiu Dai (0000-0002-1154-3276).

Author contributions: Li YS and Zou Y contributed equally to this work and should be considered as co-first authors; Li YS and Dai DQ designed the study; Li YS and Zou Y collected and analyzed the data; Li YS and Zou Y performed the experiments; all authors contributed to writing, reviewing or revising the paper; Li YS and Dai DQ submitted the final manuscript and all authors read and approved the final version; all authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Yong-Shuang Li, Ying Zou, Dong-Qiu Dai, Department of Gastrointestinal Surgery, the Fourth Affiliated Hospital of China Medical University, Shenyang 110032, Liaoning Province, China

Corresponding author: Dong-Qiu Dai, MD, PhD, Professor, Department of Gastrointestinal Surgery, The Fourth Affiliated Hospital of China Medical University, No. 4 Chongshan East Road, Shenyang 110032, Liaoning Province, China. daidq63@163.com
Telephone: +86-24-62043110
Fax: +86-24-62043110

Abstract

BACKGROUND

Ectopic expression of miRNAs promotes tumor development and progression. miRNA (miR)-320a is downregulated in many cancers, including gastric cancer (GC). However, the mechanism underlying its downregulation and the role of miR-320a in GC are unknown.

AIM

To determine expression and biological functions of miR-320a in GC and investigate the underlying molecular mechanisms.

METHODS

Quantitative real-time polymerase chain reaction (PCR) was used to determine expression of miR-320a in GC cell lines and tissues. TargetScanHuman7.1, miRDB, and microRNA.org were used to predict the possible targets of miR-320a, and a dual luciferase assay was used to confirm the findings. Western blotting was used to detect the protein levels of pre-B-cell leukemia homeobox 3 (PBX3) in GC cells and tissue samples. Cell Counting Kit-8 proliferation, Transwell, wound healing, and apoptosis assays were performed to analyze the biological functions of miR-320a in GC cells. Methylation-specific PCR was used to analyze the methylation level of the miR-320a promoter CpG islands. 5-Aza-2′-deoxycytidine (5-Aza-CdR) and trichostatin A (TSA) were used to treat GC cells.

RESULTS

miR-320a expression was lower in GC cell lines and tissues than in the normal gastric mucosa cell line GES-1 and matched adjacent normal tissues. miR-320a overexpression suppressed GC cell proliferation, invasion and migration, and induced apoptosis. PBX3 was a target of miR-320a in GC. The methylation level of the miR-320a promoter CpG islands was elevated and this was partly reversed by 5-Aza-CdR and TSA.

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CONCLUSION
miR-320a acts as a tumor suppressor and inhibits malignant behavior of GC cells, partly by targeting PBX3. DNA methylation is an important mechanism associated with low expression of miR-320a.

Key words: Gastric cancer; miRNA-320a; DNA methylation; Pre-B-cell leukemia homeobox 3; Tumor suppressor

Core tip: miRNA (miR)-320a functioned as a tumor suppressor and was downregulated in gastric cancer (GC). miR-320a overexpression suppressed proliferation, migration and invasion, and induced apoptosis through targeting Pre-B-cell leukemia homeobox 3 in GC cells. miR-320a depletion showed the opposite results. The potential mechanism of miR-320a deficiency in GC was the increased methylation level of the miR-320a promoter CpG islands.

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INTRODUCTION

Gastric cancer (GC) is a common malignant tumor. Although the incidence and mortality of GC have substantially decreased over the last decade in most countries worldwide, the prevalence of GC is still ranked fifth and GC mortality is ranked third in the world[1]. The occurrence and development of advanced GC involve a complex process. Efficient methods for early diagnosis and curative treatment for GC are currently lacking. As precision medicine is the future direction in cancer therapy, identifying an early diagnostic or therapeutic biomarker for GC is essential.

MicroRNAs (miRNAs) are short [19–25 nucleotides (nt)] noncoding RNAs that recognize specific target mRNAs and participate in the post-transcriptional regulation of gene expression by promoting degradation and inhibiting translation of mRNAs[2]. It was previously thought that only regulatory proteins encoded by genes could control physiological functions. However, in recent years, researchers have found that miRNAs play an important role in regulating cell differentiation and cell fate decisions[3]. They can act as oncogenes or tumor suppressors according to their target genes[4]. For example, miR-98 is expressed at low levels and acts as a tumor suppressor in hepatocellular carcinoma by targeting Sal-like protein 4, and the highly expressed miR-27a-3p functions as an oncogene in GC by targeting the B-cell translocation gene 2[5,6]. Abnormal expression of miRNAs occurs in many types of cancer, and miRNAs are involved in tumorigenesis and tumor progression[7]. miR-212 is significantly downregulated in GC and is involved in GC development and progression by regulating expression of oncogene *Myc*[8]. miRNAs also play an important role in regulating cancer cell proliferation, invasion, migration and apoptosis[9,10].

The function of miR-320a, located on chromosome 8p21.3, was investigated previously. miR-320a acts as a tumor-suppressive miRNA in many cancers, including blood malignancies and solid tumors. Xishan *et al*[11] showed that miR-320a is downregulated in chronic myeloid leukemia (CML) and inhibits CML cell migration, invasion and proliferation, and promotes apoptosis by targeting the *BCR/ABL* oncogene. miR-320a inhibits multiple myeloma cell proliferation and induces apoptosis by targeting pre-B-cell leukemia homeobox 3 (PBX3)[12]. miR-320a is downregulated in many solid tumors and has important functions. mir-320a plays a tumor-suppressing role in colorectal cancer, nasopharyngeal carcinoma, breast cancer, and bladder carcinoma, and overexpression of miR-320a partly inhibits tumor malignant behavior[13-16]. However, the mechanism underlying the downregulation of these miRNAs is unknown.

Epigenetic regulation plays a crucial role in the development and progression of

tumors, and DNA methylation is an important part of this process. The expression of miRNAs is regulated by DNA methylation, and abnormal DNA hypermethylation can lead to cancer suppressor gene silencing and promotion of tumor progression. Ayala-Ortega *et al*[17] showed that DNA hypermethylation at the miR-181c promoter region results in low miR-181c expression in glioblastoma cell lines compared with normal brain tissues. High methylation levels of the miR-27b promoter region downregulate expression of miR-27b, whereas demethylation restores miR-27b expression in breast cancer[18]. Downregulation of miR-320a is associated with regulation by methylation in breast cancer[19].

miRNAs are expressed in a tissue-specific manner. However, whether miR-320a acts as a tumor suppressor in GC is unknown. Wang *et al*[20] found that the expression of miR-320a was reduced in GC tissues. However, the biological function and epigenetic regulatory mechanism of miR-320a in GC remains unknown.

In this study, we identified the biological role of miR-320a in GC and clarified the relationship between miR-320a expression and DNA methylation. miR-320a expression was reduced in GC cell lines and tissues. miR-320a interacted with the 3′ untranslated region (UTR) of the oncogene *PBX3* in GC. miR-320a overexpression inhibited malignant biological actions in GC cells. These results suggest that miR-320a acted as a tumor suppressor by regulating *PBX3* expression in GC. The promoter CpG islands of miR-320a showed abnormal hypermethylation, and the methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC) partially reversed miR-320a expression. These findings demonstrated that methylation-associated silencing of miR-320a suppressed tumor progression by targeting *PBX3* in GC.

MATERIALS AND METHODS

Clinical GC samples

This study was approved by the Ethics Committee of the Fourth Affiliated Hospital, China Medical University (Shenyang, China). We obtained 84 GC tissues and matched adjacent normal tissues (located > 5 cm from the tumor) from patients who had a diagnosis of GC confirmed by histopathology at the Cancer Research Institute of China Medical University (Shenyang, China) between 2013 and 2014. These patients did not receive chemotherapy before surgical resection, and all tissues were immediately frozen in liquid nitrogen after surgery until DNA or RNA extraction. The basic patient data are listed in Table 1.

Cell types and source and cell culture

We used five cell lines, including one normal gastric mucosa cell line (GES-1) and four GC cell lines (BGC-823, MGC-803, SGC-7901 and MKN-45). All cell lines were purchased from the Institute of Biochemistry and Cell Biology, China Academy of Science (Shanghai, China). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco/BRL, Waltham, MA, USA) at 37°C in 5% CO_2.

Cell transfection

The miR-320a mimics and miR-320a scramble mimics were designed and produced by GenePharma (Shanghai, China) and transfected into MKN-45 cells. The final concentration for transfection was 50 nmol/L. The miR-320a inhibitor and miR-320a scramble inhibitor were designed and produced by GenePharma and transfected into BGC-823 cells. The final concentration for transfection was 100 nmol/L. Transfection was performed using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer's instructions. Experiments were carried out in triplicate.

RNA extraction and quantitative real-time polymerase chain reaction (PCR)

TRIzol reagent (Invitrogen) was used to extract total RNA from cell lines, GC tissues, and matched adjacent normal tissues. RNA concentration and purity were determined spectroscopically, and samples were stored at -80°C long term or -20°C short term. The miRNA RT-PCR Quantitation Kit (GenePharma) was used for miR-320a reverse transcription using the following protocol: 30 min at 25°C, 30 min at 42°C, and 5 min at 85°C. The cDNA product was subjected to quantitative real-time PCR using the following program: 3 min at 95°C, 40 cycles of 12 s at 95°C, and 40 s at 62°C. All mRNA quantification data were normalized to U6. The primers were designed and produced by GenePharma and the primer sequences are shown in Table 2.

Cell viability and apoptosis assay

GC cells (MKN-45 and BGC-823) were inoculated into 96-well plastic dishes

Table 1 Clinicopathological characteristics of patients with gastric cancer

Clinicopathological characteristics	No. of patients	miR-320a low expression	miR-320a high expression	χ^2	*P* value
Age (yr)					
≤ 60	25	15	10	0.285	0.594
> 60	59	39	20		
Gender					
Female	20	11	9	0.986	0.321
Male	64	43	21		
Location					
Gastric body	20	15	5	1.524	0.467
Gastric antrum	52	31	21		
Gastric fundus	12	8	4		
Tumor size (cm)					
< 5	25	15	10	0.285	0.594
≥ 5	59	39	20		
Differentiation					
Poor	55	40	15	4.945	0.026
Well and moderate	29	14	15		
TNM stage					
I+II	34	15	19	10.120	0.001
III+ IV	50	39	11		
Lymph node metastasis					
No	18	7	11	6.436	0.011
Yes	66	47	19		

containing 10% CCK-8 (Dojindo, Kumamoto, Japan) diluted in culture medium and incubated at 37°C in 5% CO_2 after transfection with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor, or no transfection. The OD value at 0, 12, 24, 48, and 72 h after transfection was measured using an enzyme assay. All experiments were performed three times. GC cells ($n = 10^6$) transfected with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor, or no transfection were inoculated into six-well plates after transfection for 48 h, and all cells were harvested at 72 h. The Annexin V-PE/7AAD Apoptosis Detection Kit (KeyGen, Jiangsu, China) was used to determine apoptosis.

Cell migration and invasion assays

GC cells (MKN-45 and BGC-823) were inoculated into six-well plastic dishes and transfected with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor or no transfection for 36 h. Cuts were then made using a 200-μL pipette tip. The wound healing percentage was measured at 0, 24, and 48 h after transfection using ImageJ software to evaluate the migration ability of GC cells. A Transwell assay was used to determine the invasion ability of GC cells (MKN-45 and BGC-823). A Matrigel-coated membrane matrix (UnivBio, Shanghai, China) was used to simulate a physiological matrix membrane between the upper and lower chambers. GC cells ($n = 10^5$) transfected with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor, or no transfection were inoculated into the upper chamber filled with serum-free medium. Medium containing 10% FBS was added to the lower chamber. After 24 h, cotton wool was used to remove the noninvading cells in the upper chamber, and crystal violet (Tiangen Biotech, Beijing, China) was used to stain the invasive cells on the lower surface of the chamber. The number of invasive cells was counted under an inverted microscope. All experiments were repeated three times.

5-Aza-CdR and trichostatin A (TSA) treatments

MKN-45 cells were inoculated into six-well plates. Cells were treated with 5-Aza-CdR (Sigma–Aldrich, St Louis, MO, USA) at 0.5, 1, or 1.5 μmol/L for 72 h, or with TSA (Beyotime, Beijing, China) at 300 nmol/L for 24 h. TSA was also added to the medium for the final 24 h of the 72 h 5-Aza-CdR (0.5 μmol/L) treatment period. To maintain drug effectiveness, the medium containing the drug was replaced every 24 h. Cells were then harvested, and RNA from MKN-45 cells was purified using the TRIzol reagent. cDNA synthesis was performed as described previously, and 1 mL of diluted

Table 2 Primer/mimic sequences

Name	Sequence (5'–3')
miR-320a reverse transcription primer	
miR-320a	GTCTGTATGGTTGTTCTCGACTCCTTCACATCCCTATCCAACCATACAGACTCGCCCTC
U6 snRNA	GGAACGCTTCACGAATTTG
Real-time PCR primer sequence	
miR-320a	FO:AAGGGATCGCGGGCG
	RE:TGCGTGTCGTGGAGTC
U_6 snRNA	FO:ATTGGAACGATACAGAGAAGATT
	RE:GGAACGCTTCACGAATTTG
MSP primer sequence	
Methylated	FO:ACGTCGTAATGTGAGGATTC
	RE:CGCAAACAAAACTCGATATAA
Nonmethylated	FO:ATTATGTTGTAATGTGAGGATTT
	RE:CACAAACAAAACTCAATATAACCC
miR-320a mimics sequence	
miR-320a	Sense: AAAAGCUGGGUUGAGAGGGCGA
	Antisense: GCCCUCUCAACCCAGCUUUUUU
Negative control	Sense: UUCUUCGAACGUGUCACGUTT
	Antisense: ACGUGACACGUUCGGAGAATT
PBX3 primer sequence	FO:GCCAAATTGACCCAGATCAGAC
	RE:GAAATGGGACGTGTTCTACTCTGTT

PCR: Polymerase chain reaction.

cDNA from each sample was amplified by quantitative PCR using a previously described protocol.

DNA isolation and bisulfite modification

Genomic DNA was isolated from primary GC samples (cancer tissues and matched adjacent normal tissues) and GC cells, and harvested after transfection for 48 h with miR-320a mimics, scramble mimics, or no transfection. The MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Shiga, Japan) was used for DNA isolation. The genomic DNA underwent bisulfite modification using the EZ DNA Methylation-Gold Kit (TaKaRa). DNA (400 ng) was diluted to 20 μL, and 130 μL of the conversion reagent (900 μL water, 50 μL M-dissolving buffer, 300 μL M-dilution buffer) was added. Bisulfite conversion was carried out as follows: 98°C for 10 min, 64°C for 150 min, and 4°C for 5 min, and DNA was recovered using a Zymo-Spin column (Zymo Research, Irvine, CA, USA). Bisulfite-treated DNA was used for methylation analyses.

Methylation analysis

The methylation status of the miR-320a promoter CpG islands in cell lines and tissues was analyzed using methylation-specific PCR (MSP). The MSP primers were designed using MethPrimer (http://www.urogene.org/methprimer) and are listed in Table 2. The PCR product length was 149 bp. The amplification reaction volume was 25 μL, and the reaction procedure was as follows: One cycle at 94°C for 5 min, 40 cycles at 94°C for 30 min, 58°C for 30 min, 72°C for 30 min, and one cycle at 72°C for 10 min. Agarose gel electrophoresis was performed to analyze the MSP products.

Dual luciferase assay

Luciferase activity was analyzed using a dual luciferase reporter assay system (Promega, Shanghai, China). miR-320a mimics, miR-320a negative control miRNA, wild-type PBX3 (ACAGCUUUA), or mutant PBX3 (ACACCCUUA) was transfected into MKN-45 cells for 48 h using Lipofectamine 3000 (Invitrogen). Experiments were repeated three times.

Protein isolation and Western blotting

Total protein was extracted from GC tissues and cells (MKN-45 and BGC-823) after transfection for 48 h with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor, or no transfection using RIPA buffer (Beyotime, Shanghai, China), and protein concentration was determined using a BCA Protein Assay Kit (TaKaRa,

Dalian, China). Appropriate protein samples were separated by SDS-PAGE (TaKaRa, Dalian, China), and electrophoresis was carried out for 100 min at 120 V. The proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Shanghai, China), which were blocked in bovine serum albumin (BSA) for 2 h at room temperature. Rabbit anti-PBX3 monoclonal antibody (1:3000; Abcam, Cambridge, UK; #Ab109173) was added overnight at 4°C. Rabbit anti-GAPDH monoclonal antibody (1:3000; ABclonal, Boston, MA, USA) was used as the internal reference. Goat anti-rabbit secondary antibody was added to the polyvinylidene difluoride membranes for 2 h the following day. Then, a chemiluminescence instrument (Tanon, Shanghai, China) was used to determine the MSP product levels.

Histology

All samples were incubated overnight in buffered formalin, after which they were embedded in paraffin, cut into 3-μm-thick sections, and stained with hematoxylin–eosin (HE).

Bioinformatics and statistical analysis

The miR-320a target gene was predicted by TargetScanHuman7.1 (http://www.targetscan.org/vert_71/), miRDB (http://www.mirdb.org/miRDB/), and microrna.org (http://www.microrna.org/microrna/home.do). MethPrimer (http://www.urogene.org/methprimer2/index.html) was used to predict the miR-320a promoter CpG islands.

Statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). X^2 test or Student's t test (two-tailed) were used. $P < 0.05$ was considered statistically significant.

RESULTS

miR-320a expression in GC tissues and its associations with clinicopathological characteristics

miR-320a expression in GC tissues was measured by quantitative real-time PCR. Assessment of miR-320a expression in 84 GC tissues and matched adjacent normal tissues (Figure 1A) showed that miR-320a was downregulated in 54 samples (54/84, 64%) compared with matched adjacent normal tissues, and the difference was significant (Figure 1B, 1C). To assess the association between miR-320a expression and clinicopathological characteristics, the tissues were separated into two groups: upregulated and downregulated miR-320a groups. miR-320a expression was significantly associated with TNM stage, tumor differentiation, and lymph node metastasis (Table 1 and Figure 1D). These results suggest that miR-320a is involved in GC progression.

miR-320a is downregulated in GC cell lines

miR-320a expression was assessed in four GC cell lines (BGC-823, MGC-803, SGC-7901 and MKN-45) and one normal gastric mucosa cell line (GES-1). miR-320a expression was lower in GC than in GES-1 cells, and particularly downregulated in MKN-45 cells (Figure 1E). These data suggest that miR-320a expression is associated with gastric carcinoma. MKN-45 and BGC-823 cells were used for subsequent miR-320a mimic and inhibitor transfection experiments, respectively.

miR-320a overexpression inhibits GC cell viability and induces apoptosis in vitro

To determine the role and potential biological function of miR-320a in GC, GC cells were transfected with miR-320a mimics and miR-320a inhibitor, and the effects of miR-320a up- and downregulation on cell function were evaluated. miR-320a mimics transfection significantly upregulated, whereas miR-320a inhibitor significantly downregulated miR-320a expression compared with that in the other groups (Figure 2A). The CCK-8 assay was performed to estimate the effect of miR-320a on the viability of GC cells. We found that overexpression of miR-320a inhibited MKN-45 cell viability, whereas miR-320a depletion promoted BGC-823 cell viability (Figure 2B). Because apoptosis is an important part of the cell life cycle, we determined the effect of miR-320a on the early apoptosis rate of GC cells. miR-320a mimics accelerated MKN-45 cell apoptosis, whereas miR-320a inhibitor suppressed BGC-823 cell apoptosis, compared with that in the scramble and untreated groups (Figure 2C, 2D).

miR-320a overexpression inhibits GC cell migration and invasion in vitro

Wound scratch and Transwell invasion assays were used to determine the effects of miR-320a on the metastatic capacity and invasiveness of GC cells, which are

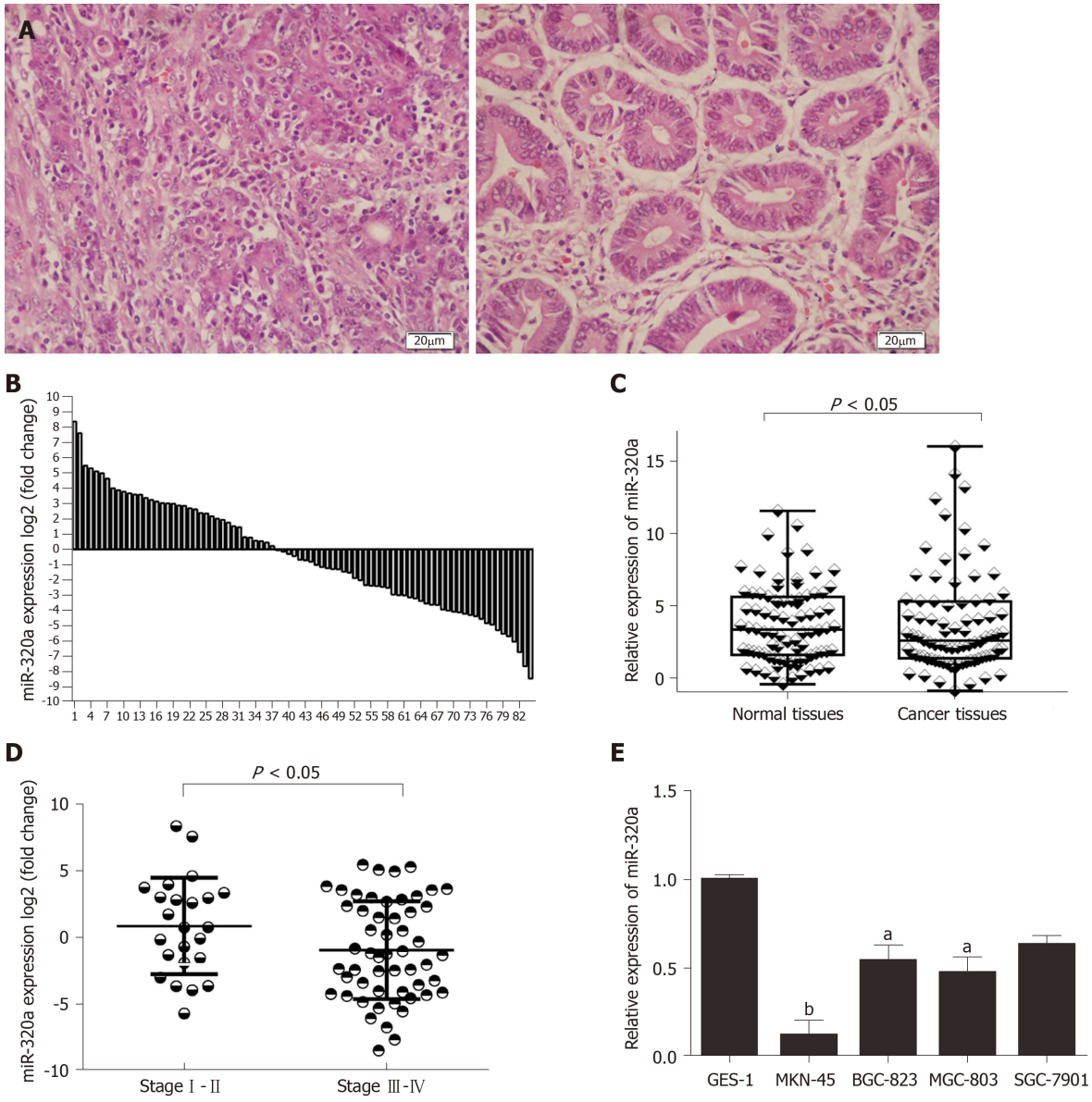

Figure 1 Expression of miR-320a in gastric cancer samples and cell lines, and miR-320a downregulation is correlated with tumor TNM stage. A: Representative tissue slices from gastric cancer (GC) patients were stained using HE; scale bars 20 μm; B: miR-320a expression in 84 pairs of GC tissues and adjacent nontumor tissues is presented as log2 of fold change. Expression of miR-320a in these 84 pairs of samples was determined by quantitative PCR; C: Comparison of expression of miR-320a in GC tissues and matched normal tissues ($P < 0.05$); D: mir-320a expression in stage I/II ($n = 23$) and stage III/IV ($n = 54$) and the results are shown as log2 of fold change in GC tissues relative to normal tissues ($P < 0.05$); E: Expression of miR-320a in GES-1 and GC cell lines (MKN-45, BGC-823, MGC-803 and SGC-7901). $^{a}P < 0.05$, $^{b}P < 0.01$. The reference genes were set using U6 snRNA. All the experiments were conducted in triplicate. Data were analyzed by ANOVA or *t* test. The data are reported as means ± SD.

important malignant activities of tumor cells. GC cells were divided into three treatment groups: miR-320a mimics/inhibitor, scramble mimics/inhibitor, and untreated. Wound healing was suppressed in cells treated with miR-320a mimics, whereas it was accelerated in cells treated with miR-320a inhibitor compared with that in the other two groups at 24 and 48 h after scratching (Figure 3A and 3B). The number of cells traversing the Matrigel matrix was lower in cells treated with miR-320a mimics, whereas it was higher in groups treated with miR-320a inhibitor than in the other two groups (Figure 3C and 3D). These data suggest that miR-320a overexpression inhibits GC cell migration and invasion to overcome the malignant properties of GC cells.

miR-320a expression is regulated by DNA methylation

To determine the cause of miR-320a downregulation, we analyzed the relationship between DNA methylation and miR-320a expression. A search of the human genome database identified CpG islands around the miR-320a promoter (Figure 4A). MSP was used to detect the methylation level of miR-320a in the four GC cell lines and normal

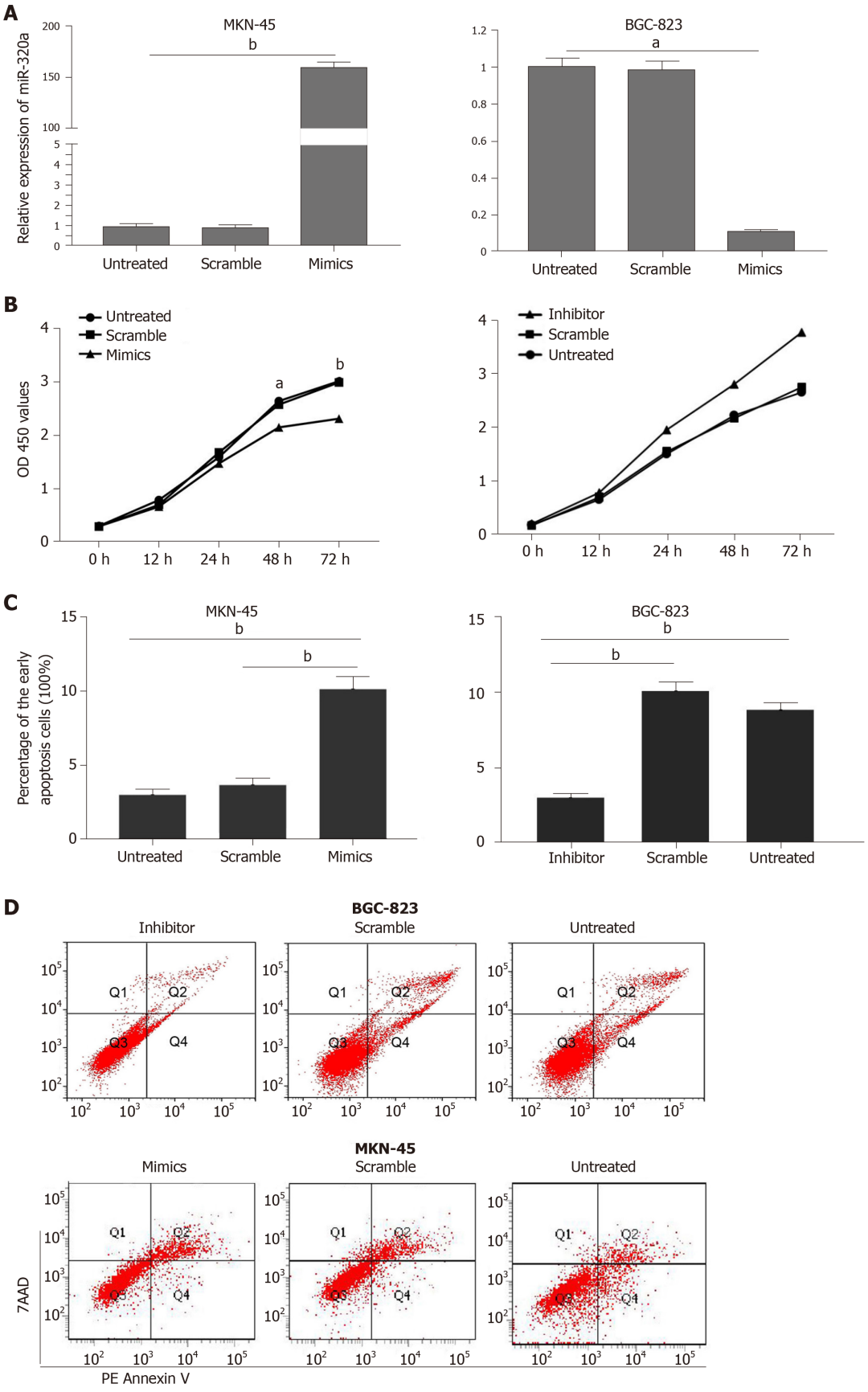

Figure 2 Overexpression of miR-320a inhibited gastric cancer cell viability and promoted apoptosis. A: Expression of miR-320a in gastric cancer (GC) cells was determined by quantitative PCR after transfection with miR-320a mimics (50 nmol/L), scramble mimics, miR-320a inhibitor (100 nmol/L), scramble inhibitor or no transfection; B: CCK8 assay was performed to determine cell viability after transfection at 0, 12, 24 and 72 h; C: Early apoptosis rate in the three groups is shown in the histogram; D: Flow cytometry was used to determine apoptosis after transfection for 48 h. The data are reported as means ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$.

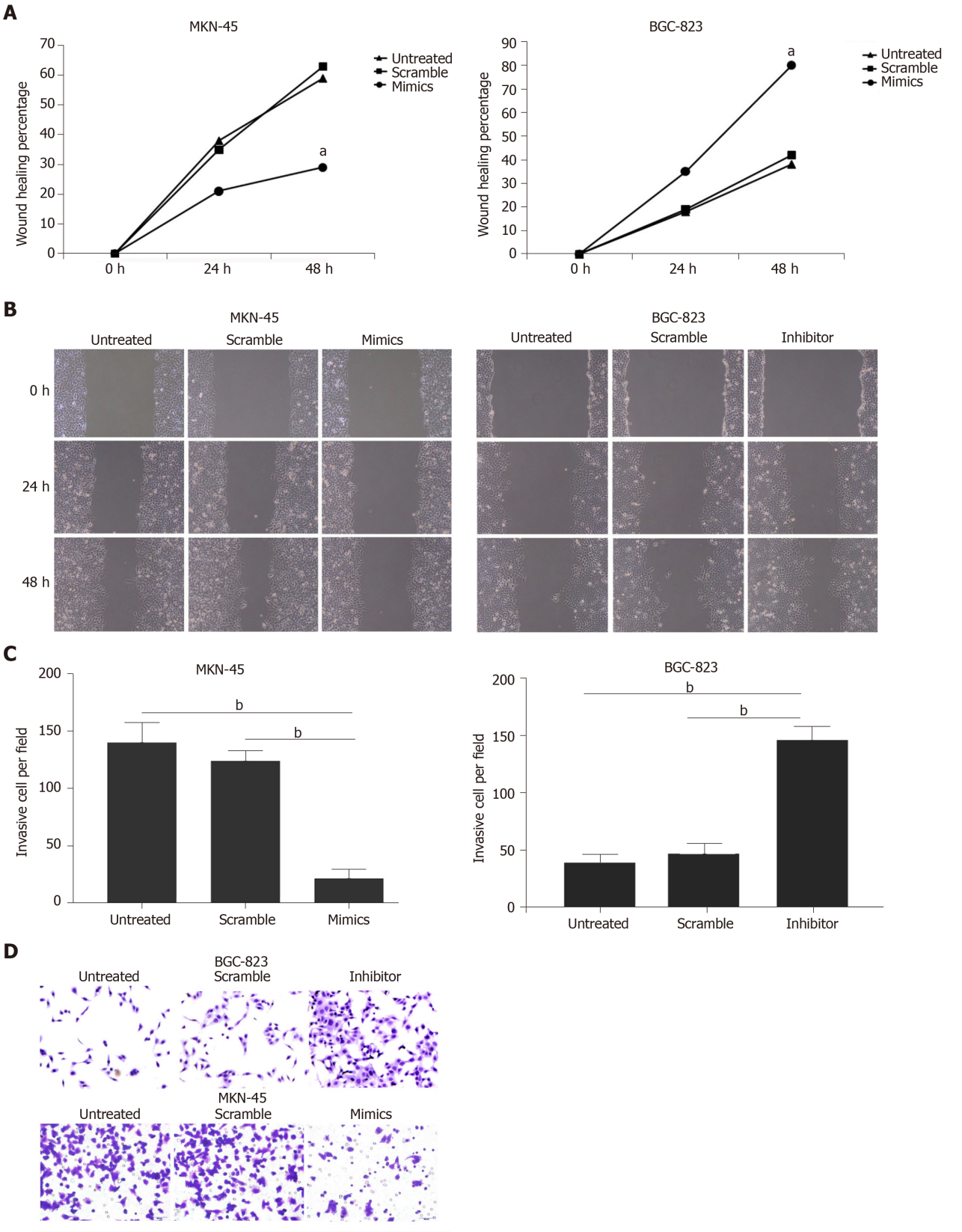

Figure 3 Overexpression of miR-320a suppressed gastric cancer cell migration and invasion. A, B: Wound scratch assay was used to assess migration of MKN-45 cells. Wound healing percentage was measured at 0, 24 and 48 h after transfection with miR-320a mimics (50 nmol/L), scramble mimics, miR-320a inhibitor (100 nmol/L), scramble inhibitor or no transfection; C, D: Transwell invasion assay was carried out to determine gastric cancer cell invasion after transfection with miR-320a mimics/inhibitor, scramble mimics/inhibitor or no transfection for 48 h. Cell invasion was assessed at 24 h. $^{a}P < 0.05$, $^{b}P < 0.01$.

GES-1 cell line. miR-320a promoter CpG islands were hypermethylated in GC cells, whereas no methylation was observed in GES-1 cells (Figure 4B). To examine further the effect of methylation, MKN-45 cells were treated with 5-Aza-CdR (DNA

methylation inhibitor) and TSA (histone deacetylase inhibitor), and the expression and methylation levels of miR-320a were determined by quantitative real-time PCR and MSP, respectively. miR-320a expression was partially reversed by 5-Aza-CdR and TSA, particularly by the combination of 5-Aza-CdR and TSA, and the methylation level of the miR-320a promoter CpG islands was markedly reduced (Figure 4C). To determine the status of miR-320a CpG islands in GC samples, methylation levels were measured in six pairs of GC samples (primary cancer tissues and matched adjacent normal tissues) with low miR-320a expression and four pairs of GC samples with high miR-320a expression levels. miR-320a CpG islands tended to be hypermethylated in the miR-320a downregulated group (83%, 5/6) compared with the miR-320a upregulated group (50%, 2/4) (Figure 4D). These findings indicated that DNA methylation plays an important role in the downregulation of miR-320a expression in GC.

miR-320a directly targets the oncogene PBX3 in GC

To examine the mechanism underlying the effect of miR-320a in GC, online software programs (TargetScanHuman7.1, miRDB, and microRNA.org) were used to predict the target gene of miR-320a. The three databases predicted the interaction of miR-320a with the 3′-UTR of *PBX3* (Figure 5A). The dual luciferase assay confirmed that *PBX3* was a direct target gene of miR-320a, and the regulatory activities are shown in Figure 5B. To determine whether miR-320a modulates PBX3 protein expression, six pairs of samples (GC tissues and matched adjacent normal tissues) with low miR-320a expression were analyzed by western blotting using anti-PBX3 antibodies. The results showed that PBX3 was significantly upregulated in five pairs of GC tissues compared with matched adjacent normal tissues (Figure 5D). Assessment of PBX3 protein expression in MKN-45 cells transfected with miR-320a mimics, scramble mimics, or no transfection showed that PBX3 was significantly downregulated in cells transfected with miR-320a mimics compared with the other two groups (Figure 5C).

DISCUSSION

miRNAs act as oncogenes or tumor suppressor genes and have important functions in the progression of many types of cancers[4]. In the present study, we found that miR-320a was significantly downregulated in GC cell lines and tissues. miR-320a overexpression inhibited GC cell proliferation, invasion and migration, and induced apoptosis in MKN-45 cells. Further studies demonstrated that low miR-320a expression was partly due to hypermethylation of the promoter CpG islands of miR-320a. *PBX3* was identified as a direct target gene of miR-320a. Our findings demonstrated that miR-320a plays a tumor-suppressing role in GC, and DNA methylation is a crucial mechanism underlying miR-320a silencing.

Recent studies show that miR-320a is downregulated in many types of cancers, including breast, colorectal and ovarian cancers, and is involved in several biological functions by regulating specific target genes[11-16]. Xie *et al*[21] showed that miR-320a is markedly decreased in hepatocellular carcinoma, and the lack of miR-320a promotes cell migration and invasion. Decreased expression of miR-320a promotes proliferation and invasion of non-small cell lung cancer by targeting voltage dependent anion channel 1[22]. These data demonstrate the involvement of miR-320a in cancer. In this study, miR-320a expression was significantly lower in GC cells and tissues than in the normal GES-1 cell line and matched adjacent normal tissues. Assessment of the effect of miR-320a on the malignant biological behavior of GC cells showed that miR-320a overexpression prevented GC cell proliferation, invasion and migration, and promoted apoptosis compared with those in control MKN-45 cells. These data demonstrated that miR-320a acted as a tumor suppressor in GC. However, these findings need to be confirmed using a larger data sample.

PBX3, located on chromosome 9q33.3, belongs to the highly conserved PBX family of proteins, which are members of the PBX class of three-amino-acid loop extension superclass of homeodomain proteins[23]. PBX3 is overexpressed in many types of cancers[24-26], and plays a crucial role by activating various signaling pathways including the MAPK/ERK signaling pathway[24,26]. These data showed the powerful function of PBX3. PBX3 can act as an oncogene, promoting cell proliferation and colony formation, and its expression is associated with the clinicopathological characteristics of GC patients[27]. In the present study, Western blot analysis showed that PBX3 was upregulated in GC tissues and cells. miRNAs function by inhibiting translation or promoting degradation of target mRNAs[2]. *PBX3* is regulated by multiple miRNAs in cancer, including let-7d, let-7c, miR-200b, miR-222, miR-424, and miR-181[25,26,28]. In multiple myeloma, *PBX3* is regulated by miR-320a and involved in

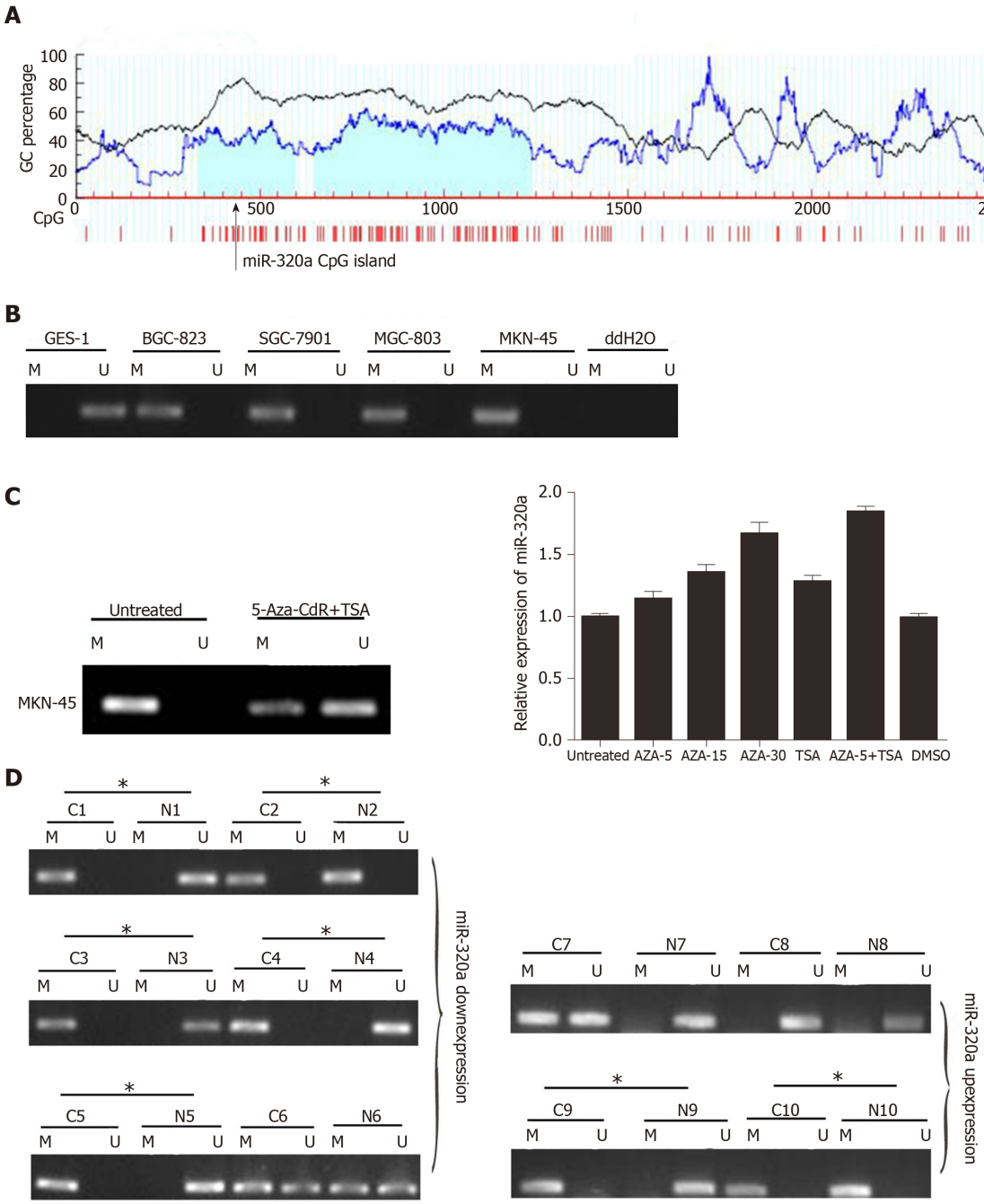

Figure 4 Expression of miR-320a and methylation status of its promoter CpG islands in gastric cancer cell lines and tissue samples. A: Schematic of the location of miR-320a promoter CpG islands obtained from the human genome database and MethPrimer 2.0 (online); B: Methylation level of miR-320a in GES-1 and four gastric cancer (GC) cell lines; C: miR-320a expression and its promoter CpG island methylation level after 5-Aza-CdR and/or TSA treatment of MKN-45 cells; D: Representative MSP results of miR-320a promoter CPG islands in GC samples (cancer tissues and matched adjacent normal tissues). Cases with hypermethylation are marked. M: Methylated; U: Unmethylated; C: Cancer tissues; N: Normal tissues; *: Hypermethylation.

disease progression[12]. However, whether *PBX3* is regulated by miR-320a in GC remains unknown. Our findings showed that miR-320a directly interacted with the 3′-UTR of *PBX3* and downregulated PBX3 protein expression in GC, suggesting a mechanism underlying the post-transcriptional control of *PBX3* by miR-320a.

DNA methylation plays an important role in the expression of tumor suppressor miRNAs[29]. Many miRNAs show hypermethylation of promoter CpG islands, including classic miRNAs such as miR-335 in GC, miR-181 in glioblastoma, and miR-342 in colorectal cancer[17,30,31]. However, the mechanism underlying the downregulation of miR-320a in GC is unknown. We found that CpG islands were present around miR-320a by searching the human genome database. He *et al*[19] reported that miR-320a was hypermethylated in colorectal cancer, which led us to

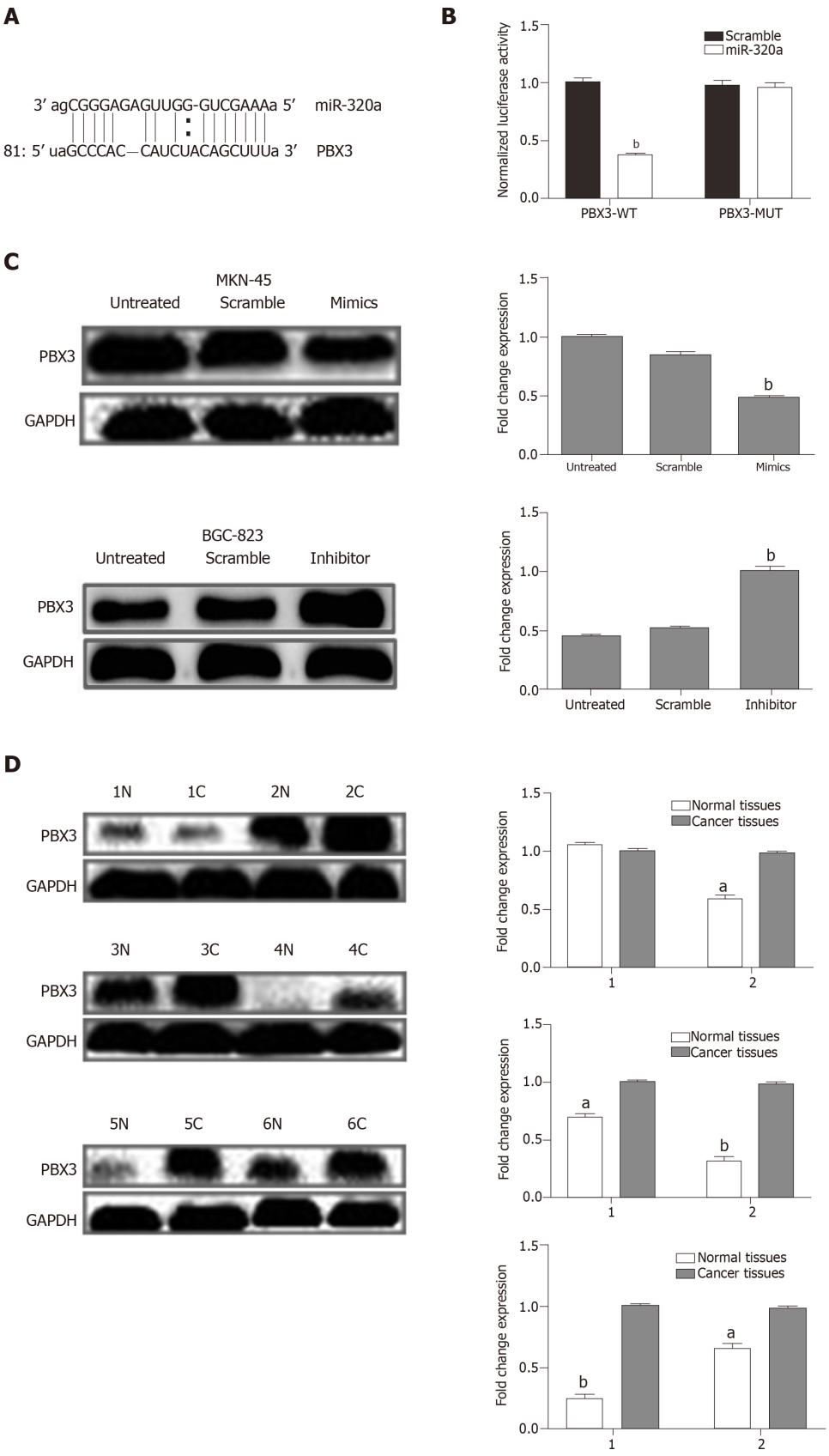

Figure 5 PBX3 was a direct target of miR-320a. A: Banding region between miR-320a and PBX3 was predicted by microRNA.org; B: Relative luciferase activities of PBX3 in wild type and mutant; C: PBX3 protein level in gastric cancer (GC) cells after transfection with miR-320a mimics, scramble mimics, miR-320a inhibitor, scramble inhibitor or no transfection for 48 h; D: Protein level of PBX3 in GC tissues and matched adjacent normal tissues. Reference protein was set using GAPDH. C: Cancer tissues; N: Normal tissues; $^{a}P < 0.05$; $^{b}P < 0.01$.

hypothesize that low miR-320a expression is associated with DNA methylation in GC.

MSP showed that, compared with normal GES-1 cells and matched adjacent normal tissues, miR-320a promoter CpG islands were hypermethylated in GC cell lines and GC tissues in which miR-320a was downregulated. Furthermore, the level of miR-320a hypermethylation was partially reversed by the demethylating agent 5-Aza-CdR. Overall, these data demonstrate that the expression of miR-320a is regulated by DNA methylation in GC.

There were some limitations in the present study. First, our study was a small, retrospective, single-institution study, and further larger, multicenter studies are required to validate our results. Second, although the expression of miR-320a is regulated by DNA methylation, the specific mechanisms remain largely undefined. Further detailed studies are currently underway to explore the participation of miR-320a in this process, to provide more comprehensive information about the functions of miR-320a in cancer.

In conclusion, we showed that miR-320a was downregulated and played a tumor-suppressing role by regulating the oncogene *PBX3* in GC. miR-320a overexpression inhibited cell proliferation, invasion and migration, and induced cell apoptosis. miR-320a expression was regulated by DNA methylation and was partially reversed by 5-Aza-CdR and TSA. These data indicate that miR-320a may be a potential therapeutic target in GC.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common type of malignant tumor with poor prognosis and presents a serious threat to human health. In February 2018 , the latest statistics from the Chinese National Cancer Center showed that although the overall incidence of GC is declining, it remains second in terms of incidence among all malignancies in China, just below lung cancer. At present, there is no efficient early diagnosis and curative treatment strategy for GC. As precision medicine is the future direction in cancer therapy, it is important to identify an early diagnostic or therapeutic biomarker of GC.

Research motivation

To explore the expression pattern, biological function and potential mechanism of miRNA (miR)-320a in GC, and to determine whether miR-320a functions as an early diagnostic or therapeutic biomarker in GCaccording to its expression and biological functions.

Research objectives

miR-320a mimic and inhibitor were transfected into GC cells for bidirectional regulation of the expression level of miR-320a. The effect of miR-320a on cell viability, migration, invasion and apoptosis was determined through a series of functional experiments. These results would provide scientific evidence for clinical treatment of GC.

Research methods

Quantitative real-time polymerase chain reaction (PCR) and Western blotting were performed to determine the levels of related factors. Methylation-specific PCR was applied for analysis of methylation status of the miR-320a promoter CpG islands in GC cell lines and tissues. CCK8, flow cytometry, Transwell invasion and wound healing assays were performed to determine the effect of miR-320a on cell behavior. Dual luciferase assay was performed to identify whether pre-B-cell leukemia homeobox (PBX) 3 was the target gene of miR-320a. TargetScanHuman7.1, miRDB, microrna.org and MethPrimer were used for bioinformatics analysis. Student's *t* test (two-tailed) and analysis of variance (ANOVA) were used for statistical analysis.

Research results

miR-320a was downregulated in GC, and its expression deficiency was partly due to hypermethylation of the promoter CpG islands. miR-320a overexpression inhibited cell viability, migration and invasion, and induced apoptosis through targeting PBX3 in GC cells. miR-320a functioned as a tumor suppressor *in vitro*. However, its biological effect in a preclinical model should be further determined.

Research conclusions

miR-320a was downregulated in GC tissues and cells, and its abnormal expression was related to GC cell behavior. We confirmed that miR-320a overexpression inhibited cell viability, migration and invasion, and induced apoptosis in GC cells. miR-320a deficiency showed the opposite results. Consistent with previous research, miR-320a functioned as a tumor suppressor. miR-320a could be a biomarker for GC diagnosis and treatment. We found that miR-320a downregulation in GC was due to the elevated methylation level of the miR-320a promoter CpG islands. The elevation was partly reversed by 5-Aza-2'-deoxycytidine and trichostatin A. In addition, PBX3 was firstly identified as the target gene of miR-320a in GC.

Research perspectives

For future research, we will focus on the related signaling pathways through which miR-320a

regulates GC cell biological behaviors. Screening for related signaling pathways of GC will be performed using the Cancer Genome Atlas database, followed by related factor detection using Western blotting and quantitative real-time PCR. Activator and inhibitor for target signaling will be applied to GC cells, and cell viability, cell cycle, cell migration, invasion and apoptosis will be determined.

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ORIGINAL ARTICLE

Retrospective Study

Retrospective review of total neoadjuvant therapy

Laila Babar, Veli Bakalov, Stephen Abel, Obaid Ashraf, Gene Grant Finley, Moses S Raj, Kristina Lundeen, Dulabh K Monga, Alexander V Kirichenko, Rodney E Wegner

ORCID number: Laila Babar (0000-0003-1520-8798); Veli Bakalov (0000-0002-2472-5546); Stephen Abel (0000-0003-4956-8900); Obaid Ashraf (0000-0002-2138-4475); Gene Grant Finley (0000-0003-1987-1781); Moses S Raj (0000-0002-4779-3306); Kristina Lundeen (0000-0002-3254-1886); Dulabh K Monga (0000-0002-2242-4643); Alexander V Kirichenko (0000-0002-4432-1039); Rodney E Wegner (0000-0003-2416-3499).

Author contributions: Babar L and Wegner R designed the study; Babar L, Bakalov V, and Abel S performed the statistical analysis; Babar L wrote the paper; Ashraf O, Finley GG, Raj MS, Lundeen K, Monga DK, Kirichenko AV, and Wegner R contributed to the scientific review; Wegner R was the faculty mentor for this project.

Institutional review board statement: The IRB has reviewed this information and finds that this protocol does not fall under the purview of the IRB as it does not meet the definition of human subject research according to the federal code of regulations: 45 CFR 46.102(f).

Informed consent statement: As the data used was accessed *via* a public national database with deidentified patients, there was no need for informed consent.

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Laila Babar, Veli Bakalov, Obaid Ashraf, Department of Internal Medicine, Allegheny General Hospital, Pittsburgh, PA 15212, United States

Stephen Abel, Alexander V Kirichenko, Rodney E Wegner, Department of Radiation Oncology, Allegheny General Hospital, Pittsburgh, PA 15212, United States

Gene Grant Finley, Moses S Raj, Kristina Lundeen, Dulabh K Monga, Department of Medical Oncology, Allegheny General Hospital, Pittsburgh, PA 15212, United States

Corresponding author: Laila Babar, MD, Doctor, Resident Physician, Department of Internal Medicine, Allegheny General Hospital, 320 E North Ave, Pittsburgh, PA 15212, United States. laila.babar@ahn.org
Telephone: +1-412-6520419

Abstract

BACKGROUND

Neoadjuvant chemoradiotherapy (nCRT) followed by resection and postoperative multi-agent chemotherapy (maChT) is the standard of care for locally advanced rectal cancer. Using this approach, maChT administration can be delayed for several months, leading to concern for distant metastases. To counteract this, a novel treatment approach known as total neoadjuvant therapy (TNT) has gained popularity, in which patients receive both maChT and nCRT prior to resection. We utilized the National Cancer Database to examine temporal trends in TNT usage, and any potential effect on survival.

AIM

To study the temporal trends in the usage of TNT and evaluate its efficacy compared to neoadjuvant chemoradiation.

METHODS

We queried the National Cancer Database for patients with locally advanced rectal cancer, Stage II-III, from 2004-2015 treated with nCRT or TNT. TNT was defined as maChT initiated ≥ 90 d prior to nCRT initiation. Overall survival was calculated from the date of diagnosis to the date of last contact or death using Kaplan-Meier curves to present the cumulative probability of survival, with log-rank statistics to assess significance. Multivariable cox regression was used to identify predictors of survival and propensity score analysis accounted for bias.

RESULTS

We identified 9066 eligible patients, with 8812 and 254 patients receiving neoadjuvant chemoradiation followed by maChT and TNT, respectively. Nodal

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involvement, stage III disease, and treatment in recent years were predictive of TNT use. There was greater use of TNT with more advanced stage, specifically > 1 node involved (odds ratio [OR] = 2.88, 95% confidence interval [CI]: 2.11-3.93, $P < 0.01$) and stage III disease (OR = 2.88, 95%CI: 2.11-3.93, $P < 0.01$). From 2010 to 2012 the use of TNT increased (OR = 2.41, 95%CI: 1.27-4.56, $P < 0.01$) with a greater increase from 2013 to 2015 (OR = 6.62, 95%CI: 3.57-12.25, $P < 0.01$). Both the TNT and neoadjuvant chemoradiation arms had a similar 5-year survival at 76% and 78% respectively. Multivariable analysis with propensity score demonstrated that increased age, high comorbidity score, higher grade, African American race, and female gender had worse overall survival.

CONCLUSION
Our data demonstrates a rising trend in TNT use, particularly in patients with worse disease. Patients treated with TNT and nCRT had similar survival. Randomized trials evaluating TNT are underway.

Key words: Total neoadjuvant therapy; Neoadjuvant chemoradiation; Multi-agent chemotherapy; Locally advanced rectal cancer; National cancer database; Colorectal cancer; Retrospective review; Gastrointestinal oncology; Temporal trends; Surgical excision

Core tip: Total neoadjuvant treatment (TNT) has been gaining favor as the treatment of choice for rectal carcinoma. It has been linked to better sphincter preservation and overall improved quality of life. Our study aim was to compare TNT with traditional chemotherapy and radiation over the last 10 years and evaluate the differences in outcomes. Our study confirms a rising trend in the of use of TNT especially in patients diagnosed with locally advanced rectal cancer. Patients treated with TNT had higher burden of disease but had similar survival outcomes as those treated with traditional chemotherapy and radiation.

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INTRODUCTION

Over the past several years, there have been several advances in the medical and surgical treatment of locally advanced, stage II and III rectal cancer. With the advent of total mesorectal excision as well as anal laparoscopic surgeries, treatment options continue to improve and result in lower rates of local disease recurrence in these patients. There has also been an ongoing paradigm shift in the approach to chemoradiation administration. The standard of care for rectal cancer is neoadjuvant chemoradiotherapy (nCRT) followed by surgery and then postoperative multiagent chemotherapy based on several randomized trials[1,2]. A novel approach called total neoadjuvant therapy (TNT) has recently gained favor whereby patients are given both nCRT as well as multiagent chemotherapy prior to surgery. TNT use has been associated with better compliance, decrease in toxicities, and higher rates of anal sphincter preservation[3-9].

This change in approach has been based on increasing evidence that there is better quality of life with improved functionality, decreased toxicity, and lower rates of recurrence with preoperative treatment[4-9]. Patients receiving adjuvant chemotherapy have delays in the administration of treatment secondary to long postoperative recovery time and inability to regain their prior functional status. Compliance is an issue in these patients, and they often do not receive their scheduled chemotherapy or it is delayed[3]. Additionally, with the use of TNT, carefully selected patients who have a clinical complete response can in some instances avoid surgery altogether and instead be followed with close observation (*i.e.* "watch and wait approach")[10].

With local disease recurrence rates falling due to the use of total mesorectal excision, which is a close dissection of the rectum and para rectal lymph nodes within the mesorectal envelope[11], there is still an increased concern for distant metastases. Over 35% of patients treated with the standard therapy for locally advanced rectal cancer (LARC) have distant recurrence of disease[12]. One reason for this is the presence of micrometastases. As surgery only addresses local disease and potentially delays the administration of chemotherapy, these micrometastases can result in distant metastasis. The administration of TNT helps tackle this problem by providing total systemic treatment prior to surgical resection. It also gives clinicians the ability to detect patients who do not respond to the treatment in a timely manner[13].

As such, we queried the National Cancer Database (NCDB) to analyze the trends in treatment approach towards locally advanced rectal carcinoma and to determine if there has been a rise in TNT use in recent years. Additionally, we identified the factors affecting the rate of utilization of TNT and how they correlate with patients from different socioeconomic strata.

MATERIALS AND METHODS

The aim of this study was to use the NCDB to evaluate the trends in treatment options for LARC, specifically with regards to TNT utilization. The NCDB is a national database cataloguing approximately 70% of all cancer cases within the United States[14]. This information is collected from over 1500 cancer treatment centers. This database details information about patients' demographics, disease, treatment, and mutations for several cancer subtypes. The data that are submitted to this database must meet established quality standards and is managed by the Commission on Cancer of the American College of Surgeons and the American Cancer Society. We used data extracted from a de-identified NCDB record. This study was exempt from review by the internal revenue board due to its deidentified and retrospective nature.

We queried the NCDB for patients diagnosed with rectal adenocarcinoma diagnosed between 2004 and 2014. Patients with stage II and III cancer were included and the data were analyzed to extract if the treatment modality was TNT or multi-agent chemotherapy (maChT). TNT entails the administration of induction chemotherapy as well as chemoradiotherapy prior to surgery, while maChT is postoperative multiagent chemotherapy, which is given in the adjuvant setting and is the current standard of care, and is often combined with neoadjuvant chemoradiation preop[1]. All patients who did not meet the inclusion criteria were excluded, leaving a total of 9066 patients (Figure 1). We analyzed the patient demographics, clinical course, treatment modalities, and survival outcome among our patient population. The patient characteristics used for analysis were age, gender, insurance status, income, facility location and type, tumor stage, treatment modality, and nodal stage.

Overall survival (OS) was calculated using the date of diagnosis and the last contact or death. This data were then analyzed using Kaplan-Meier curves for cumulative probability of survival. Log rank statistics were employed to assess for statistical significance between the two groups. Multivariate cox regression was used to identify factors associated with OS. The *P* value was set at < 0.005.

Multivariable cox regression was done for OS considering the treatment, stage, facility type, location, education status, age, gender, insurance type, race, comorbidity score, mean household income, age distribution, and year of diagnosis. The odds ratios (ORs) for the two groups, those who underwent TNT and those were treated with conventional neoadjuvant treatment, were reported with a 95% confidence interval (CI) along with the corresponding type 1 error (*P*-value). To account for any potential biases within the two treatment arms, propensity score analysis was used.

RESULTS

Using the eligibility criteria defined above, our final study population consisted of 9066 patients [5648 men (62%), 3418 women (38%)]. These patients were separated on the basis of the treatment they received: 8812 patients received nCRT and 254 patients received TNT from 2004 to 2015. Of the cohort, 3694 patients had stage II disease and 5372 had stage III disease with 2525 patients having > 1 node positive (Figure 1). The majority of patients were less than age 65 and had private payer insurance. A summary of the clinical and demographics of our study population are in Table 1.

There was greater use of TNT with more advanced stage, specifically > 1 node involved (OR = 2.88, 95%CI: 2.11-3.93, $P < 0.01$) and stage III disease (OR = 2.88, 95%CI: 2.11-3.93, $P < 0.01$). From 2010 to 2012, the use of TNT increased (OR = 2.41,

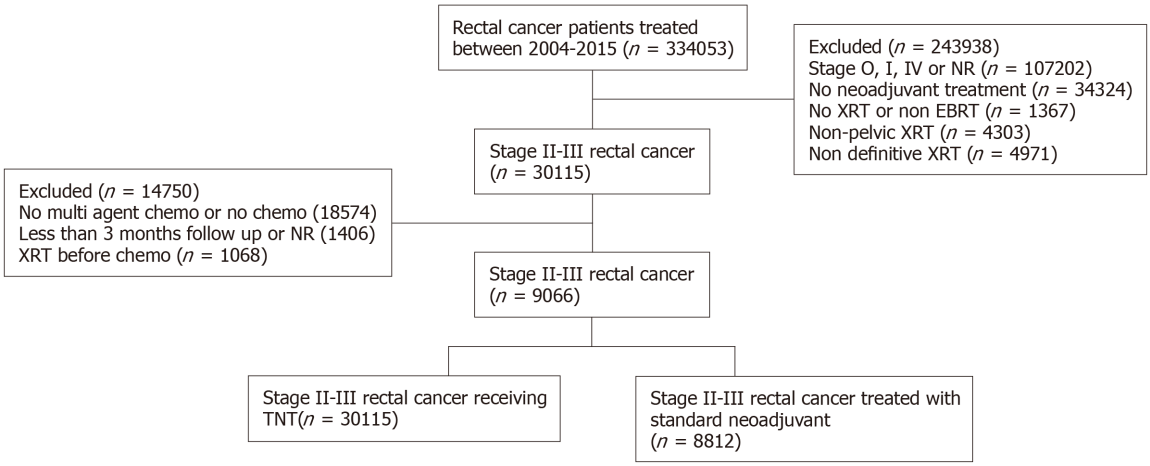

Figure 1 Flow chart for filtering data.

95%CI: 1.27-4.56, $P < 0.01$) with a greater increase from 2013 to 2015 (OR = 6.62, 95%CI: 3.57-12.25, $P < 0.01$) (Table 1). The 5-year survival for both treatment arms was similar at 76% and 78%, respectively. Multivariable analysis found age > 58, higher income, urban location, academic treatment facility, and lower co-morbidity score as predictors for worse OS. Propensity score matching demonstrated increased age, higher comorbidity score, higher tumor grade, African American race, and gender as predictive of worse OS.

DISCUSSION

Colorectal cancer is the fourth highest occurring cancer in the United States and is the second leading cause of cancer-related deaths as per the Surveillance, Epidemiology, and End Results data[15]. Patients with locally advanced rectal carcinoma, which includes patients with T2 or T3 disease with or without nodal involvement, benefit from a multidisciplinary treatment approach. These patients are treated with multiagent chemotherapy, radiation, and surgery to decrease the incidence of local and systemic recurrence. The German Rectal Cancer Study compared neoadjuvant and adjuvant chemotherapy and found improved outcomes with neoadjuvant chemotherapy[1]. This can be done in the form of a long course of chemoradiotherapy, a short course radiotherapy or TNT.

When TNT adjuvant chemotherapy is moved to the neoadjuvant setting and given preoperatively, it improves compliance, decreases treatment-associated toxicity, and results in tumor down staging leading to better sphincter preservation[16,17]. Randomized clinical trials have demonstrated that there is no benefit to adjuvant chemotherapy in LARC and its use does not improve outcomes in patients with stage II and III disease who receive chemoradiation in the neoadjuvant setting[7-9]. The EORTC 22921 trial was conducted to evaluate the utility of adjuvant chemotherapy in patients with T3-T4 tumors, and it found that there was no additional benefit of giving adjuvant chemotherapy to patients who had been treated with neoadjuvant chemoradiation[6]. Patients who underwent adjuvant chemotherapy showed poor compliance with only 43%-73.6% of them receiving the planned doses and an overall delay of over 18 wk in receiving systemic therapy[3]. They also had higher incidence of chemotherapy related toxicity and poor surgical outcomes[4-9]. Prior studies have shown that over 10%-25% of patients receiving neoadjuvant chemotherapy have complete pathological response, which is an established predictor of improved survival[18-23] (Table 2).

In light of these findings, the use of alternate treatment strategies such as TNT is gaining favor and its use is becoming increasingly common. Our data showed an increase in recent years in the use of TNT, which coincided with the initiation of several clinical trials evaluating the outcomes of using TNT over traditional CRT. The number of patients treated with TNT was small in our dataset, however, this was to be expected as it was compiled over 10 years from 2004 to 2015, and TNT use is a rather recent phenomenon. Our data showed a rising trend in TNT use, with patients diagnosed from 2013 to 2015 having the highest frequency of use. We expect this use to further increase as more data about benefits of TNT over traditional treatment comes to light.

Table 1 Comparative use of total neoadjuvant treatment *vs* conventional neoadjuvant treatment approach by baseline characteristics in patients receiving treatment for rectal adenocarcinoma

Characteristic	TNT, *n* = 254 (%)	Conventional neoadjuvant treatment, *n* = 8812 (%)	OR	95%CI	*P* value
Sex					
Male	151 (59)	5497 (62)	1	Ref	
Female	103 (41)	3315 (38)	1.13	0.88-1.46	0.34
Race					
White	205 (81)	7757 (88)	1	Ref	
African American	22 (8)	624 (7)	1.33	0.85-2.09	0.21
Other	27 (11)	431 (5)	2.37	1.57-3.58	< 0.0001
Comorbidity score					
0	224 (88)	7175 (81)	1	Ref	
1	27 (11)	1366 (16)	0.63	0.42-0.95	0.026
≥ 2	3 (1)	271 (3)	0.35	0.11-1.11	0.08
Insurance					
Not Insured	9 (3)	417 (5)	1	Ref	
Private Payer	178 (70)	5313 (60)	1.55	0.79-3.05	0.20
Government	63 (25)	2981 (34)	0.98	0.48-1.98	0.95
Unrecorded	4 (2)	101 (1)	1.84	0.55-6.08	0.32
Education %					
≥ 29	39 (15)	1262 (14)	1	Ref	
20 to 28.9	53 (21)	2231 (25)	0.77	0.51-1.17	0.22
14 to 19.9	84 (33)	2960 (34)	0.92	0.62-1.35	0.66
Locations					
Metro	209 (82)	6915 (78)	1	Ref	
Urban	16 (6)	1474 (17)	0.36	0.22-0.60	0.0001
Rural	4 (2)	207 (2)	0.64	0.24-1.74	0.38
Unrecorded	25 (10)	216 (2)	3.83	2.48-5.92	< 0.0001
Income, United States dollars					
< 30000	25 (10)	1391 (16)	1	Ref	
30000 to 35000	37 (15)	2086 (24)	0.99	0.59-1.65	0.96
35000 to 45999	53 (20)	2510 (29)	1.17	0.73-1.90	0.51
> 46000	139 (55)	2773 (31)	2.79	1.81-4.29	< 0.0001
Distance to treatment facility, miles					
≤ 8.5	82 (32)	3561 (41)	1	Ref	
> 8.5	172 (68)	5205 (59)	1.44	1.10-1.87	0.0079
Age distribution in yr					
≤ 65	208 (82)	6734 (76)	1	Ref	
> 65	46 (18)	2078 (24)	0.72	0.52-0.99	0.04
Year of diagnosis					
2004-2006	11 (4)	1125 (13)	1	Ref	
2007-2009	19 (7)	2256 (26)	0.86	0.41-1.82	0.69
2010-2012	73 (29)	3097 (35)	2.41	1.27-4.56	0.0068
2013-2015	151 (59)	2334 (26)	6.62	3.57-12.25	<0.0001
Stage grouping					
2	50 (20)	3644 (41)	1	Ref	
3	204 (80)	5168 (59)	2.88	2.11-3.93	< 0.0001
Nodes					
0	170 (67)	5277 (60)	1	Ref	
1	25 (10)	1089 (12)	0.71	0.47-1.09	0.12

TNT: Total neoadjuvant therapy.

Our study was a large analysis of multi-institutional data to compare the use of conventional chemoradiation with TNT. Due to the large sample size in this database,

Table 2 Different surgery/chemoradiation protocols used per standard guidelines

Treatment type	Protocol used
nCRT	50-55Gy/25-28 fx with concurrent 5-fluorouracil (5-FU) or capecitabine1
Post-op MaChT	Excisional surgery followed by postoperative (i.e. adjuvant) chemotherapy with 5-FU based regimens1
TNT	25-35Gy/5 fx followed by CAPOX or FOLFOX chemotherapy

1nCRT and MaChT are usually used in conjunction with each other in traditional chemoradiation therapy. nCRT: Neoadjuvant chemoradiotherapy; MaChT: Multi-agent chemotherapy; TNT: Total neoadjuvant therapy.

we were afforded the statistical power to conduct retrospective analyses of different treatment strategies. Our analysis of the NCDB database showed that there has been an increase in TNT use and this trend has been increasing in recent years. This trend was most apparent in patients with stage III disease (Figure 2).

The total number of patients who received TNT is comparatively much lower than the number of patients undergoing CRT, which was a possible limitation of our study. However as prospective data matures, we will see a greater number of patients being treated with TNT. There were limitations to our study, as the data collected by NCDB is retrospective; thus selection biases certainly existed. To counteract this possible bias, propensity score adjustment was performed. Second, as NCDB is coded, there is potential for incomplete coded or miscoded variables, which may have impacted our results and some statistics. One such variable is the incidence of colorectal cancer divided by gender. Per the Surveillance, Epidemiology, and End Results data, men have a slightly increased lifetime risk of developing colorectal cancer (4.5%) compared to women (4.15%). This difference in risk may explain why as per the NCDB database, roughly 60% of our population was male compared to approximately 40% women. It is imperative to note that although the NCDB accounts for cancer treatment all over the United States, only about 70% of the patients treated for cancer are entered into the database; this may also explain why our population had a higher percentage of male patients.

In conclusion, this study was significant, despite its limitations, as it was a large-scale analysis of treatment selection for more than 70% of patients diagnosed with rectal cancer from 2004 to 2015 in the United States. It showed an increase in the use of TNT, concordant with current research demonstrating its benefits over the use of conventional neoadjuvant chemoradiation. The rate of use of TNT has been steadily increasing and is most prevalent in patients diagnosed with LARC in recent years. As clinical trials studying the use of TNT near completion, the benefits of this approach are becoming more widely accepted and the use of TNT is increasing.

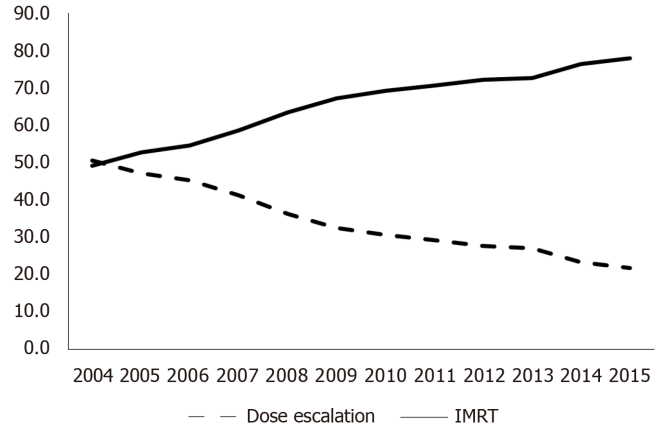

Figure 2 Line graph comparing use of total neoadjuvant therapy and neoadjuvant radiation from 2004 to 2014.

ARTICLE HIGHLIGHTS

Research background

Locally advanced rectal cancer (LARC) treatment has been evolving for several years, specifically to improve standard of living after treatment. Total neoadjuvant treatment (TNT) has been gaining favor in recent years as it allows for better sphincter preservation and an overall improved quality of life. Our study analyzes the use of TNT over a 10-year period and compares the overall survival (OS) to that of patients treated with traditional neoadjuvant chemoradiation.

Research motivation

The focus of our study was to evaluate the OS in patients with LARC when treated with either TNT or traditional chemoradiation. It compared the two modes of treatment, which will help clinicians decide between the two modalities. It also sets up the stage for ongoing and future clinical trials that aim to study the benefit of using one modality over the other.

Research objectives

The main objective of our research was to analyze differences in OS in patients treated with TNT or traditional chemoradiation. We did not find a statistically significant difference in OS between the two modalities. An equivocal OS allows researchers to further design clinical trials comparing both treatment options prospectively and establish stronger guidelines that will further help clinicians decide what treatment will benefit their patients most.

Research methods

This was a retrospective review of data extracted from the national cancer database. We queried the National Cancer Data Base to find patients with LARC, stages II and III, who were treated with either TNT or traditional chemoradiotherapy. The standard of care, currently, utilizes a combination of adjuvant chemoradiation and surgery, followed by postoperative multi-agent chemotherapy. We analyzed the differences in OS between the two arms as our primary goal. For our secondary goal, we established what patient characteristics were likely to be associated with TNT use. These characteristics included age, race, gender and comorbidity score.

Research results

Using univariate and multivariate cox regressions, we analyzed our data for both primary and secondary goals. There was no statistically significant difference in OS between the two arms. We also found that patients with stage III disease, higher nodal involvement or treatment within recent years were more likely to have been treated with TNT. Patients in both arms had poor OS with higher comorbidity score, older age, African American race and female gender. Our results further solidify the theory that TNT is non inferior to traditional chemoradiotherapy, and thus must be studied in more detail with prospective trials.

Research conclusions

OS is similar in patients treated with either TNT or traditional chemoradiation. TNT has been associated with better quality of life. As OS is similar to that of neoadjuvant chemoradiation, using TNT may be preferable, to allow for a better standard of living. The 5-year OS is similar for both TNT and traditional chemoradiation. First, in patients with LARC, TNT and neoadjuvant chemoradiation have similar rates of OS. Second, TNT use may be linked to a better quality of life however more studies are needed in this area. There were no new methods as this was a retrospective review of a national database. There was no new phenomenon found as this was a retrospective analysis of an existing database. We queried the database to establish differences in OS for patients treated with two different chemoradiation protocols. Our retrospective analysis showed that the rate of OS was equivocal in patients treated with either TNT or traditional chemoradiation. It will allow clinicians and researchers to further compare the two modalities

and may increase the treatment options available to patients suffering from LARC.

Research perspectives

New techniques that allow for radiation treatment to be completed prior to surgery, such as TNT, have yielded non- inferior results and therefore must be studied in greater detail. Future research should aim to conduct prospective clinical trials comparing TNT to traditional chemoradiation. This will help clinicians better decide what treatment is best suited to their patient population. Clinical trials exploring the survival, quality of life and toxicities associated with both TNT and neoadjuvant chemoradiation will help further the research in this field and provide concrete answers to many of the questions raised.

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ORIGINAL ARTICLE

Retrospective Study

Evaluation of the safety and effectiveness of direct oral anticoagulants and low molecular weight heparin in gastrointestinal cancer-associated venous thromboembolism

Alejandro Recio-Boiles, Sumana Veeravelli, Jessica Vondrak, Hani M Babiker, Aaron J Scott, Rachna T Shroff, Hitendra Patel, Emad Elquza, Ali McBride

ORCID number: Alejandro Recio-Boiles (0000-0002-5423-3112); Sumana Veeravelli (0000-0002-7179-6996); Jessica Vondrak (0000-0001-7570-8538); Hani M Babiker (0000-0002-9368-6296); Aaron J Scott (0000-0002-0301-3072); Rachna T Shroff (0000-0003-0066-2672); Hitendra Patel (0000-0001-8746-2600); Emad Elquza (0000-0003-3383-7950); Ali McBride (0000-0002-9430-4675).

Author contributions: Recio-Boiles A, Patel H, Elquza E, and McBride A designed and drafted the study concept. Recio-Boiles A, Vondrak J, and Veeravelli S collected the data. Recio-Boiles A, Elquza E, and McBride A performed data analysis and interpretation. Recio-Boiles A, Babiker HM, Scott AJ, Shroff RT, Elquza E, and McBride A contributed to writing, revising and editing the manuscript. All authors helped to perform the research, revision of the manuscript and have approved the final version.

Institutional review board statement: This study has a statement on ethics approval by the Institutional Review Board of University of Arizona Cancer Center, which is a part of the University of Arizona The Human Subjects Protection Program.

Informed consent statement: Patients were not required to give

Alejandro Recio-Boiles, Hani M Babiker, Aaron J Scott, Rachna T Shroff, Emad Elquza, Department of Medicine, Hematology and Medical Oncology, University of Arizona Cancer Center, Tucson, AZ 85724, United States

Sumana Veeravelli, Jessica Vondrak, Department of Medicine, Internal Medicine Residency Program, University of Arizona, Tucson, AZ 85725, United States

Hitendra Patel, UC San Diego Health Moores Cancer Center, La Jolla, CA 92093, United States

Ali McBride, University of Arizona College of Pharmacy, Tucson, AZ 85725, United States

Corresponding author: Alejandro Recio-Boiles, MD, Assistant Professor of Medicine, Department of Medicine, Hematology and Medical Oncology, University of Arizona Cancer Center, 1515 N Campbell Ave, Tucson, AZ 85724, United States.
areciomd@email.arizona.edu
Telephone: +1-520-6264703
Fax: +1-520-6268095

Abstract

BACKGROUND

Gastrointestinal cancer (GICA) is associated with a higher incidence of venous thromboembolism (VTE) compared to other solid tumors, moreover, recurrent VTE and major bleeding (MB) complications during anticoagulation treatment have an associated increase rate. GICA-VTE remains a challenging clinical scenario with MB concerns for utilization of direct oral anticoagulants (DOAC), especially with active cancer therapies.

AIM

To evaluate patient risk factors, effectiveness (VTE) and safety (MB) of DOACs and low molecular weight heparin (LMWH) in patients with active GICA-VTE.

METHODS

A retrospective chart review of patients receiving DOACs and LMWH with GICA and symptomatic or incidental VTE treated at comprehensive cancer center from November 2013 to February 2017 was performed. Inclusion criteria included active GI cancer diagnosed at any stage or treatment +/- 6 mo of VTE diagnosis,

informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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whom were prescribed 6 mo or more of DOACs or LMWH. The Chi-squared test was used for overall and the Fisher exact test for pairwise comparisons of the proportions of patients experiencing recurrent VTE and MB events. Odds ratios were used to compare the relative odds of the occurrence of the outcome given exposure to the risk factor.

RESULTS

A total of 144 patients were prescribed anticoagulation, in which 106 fulfilled inclusion criteria apixaban (27.3%), rivaroxaban (34.9%) and enoxaparin (37.7%), and 38 were excluded. Patients median age was 66.5 years at GICA diagnosis and 67 years at CAVTE event, with 62% males, 80% Caucasian, 70% stage IV, pancreatic cancer (40.5%), 30% Khorana Score (≥ 3 points), and 43.5% on active chemotherapy. Sixty-four percent of patients completed anticoagulation therapy (range 1 to 43 mo). Recurrent VTE at 6 mo was noted in 7.5% (n = 3), 6.8% (n = 2) and 2.7% (n = 1) of patients on enoxaparin, apixaban and rivaroxaban, respectively (all P = NS). MB at 6 mo were 5% (n = 2) for enoxaparin, 6.8% (n = 2) for apixaban and 21.6% (n = 8) for rivaroxaban (overall P = 0.048; *vs* LMWH P = 0.0423; all other P = NS). Significant predictors of a primary or secondary outcome for all anticoagulation therapies included: Active systemic treatment (OR = 5.1, 95%CI: 1.3-19.3), high Khorana Score [≥ 3 points] (OR = 5.5, 95%CI: 1.7-17.1), active smoker (OR = 6.7, 95%CI: 2.1-21.0), pancreatic cancer (OR = 6.8, 95%CI: 1.9-23.2), and stage IV disease (OR = 9.9, 95%CI: 1.2-79.1).

CONCLUSION

Rivaroxaban compared to apixaban and enoxaparin had a significantly higher risk of MB on GICA-VTE patients with equivocal efficacy.

Key words: Direct oral anticoagulants; Low molecular weight heparin; Gastrointestinal cancer; Venous thromboembolism; Cancer associated thrombosis; Clinical risk

Core tip: Our study shows similar efficacy of low molecular weight heparin as compared to apixaban and rivaroxaban. However, side effect profiles of these new direct oral anticoagulants (DOAC)'s may lead to a preferred use of apixaban, which had lower bleeding events in the highly sensitive gastrointestinal cancer (GICA) patient population. GICA-venous thromboembolism (VTE) is a high-risk patient subpopulation and warrants additional dedicated prospective clinical analysis of the efficacy and safety of DOACs. In addition, evaluation of clinical predictors that may influence the risk of VTE recurrence and major bleeding should include GICA as a high-risk group.

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INTRODUCTION

Venous thromboembolism (VTE) is a common occurrence in cancer patients, specifically in those patients with advanced disease. Cancer-associated VTE (CAVTE) has a three-fold higher risk of recurrent VTE, poorer prognosis and carries with it a significant morbidity and mortality burden as compared to those patients without malignancy[1,2]. The goal of anticoagulation therapy is to prevent VTE recurrence while balancing the risk of bleeding events. Two major randomized controlled clinical trials (RCTs) (CLOT[3] and LITE[4]) demonstrated improved outcomes without secondary safety signals for low molecular weight heparin (LMWH), although 3 other RCTs (CANTHANOX[5], ONCENOX[6] and CATCH[7]) showed equivocal outcomes compared to vitamin K antagonists (VKA) (Table 1)[3-7]. The CLOT trial is the only RCT with mortality benefit at 12 mo in the non-metastatic subgroup (HR = 0.50, 95%CI:

0.27-0.95). Several guidelines including the American Society of Oncology (ASCO)[8], the European Society of Medical Oncology[9], the American College of Chest Physicians[10], and the National Cancer Comprehensive Network[11], recommend LMWH-based therapy over warfarin as the preferred VTE treatment in cancer patients.

Gastrointestinal cancer (GICA) is associated with a higher incidence of VTE compared to other solid tumors[12]. The VTE incidence (per 100 person-years/percent of patients) based on GI site is gastroesophageal 50/7-14, pancreatic 20/5-60, colorectal and anal 13.7/3-10, and hepatobiliary cancer 4.6/2-15[13]. Moreover, recurrent VTE and major bleeding (MB) complications during anticoagulation treatment of GICA patients are increased as compared to other cancer types. (HR = 5.1, 95%CI: 2.3-11.3 and 1.3, 95%CI: 0.3-5.6, VTE and MB respectively)[14]. Several therapies contribute to increased bleeding while on chemotherapy as noted with angiogenesis inhibitors (*e.g.*, bevacizumab[15] and chemotherapy-induced thrombocytopenia (*e.g.*, gemcitabine)[16]. Recurrent VTE and MB complications due to secondary VTE prophylaxis remain a salient issue in treating patients with CAVTE with VKA and LMWH.

Direct oral anticoagulants (DOACs) have been shown to be non-inferior to warfarin for VTE treatment (Table 2)[17-26]. Four major phase III trials with pooled-analysis of patients with active cancer and VTE suggest that apixaban, rivaroxaban, dabigatran, and edoxaban have independently shown similar efficacy to LMWH plus VKA while having less associated MB[27-30]. LMWH remained the preferred treatment regimen for CA-VTE until 2018 when it was challenged by edoxaban in a positive non-inferior open-label RCT for primary-VTE recurrence outcome albeit at the expense of higher MB rate and increased bleeding events in patients with upper GICA[31]. A recent study evaluated rivaroxaban to dalteparin to cancer patients with VTE that yielded a 6-mo non-significant VTE recurrence rate and the safety profile for major bleeds in both arms were not significant. However, noticeable MB was seen with upper GI malignancies and these patients were subsequently excluded from trial enrollment[32]. Cancer-associated VTE in GI malignancy remains a challenging clinical scenario with a lack of data for utilization of DOACs in the setting of primary treatment and secondary prophylaxis, thus the need for a safer DOAC in patients with active GI-CAVTE remains an open question.

Our objective was to retrospectively evaluate cancer patient risk factors, effectiveness, and safety of DOACs and LMWH in patients with active GICA-VTE at The University of Arizona Cancer Center (UACC).

MATERIALS AND METHODS

A retrospective chart review of patients receiving DOACs and LMWH with GICA and symptomatic or incidental VTE treated at UACC between November 2013-February 2017 was performed. We obtained prior to initiating research an Institutional Review Board approval Protocol Number: 1508054987 and further obtained a waiver of personal health information authorization [45 CFR 164.512(i)(2)(ii)]: As the use or disclosure of protected health information involves no more than minimal risk to the individuals and the research could not practicably be conducted without the waiver. GI malignancy included any Gastro-esophageal or junction cancer (squamous and adenocarcinoma), pancreatic cancer (adenocarcinoma), neuroendocrine tumors, colorectal cancer (including appendix and cecum), anal cancer, and hepatobiliary cancer (pathology-confirmed). Any treatment for cancer, prior to, current with, or posterior to CAVTE diagnosis and any stage including recurrent or metastatic cancer were included. Acute symptomatic or incidental deep vein thrombosis (DVT) or pulmonary embolism (PE) diagnosed by venous duplex ultrasound, computed tomography (CT) with intravenous (IV) contrast, ventilation/perfusion (V/Q) scan and/or PE pulmonary angiography protocol by CT or magnetic resonance imaging with IV contrast was required. Primary endpoints included recurrent DVT, nonfatal PE, or fatal PE. Adverse events such as MB included a Hg drop of ≥ 2 g/dL, transfusion of ≥ 2 units of pack of red blood cells (PRBC), bleeding in a critical site, or bleeding contributing to death[33].

Patients who were prescribed 6 mo or more of DOACs [rivaroxaban and apixaban) or LMWH (enoxaparin)] at UACC by retrospective review of medical records were included. Only patients receiving Federal Drug Administration (FDA)-approved VTE dosage were included: Rivaroxaban (Xarelto FDA-approved November 2, 2012) at 15 mg BID for 3 wk, then 20 mg daily and Apixaban (Eliquis FDA-approved August 21, 2014) at 10 mg BID for 7 d, then 5 mg twice daily, and Enoxaparin (Lovenox) at 1 mg/kg/dose every 12 h or 1.5 mg/kg once daily). Crossover of up to a month of

Table 1 Clinical trials for low molecular weight heparin primary efficacy and secondary safety compared to vitamin K antagonist

Study (yr)	InterventionLMWH	Reported efficacy (rVTE) (%)	Reported safety (MB) (%)	Control VKA	Reported efficacy (rVTE) (%)	Reported safety (MB) (%)	Mortality Benefit at 12 mo
CLOT (2003)[3]	Dalteparin	9.0	6.0	Warfarin	17.0	4.0	HR 0.50, 95%CI: 0.27-0.95
LITE (2006)[4]	Tinzaparin	7.0	0.0	Warfarin	16.0	2.1	NS
CANTHANOX (2002)[5]	Enoxaparin	10.5	5.0	Warfarin	21.1	12.0	NS
ONCENOX (2006)[6]	Enoxaparin	6.3	6.5	Warfarin	10.0	2.1	NS
CATCH (2015)[7]	Tinzaparin	7.2	2.6	Tinzaparin + Warfarin	10.5	2.4	NS

LMWH: Low molecular weight heparin; VKA: Vitamin K antagonist; rVTE: Recurrent venous thromboembolism; MB: Major bleeding.

LMWH to DOACs was allowed.

Patients were excluded if DOACs or LMWH were prescribed for any other reason not related to GI-CAVTE (*e.g.*, atrial fibrillation, VTE prophylaxis), when anticoagulation was contraindicated (*e.g.*, active bleed, high bleeding risk, thrombocytopenia, palliative, and hospice care) and in patients with other malignancy not related to a gastrointestinal site. Dabigatran was excluded due to an N of one patient. Edoxaban, tinzaparin, and dalteparin were not prescribed at UACC. Betrixaban was not included due to FDA approval on June 23, 2017, after the conclusion of the review.

GICA subgroup extracted from clinical trial delineations followed: Active cancer, defined as cancer diagnosed at any stage +/- 6 mo of VTE diagnosis. The Chi-squared test was used for overall and the Fisher exact test for pairwise comparisons of the proportions of patients experiencing VTE and MB events. Odds ratios (OR) were used to compare the relative of the occurrence of the outcome given exposure to the risk factor. The 95%CI was used to estimate the precision of the OR. Results were determined to be "statistically significant" when this value was less than or equal to 0.05.

RESULTS

A total of 144 patients were prescribed anticoagulation, in which 106 fulfilled inclusion criteria and 38 were excluded non-malignant indication (atrial fibrillation n = 13), palliative and hospice treatment interruption (n = 8), VTE prophylaxis (n = 2), other anticoagulation (VKA n = 7 and IVC filter n = 1), other concurrent malignancy (n = 5), and outside records (n = 2). Our analysis included patients on Apixaban (n = 29), Rivaroxaban (n = 37) and Enoxaparin (n = 40).

Patients median age was 66.5 years old (range 37-83) at GICA diagnosis and 67 years old (range 37-83) at CAVTE event, and compromised of 62% males and 80% Caucasian (Table 3). The population was typical for those presenting with GI-CAVTE, with 70% having recurrent or metastatic disease, predominately composed of pancreatic cancer (40.5%), with a 30% predictive High Khorana Score (≥ 3 points), and with 43.5% on active chemotherapy. The VTE distribution was 65%, 15%, and 20% for DVT, PE and DVT/PE, respectively. Identifiable risk factors for VTE were seen in 6 patients with recent surgery/hospitalization and 8 patients with diagnosed catheter-related VTE. Approximately, patients were 20% current smokers (n = 10), 40% on active antiplatelet therapy (n = 53) and 7.5% had previous VTE (n = 9). Sixty-four percent of patients completed anticoagulation therapy (range 1 to 40 mo). Patients had similar baseline characteristics compared to Hokusai (Dalteparin n = 140) (Raskob *et al*[31], 2018), AMPLIFY (Apixaban n = 81) (Agnelli *et al*[27], 2015), and pooled-EINSTEIN (Rivaroxaban n = 71) (Prins *et al*[28], 2014) (Supplemental Table 1).

Recurrent VTE at 6 mo was noted in 7.5% (n = 3), 6.8% (n = 2) and 2.7% (n = 1) of patients on enoxaparin, apixaban and rivaroxaban, respectively (all P = NS, P = 0.0623 for rivaroxaban *vs* LMWH, for rivaroxaban vs apixaban P = 0.1659, for apixaban *vs* LMWH P = 1.000). VTE historical cancer subgroups comparison to Hokusai (11.3%), AMPLIFY (3.7%), and EINSTEIN (2.8%) showed no significant difference (all P = NS). MB at 6 mo were 5% (n = 2) for enoxaparin, 6.8% (n = 2) for apixaban and a significantly higher 21.6% (n = 8) for rivaroxaban (overall P = 0.048; *vs* LMWH

Table 2 Clinical trials for direct oral anticoagulants reported recurrent venous thromboembolism and reported mayor bleed outcomes compared to cancer subgroup

Study	Intervention DOAC	Control	Reported efficacy (rVTE) (%)	Cancer subgroup efficacy (%)	Reported safety (MB) (%)	Cancer subgroup safety (%)	Yr
AMPLIFY[17]	Apixaban	Enoxaparin + Warfarin	2.30	3.70	0.6	2.3	2013
AMPLIFY-EXT[18]	Apixaban	Placebo	4.00	NA	0.2	NA	2013
RE-COVER[19]	Dabigatran	Heparin + Warfarin	3.10	3.10	1.60	4.20	2009
RE-COVER II[20]	Dabigatran	Heparin + Warfarin	2.30	2.40	1.20	< 1	2013
RE-MEDY[21]	Dabigatran	Warfarin	1.80	3.30	0.90	NA	2013
RE-SONATE[21]	Dabigatran	Placebo	0.40	NA	0.30	NA	2013
HOKUSAI-VTE[22]	Edoxaban	Warfarin	3.20	3.70	1.40	4.50	2013
EINSTEIN-Choice[25]	Rivaroxaban	Aspirin	1.50	NA	0.5	NA	2017
EINSTEIN-DVT[23]	Rivaroxaban	Enoxaparin+ Warfarin	2.10	3.40	0.8	14.4	2010
EINSTEIN-EXT[26]	Rivaroxaban	Placebo	1.30	2.10	0.7	12.3	2016
EINSTEIN-PE[24]	Rivaroxaban	Enoxaparin+ Warfarin	2.10	2	1.1	12.3	2012

DOAC: Direct oral anticoagulant; LMWH: Low molecular weight heparin; VKA: Vitamin K antagonist; rVTE: Recurrent venous thromboembolism; MB: Major bleeding.

pairwise P = 0.0423; all other P = NS). Historical CAVTE major bleed rate comparison was significantly different for rivaroxaban reported as 2.8% in the EINSTEIN trial (P = 0.0027), and not different as reported in the Hokusai (4%), and AMPLIFY (2.3%), respectively.

Rivaroxaban had one recurrent non-fatal PE event and a significantly worse safety profile with 3 major bleed requiring PRBC, 2 critical bleeding sites (subarachnoid hemorrhage and retroperitoneal), and 3 fatal bleeds [hemopericardium (n = 2) and upper GI bleed] (P = 0.0423), whereas Apixaban had 2 recurrent DVT and 2 major bleeds. LMWH had 1 recurrent non-fatal bleed, 2 DVT, 1 major bleed and 1 fatal bleed [altered mental status presumed intracranial bleed, the family declined further investigation]. Including those events beyond 6 mo, 21.1% of DOACs patients had recorded bleeding events [2 additional major bleeds for apixaban and 2 for rivaroxaban (including another intra-operative fatal bleed following an urgent small bowel resection)] compared to 10% events of LMWH patients (2 more major bleeds) with non-significant difference (P-value 0.5610).

Significant predictors of a primary or secondary outcome for all anticoagulation therapies included: active systemic treatment (OR = 5.1, 95%CI: 1.3-19.3, P = 0.016), high Khorana Score (≥ 3 points) (OR = 5.5, 95%CI: 1.7-17.1, P = 0.003), active smoker (OR = 6.7, 95%CI: 2.1-21.0, P = 0.012), pancreatic cancer (OR = 6.8, 95%CI: 1.9-23.2, P = 0.002), and stage IV disease (OR = 9.9, 95%CI: 1.2-79.1, P = 0.03)(Table 4). Those who suffered a primary or secondary outcome were 17.4 times more likely to die within a month period, compared to those who didn't experience an event (CI: 4.7-63.4, P = 0.0001). Antiplatelet therapy may have affected on four major bleeds (2 rivaroxaban and 2 enoxaparin), although was not a significant risk factor as 11 patients completed therapy without any outcome event (P = 0.479).

DISCUSSION

Review of anticoagulation in cancer treatment

The treatment of VTE in cancer patients aims at reducing mortality and morbidity and improving quality of life, but there are potentially life-threatening challenges - namely hemorrhagic risk and the high rate of recurrence. Until the mid-2000s, the standard treatment for acute CAVTE consisted of initial therapy with LMWH or unfractionated

Table 3 Baseline characteristics of the study population

Baseline characteristics	LMWH (%)	DOACs (%)	Apixaban (%)	Rivaroxaban (%)
N (%)	40	66	29 (44)	37 (56)
Age at cancer diagnosis (median years)	66	67	68	65
(range)	37-80	37-83	43-83	37-79
Age at VTE event (median years)	66	68	68	65
(range)	40-80	37-83	43-83	37-79
Weight (median kg)	71.0	73.0	69.5	77.0
(range)	42-130	42-168	42-168	56-130
Gender (Male)	18 (45)	41 (62)	17 (59)	24 (65)
Race (white)	32 (80)	52 (79)	21 (72)	31 (84)
Current smoker	10 (25)	12 (18)	6 (21)	6 (15)
Antiplatelet therapy	5 (12.5)	8 (12)	5 (17)	3 (8)
Prior treated VTE	2 (5)	7 (11)	2 (7)	5 (13)
Cancer diagnosis				
Pancreas	15 (37.5)	28 (42)	14 (50)	14 (38)
Colon	8 (20)	18 (27)	8 (25)	10 (27)
Rectal	2 (5)	5 (8)	1 (3.5)	4 (11)
NET	3 (7.5)	4 (6)	1 (3.5)	3 (8)
Gastric	4 (10)	3 (5)	1 (3.5)	2 (5)
Esophageal	0	3 (5)	1 (3.5)	2 (5)
Appendix	1 (2.5)	3 (5)	2 (7)	1 (3)
Biliary	3 (7.5)	1 (1)	1 (3.5)	0
GEJ	1 (2.5)	1 (1)	0	1 (3)
HCC	3 (7.5)	0	0	0
Stage at VTE diagnosis				
I	1 (2.5)	1 (1)	1 (3.5)	0
II	7 (17.5)	7 (11)	3 (10.75)	4 (10)
III	3 (7.5)	12 (19)	3 (10.75)	9 (25)
IV	29 (72.5)	46 (69)	22 (75)	24 (65)
Prior chemotherapy	24 (40)	24 (36)	11 (38)	13 (35)
Current chemotherapy	24 (40)	29 (47)	18 (62)	13 (35)
VTE diagnosis				
PE/DVT	11 (27.5)	8 (12)	5 (17)	3 (8)
PE	7 (17.5)	8 (12)	2 (7)	6 (16)
DVT	22 (55)	50 (75)	22 (75)	28 (76)
Identifiable risk factor		10 (16)	5 (17)	5 (14)
Recent surgery/Hospitalization	2 (5)	4 (6)	3 (10)	1 (3)
Central venous catheter	2 (5)	6 (9)	2 (7)	4 (11)
Khorana score				
Low	11 (27.5)	19 (28)	7 (24)	12 (32)
Intermediate	18 (45)	25 (38)	11 (38)	14 (38)
High	11 (27.5)	22 (34)	11 (38)	11 (30)
Therapy completion	25 (62.5)	43 (65)	20 (69)	23 (62)
Anticoagulation length (median mo)	4	6.5	8	6
(range)	1-33	0.3-40	2-29	0.3-40

VTE: Venous thromboembolism; PE: Pulmonary embolism; DVT: Deep vein thrombosis; NET: Neuroendocrine tumor; GEJ: Gastro-esophageal junction; HCC: Hepatocellular carcinoma; DOAC: Direct oral anticoagulant; LMWH: Low molecular weight heparin.

heparin followed by a transition on long-term therapy with an oral anticoagulant with a VKA as the standard of care. The first study (CLOT trial) to challenge this paradigm showed that a specific LMWH, namely dalteparin, was more effective than oral anticoagulation in reducing the risk of recurrent thromboembolism in cancer patients, with a HR 0.48 (95%CI: 0.30-0.77; P = 0.002) over the 6-mo study period. There were

Table 4 Clinical risk factors of a primary or secondary outcome for all anticoagulation therapies

Clinical risk factor	Odds ratio	95%CI	Significance level
Active treatment	5.1	1.3-19.3	$P = 0.0167$
Khorana score high	5.5	1.7-17.1	$P = 0.0033$
Active smoker	6.7	2.1-21.0	$P = 0.0012$
Pancreatic cancer	6.8	1.9-23.2	$P = 0.0023$
Stage IV	9.9	1.2-79.1	$P = 0.0306$
Death after an event	17.4	4.7-63.4	$P < 0.0001$

no differences between groups regarding bleeding rates (14% *vs* 19%; $P = 0.09$) or mortality rates at 6 mo (39% *vs* 41%; $P = 0.53$)[34]. After a plethora of supportive research, LMWH became the new standard of care with significantly lower primary recurrent VTE events balanced by an improved secondary MB profile, although not cancer-site specific.

Effectiveness and safety of DOAC vs LMWH

The utilization of DOACs in cancer patients provides another form of primary and secondary VTE prophylaxis, which must be weighed, based on safety profiles in our study population. In 2019 a systemic review and network meta-analysis, extracted data for "active cancer patient subpopulation" from major DOACs RCT have reported similar rates of VTE recurrence (HR = 0.74, 95%CI 0.54-1.01) and MB (HR = 1.78, 95%CI 1.11-2.87) in DOACs as compared to LMWH, although lower to VKA[35]. To our knowledge, we present the first and largest retrospective analysis with long-term outcome data of DOACs in patients with GICA and VTE, which showed a non-significant risk of recurrent VTE and worse safety profile compared to rivaroxaban, *vs* apixaban or enoxaparin, by indirect comparison[27-29]. Our reported safety profile is consistent with the clinical practice experience literature among non-valvular atrial fibrillation patients on DOACs indirect comparison, whereas apixaban appears to have a lower risk of bleeding than rivaroxaban and any other DOACs[35]. Furthermore, rivaroxaban matched to other DOACs patients had a significantly higher risk of MB (HR = 1.82, 95%CI: 1.36-2.43) compared to apixaban patients[36]. The most recently published systemic review and meta-analysis which included the only 2 published RCTs on this topic to date[31,32] showed that DOACs have a higher incidence of 6-mo major bleeds compared to LMWH for CAVTE (RR: 1.74 (95%CI: 1.05-2.88)[37].

There are significant safety concerns for MB with edoxaban[31], and rivaroxaban[32] in the treatment of GICA-VTE, despite demonstrated non-inferiority in recurrent VTE efficacy to LMWH. Edoxaban was non-inferior for clinically relevant nonmajor bleeds (HR = 1.38; 95%CI: 0.98), however it had a significant risk for major bleeds (HR = 1.77, 95%CI: 10.3-3.04, $P = 0.04$), where upper GI bleeds were associated with half of all major bleeds events and were mainly in patients with GICA with (17 of a total 33 events, $P = 0.02$ for interaction in the safety population)[31]. In our cohort, 4 out of 10 upper GI major bleed events occurred while on DOACs through the 6-mo interval. Although, safety profile for major bleeds on the rivaroxaban study was not significant, a noticeable difference in MB events was identified in patients with upper GI malignancies and consequently a midterm safety analysis elected to exclude their enrollment[32]. It is worth noting that in the Select-D trial, major bleeds occurred more frequently in GICA than in all other included malignancies (13 *vs* 4, $P = 0.0102$) in both arms 5/6 (83%) events on dalteparin and 8/11 (73%) events in rivaroxaban. Surprisingly, pancreatic cancer had no major bleeds contrary to our experience (8/10 rivaroxaban bleeds). Moreover, Mcbane *et al*[38] have presented at 2018 American Society of Hematology preliminary data with regards of oral apixaban therapy associated with significant low MB and VTE recurrence compared to dalteparin in treatment of CAVTE although unknown patient characteristics at present (ADAM-VTE trial pending publication). Cancer-associated VTE in GI malignancy remains a challenging clinical scenario with a lack of data for utilization of DOACs in the setting of primary treatment and secondary prophylaxis, thus the need for a safer DOAC in patients with active GI-CAVTE remains an unmet need.

Risk of VTE/Bleed treatment failure

Our study had similarly high numbers of patients with GI-CAVTE, particularly with cancers of the pancreas (40.5%), consistent with the predictive Khorana risk score for VTE based model by cancer site (Khorana Score stomach and pancreatic cancer site OR = 4.3, 95%CI: 1.2-15.6)[39]. We hypothesize that a high Khorana score may also

predict anticoagulation treatment failure and worse outcomes. Interestingly, during the development of, a clinical predictor of recurrent VTE Ottawa score, the derivation population sample recognized GICA and Stage IV cancer to be at an increased risk for efficacy failure, like our report, although only Lung Cancer and Stage I were included in the validation tool due to a potential statistical limitation[40]. Similar to our cancer center exploratory DOAC cohort, another population-based study has documented Stage IV pancreatic cancer as the strongest predictor of VTE recurrence and bleeding among active cancer patients on LMWH followed by VKA (HR = 6.38, 95%CI: 2.69-15.13)[41].

There is a gap in knowledge of predictive variables for MB in active cancer patients that was addressed by the RIETE group's bleed risk stratification of the general population which included all cancer (OR = 1.7, 95%CI: 1.4-2.2) among others clinical risk items, receiving LMWH plus VKA[42]. Kamphuisen *et al*[43] on behalf of the CATCH trial presented the first pre-specified second analysis were a metastatic stage, older age (> 75 years old) and intracranial lesions described on clinical risk considerations in CAVTE with LMWH (tinzaparin) for major bleed. We found that active systemic treatment and active smoker significantly contributed to treatment failure, regardless of the therapy modality or packs per year smoked, respectively (Table 4). Antiplatelet therapy in "real world" appeared not to contribute to an excess bleed, although we do not recommended combination use unless there is a premising cardiovascular indication. Due to LMWH treatment failure (recurrent VTE or MB) and other patient referred inconveniences (*e.g.*, subcutaneous administration), an unmet need remains to be filled by emerging, effective, safe and more convenient therapies like DOAC.

Limitations

A major limitation of this study is the small sample size of the GICA tumor types, which resulted in wide confidence intervals. Another limitation is the retrospective nature of our analysis, which is unable to capture clinically relevant non-major bleeds due to a lack of detailed documentation to further delineate unscheduled contacts physician recommendations, interruptions in treatment, motives for discontinuation or transition, and patients' preference or discomfort with their treatment. Current clinical trials from multi-center participation will maximize sample size and appropriately power comparison of DOACs with LMWH in which the primary safety outcome should be the rate of major bleed (*e.g.*, apixaban *vs* dalteparin, ADAM-VTE; NCT02585713, pending publication)[38]. Furthermore, GI-CAVTE is a high-risk subpopulation deserves additional prospective clinical analysis of the efficacy and safety of DOACs. In addition, evaluation of clinical predictors that may influence the risk of VTE recurrence and MB could include GICA as a high-risk group. At the present time, ASCO clinical practice guidelines update prefers LMWH on patients with an increase risk for bleeding (*e.g.*, GI malignancy) due to the increased of the reported major bleeds events with DOACs when treating existing CAVTE, until data from ongoing trials and real-world practice provides more safety information[44].

ARTICLE HIGHLIGHTS

Research background

Venous thromboembolism (VTE) is a common occurrence in cancer patients, specifically in those patients with advanced disease. The goal of anticoagulation therapy is to prevent VTE recurrence while mitigating the safety side effects of therapy, mainly major bleed (MB). Gastrointestinal (GI) cancers are associated with a high incidence of thromboembolic events and an even higher risk of bleeding events while on active chemotherapy. Recurrent VTE efficacy and MB safety complications due to secondary VTE prophylaxis remain a noticeable limitation in treating patients with cancer-associated VTE (CAVTE) with vitamin K antagonist and low molecular weight heparin (LMWH). Direct oral anticoagulants (DOACs), a newer set of agents with easier access and administration for CAVTE, have promising effectiveness outcomes although there is a safeness hesitance to utilize these agents in select subsets of high-risk cancer patients.

Research motivation

The current role of DOACs in cancer patients is still unfolding and current treatment guidelines recommend them as a preferred option. Since the advent of DOACs, our clinical practice has noticed an unusual safety profile often having to be addressed by changes in administration, holding of therapy, cessation of therapy or switching to another treatment regimen. We wanted to analyze the efficacy and safety outcome of our own institutional real-world experience with DOAC's in the GI cancer setting.

Research objectives

The goal of our study was to evaluate our institutional outcomes of DOACs and LMWH in patients with active GICA-VTE at The University of Arizona Cancer Center based on safety and

efficacy reported events.

Research methods
Subjects were extracted from a retrospective chart review of GI cancer patients treated at our comprehensive cancer center for incidental or symptomatic VTE with either DOACs or LMWH. Outcomes events, recurrent VTE and MB, were recorded from patients with an active GI malignancy and concurrent anticoagulation therapy at and beyond 6 mo.

Research results
Patients on apixaban (n = 29), rivaroxaban (n = 37) and LMWH (n = 40) met inclusion criteria. Recurrent VTE at 6 mo was noted in 7.5% (n = 3), 6.8% (n = 2) and 2.7% (n = 1) of patients on LMWH, apixaban and rivaroxaban, respectively (all P= NS). MB at 6 mo was 5% (n = 2) for LMWH, 6.8% (n = 2) for apixaban and 21.6% (n = 8) for rivaroxaban (overall P = 0.048; *vs* LMWH P = 0.0423; all other P = NS). Beyond six-months, MB rates were 21% and 10% for DOACs and LMWH (P = NS), respectively, while maintaining efficacy. Significant predictors of any outcome for all anticoagulation therapies included: active systemic treatment (OR - 5.1, 95%CI: 1.3-19.3), high Khorana Score (≥ 3 points) (OR = 5.5, 95%CI: 1.7-17.1), active smoker (OR = 6.7, 95%CI: 2.1-21.0), pancreatic cancer (OR = 6.8, 95%CI: 1.9-23.2), and stage IV disease (OR = 9.9, 95%CI: 1.2-79.1).

Research conclusions
Rivaroxaban compared to apixaban and LMWH had a significantly higher risk of major bleeding on GICA-VTE patients with equivocal efficacy.

Research perspectives
Our study shows similar efficacy of LMWH as compared to apixaban and rivaroxaban. Nonetheless, the safety profiles of these new DOACs have to lead to the preferred use of apixaban, which had lower bleeding events in the high-risk GI cancer patient population.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Fat clearance and conventional fixation identified ypN0 rectal cancers following intermediate neoadjuvant radiotherapy have similar long-term outcomes

Nan Chen, Ting-Ting Sun, Zhong-Wu Li, Yun-Feng Yao, Lin Wang, Ai-Wen Wu

ORCID number: Nan Chen (0000-0002-0085-7472); Ting-Ting Sun (0000-0001-5719-7236); Zhong Wu Li (0000-0003-3440-9077); Yun-Feng Yao (0000-0001-8433-0688); Lin Wang (0000-0002-5313-5297); Ai-Wen Wu (0000-0002-9650-1962).

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Nan Chen, Ting-Ting Sun, Yun-Feng Yao, Lin Wang, Ai-Wen Wu, Department of Gastrointestinal Surgery, Peking University Cancer Hospital, Beijing 100142, China

Zhong-Wu Li, Department of Pathology, Peking University Cancer Hospital, Beijing 100142, China

Corresponding author: Lin Wang, MD, Assistant Professor, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Gastrointestinal Center, Peking University Cancer Hospital and Institute, 52 Fucheng Road, Haidian District, Beijing 100142, China. wanglinmd@foxmail.com
Telephone: +86-10- 88196086
Fax: +86-10-88196086

Abstract

BACKGROUND

As a prognostic factor for colorectal cancer, lymph node (LN) status, particularly the number of LN harvested, has been demonstrated to be essential in the evaluation of quality control in terms of surgical specimen. Neoadjuvant chemoradiation, however, decreases the LN harvest. Therefore, certain approaches (such as fat clearance or methylene blue) has drawn significant attention in order to raise LN yield.

AIM

To compare the long-term oncologic outcome of ypN0 rectal cancer identified using fat clearance (FC) or conventional fixation (CF) following 30 Gy in 10 fractions (30 Gy/10f) of neoadjuvant radiotherapy (nRT).

METHODS

Three hundred and eighty-two patients with resectable and locally advanced rectal cancer were treated by 30 Gy/10f intermediate nRT (biologically equivalent dose of 36 Gy) plus total mesorectal excision. Two specimen fixation methods (FC or CF) were non-randomly used. The ypN0 status was identified in 124 and 101 patients in the FL and CF groups, respectively. Primary endpoints were local recurrence-free survival (LRFS) and cancer-specific survival (CSS).

RESULTS

The median follow-up of patients was 5.1 years. The median numbers of

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retrieved LNs in the FC and CF groups were 19.5 (range, 4-47) and 12 (range, 0-44), respectively, with a significant difference (P = 0.000). The percentages of patients with 12 or more retrieved nodes were 82.3% and 50.5% (101/159) in the FC and CF groups, respectively, with a significant difference (P = 0.000). The LRFS at 5 years were 95.7% and 94.6% in the FC and CF groups, respectively, without statistical difference (P = 0.819). The CSS at 5 years were 92.0% and 87.2% in the FC and CF groups, respectively, without statistical difference (P = 0.482).

CONCLUSION
For patients with ypN0 rectal cancer who underwent 30 Gy/10f preoperative radiotherapy, the increased retrieval of LNs using fat clearance is not associated with survival benefit. This time-consuming fixation method has a low efficacy as a routine practice.

Key words: Neoadjuvant radiotherapy; Rectal cancer; Fat clearance; Survival; Lymph node; Conventional fixation

Core tip: Enhanced lymph node (LN) yield has been noticed to be associated with increasing accuracy in tumor staging and putative prognosis. By the means of fat-clearance technique, the LN retrieval was significantly higher in the fat-clearance group, compared with convention fixation. In terms of survival, however, for patients with negative LN, increased LN harvest was not associated with prolonged survival.

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INTRODUCTION

Neoadjuvant radiotherapy (nRT) followed by total mesorectal excision (TME) has significantly improved the local control of patients with rectal cancer, and it has become the standard treatment for locally advanced rectal cancers[1,2]. Short-course nRT, compared with long-course chemoradiotherapy, has been demonstrated with the advantages of safety, high efficiency, good compliance, and improved oncological outcomes[3,4]. In two randomized trials[5], the short-course nRT is shown to be comparable with long-course chemoradiation for either local control or survival. In 2016, we reported the local control and survival data of intermediate nRT (30 Gy in 10 fractions; 30 Gy/10f) plus TME[6].

As the most important determinant of local recurrence and overall survival, lymph node (LN) status is critical in patients with rectal cancer. Inadequate LN staging may result in excessive or insufficient treatment. Several guidelines recommend a minimum examination of 12 LNs with the goal of accurately identifying the status of pN0 for colorectal cancers. However, this '12 LN' threshold for precise histological examination of rectal cancer remains unclear, especially for patients undergoing neoadjuvant radiotherapy[1].

We previously reported the LN distribution and pattern of metastases in the mesorectum using the modified fat clearing technique. This technique can reveal small LNs (1-3 mm) and increase the LN harvest in the specimens of rectal cancers following 30 Gy/10f nRT. In the present study, we aimed to compare the long-term oncologic outcome of ypN0 rectal cancer identified using the modified fat clearance or conventional fixation method following 30 Gy/10f nRT.

MATERIALS AND METHODS

Patient selection

Data were collected from patients who underwent intermediate nRT followed by

TME surgery at Peking University Cancer Hospital from August 2002 to March 2011. In this study, the nRT regimen used was previously approved by the Ethics Committee of Peking University Cancer Hospital. Informed consent was obtained from all participants before treatment.

Each patient enrolled in the study conformed to the following criteria: (1) The patient was diagnosed with rectal adenocarcinoma by biopsy; (2) The lesion was located within 10 cm from the anal verge; (3) The cancer was staged as T3-4 or any T and N+ by pelvic magnetic resonance imaging (MRI) or computed tomography (CT); (4) Patients with distant metastases were excluded by imaging examinations; (5) The patient underwent neoadjuvant RT of 30 Gy/10f; (6) The patient underwent the surgery with the intent to cure, according to the TME principle; and (7) The patient was diagnosed with ypN0 following postoperative pathological evaluation.

Patients with the following characteristics were excluded: (1) The patient had undergone previous chemotherapy or pelvic radiation; (2) The patient had a malignant tumor history within 5 years; (3) Inflammatory bowel disease; (4) The presence of acute obstructive symptoms or serious comorbidities deemed not suitable for neoadjuvant radiation; and (5) The presence of unresectable cancer.

Treatment

All patients enrolled underwent nRT followed by curative TME. The radiotherapy regimen included 30 Gy delivered in 10 fractions for 2 wk. The biological equivalent dose (BED) of this regimen is 36 Gy. Three-dimensional conformal radiotherapy (3D-CRT) was routinely employed. Patients underwent radical curative surgery according to the principles of TME at a median interval of 2 wk (range: 2 to 8 wk) from the completion of nRT. After surgery, the patients underwent 5-fluorouracil-based chemotherapy if they were able to tolerate the therapy. Capecitabine alone, mFOLFOX6, or CapeOX was equivalently preferred regimen, as recommended by the National Comprehensive Cancer Network (NCCN) guidelines.

Specimen fixation and fat clearance

The rectum specimen was fixed with modified LN revealing solution (LNRS) in order to facilitate LN yielding. The LNRS was prepared according to a modified Koren solution with a mixture of 40% ethanol, 40% ether, 10% acetic acid, and 10% formaldehyde. The entire rectal specimen was submerged in LNRS for 48 h. After fat clearance, the LNs appeared as chalk white foci against a yellow and translucent adipose background[7]. Tumor sliced samples and retrieved LNs were submitted for routine paraffin embedding and hematoxylin and eosin staining. Small LNs with a diameter of 1-3 mm could be clearly revealed through fat clearance (Figures 1 and 2).

Pathologic evaluation

The 8th edition of the American Joint Committee on Cancer TNM system was employed for staging. Postoperatively, the results of the histopathologic examination of the specimens were reviewed by the same group of experienced pathologists, and circumferential resection margin (CRM) involvement was assessed using the protocol of Kitz *et al*[8]. The negative status of N staging was identified through a routine microscopic evaluation. More intense histologic or immunohistochemical investigations (such as cytokeratin staining) to detect the presence of metastatic carcinoma were not employed in the present study.

Endpoints

The primary endpoints were local recurrence-free survival (LRFS) and cancer-specific survival (CSS). The LRFS was defined as the time from the date of nRT completion to the date of local recurrence. The CSS was defined as the time from the date of nRT completion to the death from the same cancer or from other related causes. The secondary endpoints included the median number of retrieved LNs and the proportion of patients who achieved the 12 LN thresholds.

Follow-up

Patients were routinely followed at three-month intervals in the first two years after surgery and then at six-month intervals for the next three years. Evaluations included physical examination, serum CEA levels, complete blood count, blood chemical analysis, proctoscopy, abdominal ultrasonography, abdominal and pelvic CT, and chest radiography.

Statistical analysis

The IBM SPSS Statistics for Macintosh, Version 22.0 (Armonk, NY, IBM Corp.) was used for analyses. The enumeration variables were analyzed using the Mann-Whitney *U* nonparametric test. The categorical variables were analyzed using the Pearson chi-

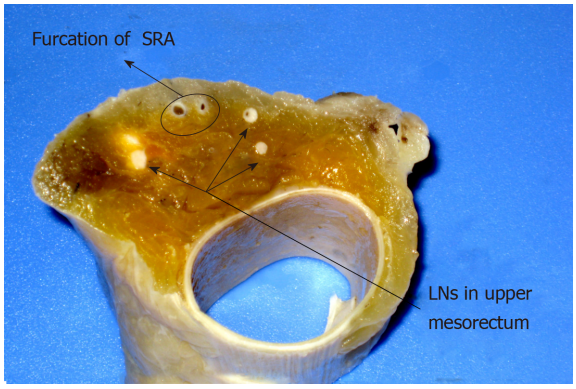

Figure 1 The effect of lymph nodes revealing after fat clearance. Small lymph nodes appeared as chalk white foci against a yellow and translucent adipose background. SRA: Superior rectal artery; LN: Lymph node.

squared or Fisher's exact test. The Kaplan-Meier survival curve was used to estimate the proportion of patients surviving or remaining disease-free at each time interval. The log-rank test was used for comparison of the Kaplan–Meier curves. The level of significance was set at 0.05.

RESULTS

Clinicopathological and demographic data

In the corresponding time period, 382 patients with rectal cancer underwent 30 Gy/10f nRT plus TME at our center, and 212 patients underwent fat clearance. A total of 225 consecutive patients with ypN0 stage were analyzed, including 101 of 170 (59.1%) patients who had conventional fixation (CF group) and 124 of 212 (58.5%) patients who had fat clearance (FC group). The median patient age was 62 years (range, 28-83 years) and 58 years (range, 32-84 years) in the CF and FC groups, respectively. The percentages of male patients in the CF and FC groups were 63.4% and 55.6%, respectively. The baseline clinicopathological factors, including clinical T and N stages, tumor distance to anal verge, prestaging methods, resection types, ypT stage distribution, and CRM status, were well matched and comparable between the two groups. The percentage of patients who underwent adjuvant chemotherapy at our center was 30.7% (n = 69), and additional data were unavailable for other patients who received postoperative care in peripheral hospitals. Moreover, the use of adjuvant chemotherapy was not analyzed in this study. All patient characteristics, pre-staging methods, and pathological findings are listed in Table 1.

LN retrieval

The median number of retrieved LNs in the FC group was significantly higher than that in the CF group (19.5 and 12, P = 0.000), which is similar to the difference found in the ypT0-2 stages (19 and 9, P = 0.000) and ypT3-4 stages (21.5 and 13, P = 0.000).

The proportions of patients who achieved the 12 LN threshold were 82.3% and 50.5% in the FC and CF groups, respectively, with a statistical difference (P = 0.000), which is similar to the difference found in ypT0-2 stages (81.6% and 34.6%, P = 0.000). The proportion of patients who achieved the 12 LN threshold was not statistically different in ypT3-4 stages between the FC and CF groups (83.3% and 67.3%, P = 0.068).

LRFS and CSS

Last follow-up was implemented in December 2014. The median follow-up period was 5.1 years. The estimated 5-year LRFS were 95.7% and 94.6% in the FC and CF groups, respectively, without significant difference (P = 0.819) (Figure 3; Table 2). The CSS at 5 years were 92.0% and 87.2% in the FC and CF groups, respectively, without statistical difference (P = 0.482) (Figure 3B).

DISCUSSION

The ideal threshold for retrieved LNs in patients with rectal cancer has been unclear for years, and the 12 LN threshold is extrapolated from the recommendation of

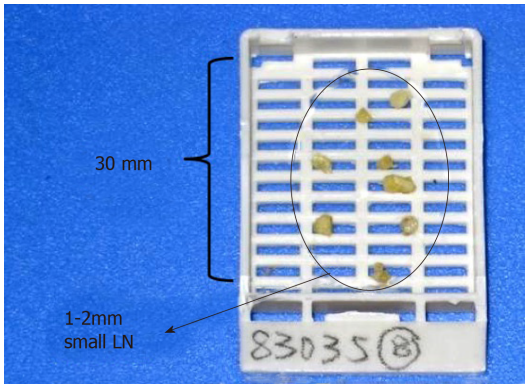

Figure 2 Small lymph nodes could be retrieved after fat clearance. The length of the sampling cassette is 30 mm.

pathological identification for stage II colon cancer. For patients with rectal cancer who underwent nRT, the retrieved LN number significantly decreases from 7% to 53% compared to those who did not undergo nRT[9,10]. After nRT, the proportion of patients who achieved more than 12 LNs is also low, from 31% to 37%[11].

Short-course nRT is recommended as routine care for low- or moderate-risk patients with rectal cancer according to the NCCN and European Society for Medical Oncology (ESMO) guidelines[12,13]. Moreover, short-course nRT decreases local failure than long-course postoperative chemoradiotherapy[3]. We previously reported the survival data of patients who underwent intermediate nRT plus TME for locally advanced rectal cancer, which has a similar biological equivalent dose (BED) and treatment schedule to short-course nRT[14]. We previously used the fat clearance as an intensive LN revealing method to reduce the difficulty of finding small LNs in the mesorectal fat tissue and to harvest more LNs.

In the present study, we focused on testing the effect of fat clearance to identify the true ypN0 rectal cancer and to observe whether the fat clearance-confirmed ypN0 rectal cancer could have survival benefit than those diagnosed using conventional fixation. Initially, we hypothesized that the ypN0 cases identified by fat clearance could eliminate those under-staged cases with small metastatic LNs. From the data of this study, the fat clearance could significantly increase the retrieval of LNs for ypN0 rectal cancer following 30 Gy/10f preoperative radiotherapy; however, the final comparison showed that the survival rates are similar between the FC and CF groups. The findings of our study demonstrated the fact that the increased LN retrieval is not associated with survival benefit in patients with ypN0 rectal cancer and might provide piece of evidence to question the necessity of pursuing higher number LN retrieval after nRT.

The ideal cut-off value of LN retrieval is highly controversial in colorectal surgery, especially for rectal cancer following nRT. In previous studies, the aim of retrieving more LNs is to discriminate positive LNs, since the positive ypN stage status is one of the most influential factors of long-term outcome[15,16]. Moreover, more retrieved LNs seem to be associated with better survival even in N0 or ypN0 patients[17]. Thus, a cut-off number of 3 or 7 or 8 or 11 LNs, or a range from 7-11 LNs based on survival stratification was recommended in various retrospective studies[18]. In the present study, the median LN retrieval number was 12 and 19.5 in the CF and FC groups, respectively, which is similar to other reports. However, data from other studies indicated that the number of retrieved LNs failed to be demonstrated as a prognostic factor for either overall or disease-free survival[19]. Furthermore, the absence of LNs (ypNx) in the resected rectum after nCRT seems to be associated with good disease-free survival rates and reflect improved response to nCRT rather than inappropriate or suboptimal radicality of resection. Finally, many studies using survival data to confirm the cut-off value of LN number conclude that the ypN status is a more stronger prognostic factor than LN retrieval itself[20,21].

Apart from the controversy over the cut-off value of LNs, more issues were raised in this field. First, the point that more LN counts could increase the N positive rate is challenged. The 12 LN threshold is not a universal standard among hospitals, and efforts to increase node examination rates have a limited value as a public health intervention[22]. Second, data from a large population of patients with colorectal cancer also demonstrated that the number of LNs for colorectal cancer experienced a markedly increase in the last two decades but was not associated with an overall shift to higher-staged tumors, leading to the controversy over the upstaging mechanism as

Table 1 Patient characteristics, pre-staging methods, and pathological stages of conventional fixation group versus fat clearance group, *n* (%)

Characteristic	Conventionalfixation (*n* = 101)	Fat clearance (*n* = 124)	*P* value
Sex			
Male	64 (63.4)	69 (55.6)	0.241
Female	37 (36.6)	55 (44.4)	
Age (yr)			
Median	62	58	0.496
Range	28-83	32-84	
cT stage			
T0-2	4 (4.0)	9 (7.3)	0.170
T3	93 (92.1)	114 (91.9)	
T4a	4 (4.0)	1 (0.8)	
cN stage			
N0	26 (25.7)	30 (24.2)	0.789
N+	75 (74.3)	94 (75.8)	
Distance from anal verge (cm)			
Median	5	5	0.299
Range	2-10	1-9	
Pre-treatment staging			
MRI + ERUS	27 (26.7)	22 (17.7)	0.428
MRI	15 (14.9)	23 (18.5)	
CT + ERUS	15 (14.9)	19 (15.3)	
ERUS	30 (29.7)	35 (28.2)	
CT	14 (13.9)	25 (20.2)	
Interval from RT to surgery			
Median	2	2	0.702
Range	1-8	1-6	
Type of resection			
Non-APR	68 (67.3)	90 (72.6)	0.391
APR	33 (32.7)	34 (27.4)	
ypT stage			
ypCR	7 (6.9)	14 (11.3)	0.550
T1	8 (7.9)	11 (8.9)	
T2	37 (36.6)	51 (41.1)	
T3	47 (46.5)	47 (37.9)	
T4a	2 (2.0)	1 (0.8)	
CRM status			
Positive	6 (5.9)	8 (6.5)	0.875
Negative	95 (94.1)	116 (93.5)	

MRI: Magnetic resonance imaging; ERUS: Endorectal ultrasound; CT: Computed tomography; RT: Radiotherapy; CRM: circumferential resection margin.

the primary basis for improved survival in patients with more LNs evaluated[23]. In fact, Ervine *et al*[24] concluded that only 1% of colorectal cancers were upstaged using an enhancing method for LN examination. Finally, the complexity of the LN count should be considered, in terms of mesenteric LN anatomy, molecular aspects, tumor characteristics, surgical procedure, and utilization of different sampling techniques[25,26]. In the present study, the ypN0 rate was 60% in both the FC and CF groups of all rectal cancers after 30 Gy/10f nRT. These data consolidate the marginal utility of retrieving more small LNs and might support the hypothesis of the constant nodal positivity of 40% across a wide range of studies.

Compared with colon cancer, LN retrieval for rectal cancer is also influenced by the intensity and schedule of nRT/nCRT and patients' intrinsic sensitivity to nRT. Therefore, several enhancing methods for LN examination, including various LN revealing solutions, meticulous sampling/resampling procedure or maneuver, and

Table 2 Lymph nodes retrieval, local recurrence-free survival, and cancer-specific survival of conventional fixation group versus fat clearance group

Characteristic	Fat clearance	Conventionalfixation	*P* value
LNs retrieved [median (range)]			
All T stages	19.5 (4-57)	12 (0-44)	0.000
ypT0-2	19 (5-57)	9 (0-30)	0.000
ypT3-4	21.5 (4-55)	13 (1-44)	0.000
Lymph nodes ≥ 12			
All T stages	82.3%	50.5%	0.000
ypT0-2	81.6%	34.6%	0.000
ypT3-4	83.3%	67.3%	0.068
5 yr-LRFS rate	95.7%	94.6%	0.819
5 yr-CSS rate	92.0%	87.2%	0.482

LN: Lymph node; LRFS: Local recurrence-free survival; CSS: Cancer-specific survival.

some staining methods such as methylene blue injection, were used in rectal cancer[27,28]. In this study, we used a modified Koren solution to reveal more small LNs. We obtained significantly more LNs than using the conventional fixation; nevertheless, this effort neither identified truer ypN0 rectal cancers nor achieved survival benefit in fat clearance-confirmed ypN0 patients. For this reason, we conclude that fat clearance is not feasible to be routinely used for rectal cancers following 30 Gy/10f nRT. We do not also advocate using this method for resampling in rectal cancers with ≤ 12 LNs, because fat clearance is time-consuming and with potential toxicity, and the ideal cut-off value of LN retrieval is still unclear.

This study has several limitations. First, the retrospective nature and long time span of the present study limited its strength. Second, the unique nRT schedule in this study has lower BED and shortened interval than the conventional long-course nCRT, which limited the utilization of the finding in other series. Despite these limitations, the sample size and efforts of pathological management remain convincing when compared with the literature. Although the conclusion is a negative result, we believe that this article could add useful and referable information for study of LN retrieving after nRT in future.

In conclusion, for patients with ypN0 rectal cancer who underwent 30 Gy/10f preoperative radiotherapy, the practice of increased retrieval of LNs using fat clearance might not be an essential factor associated with survival benefit. The efficacy of this time-consuming fixation method remains controversial, compared with the conventional practice.

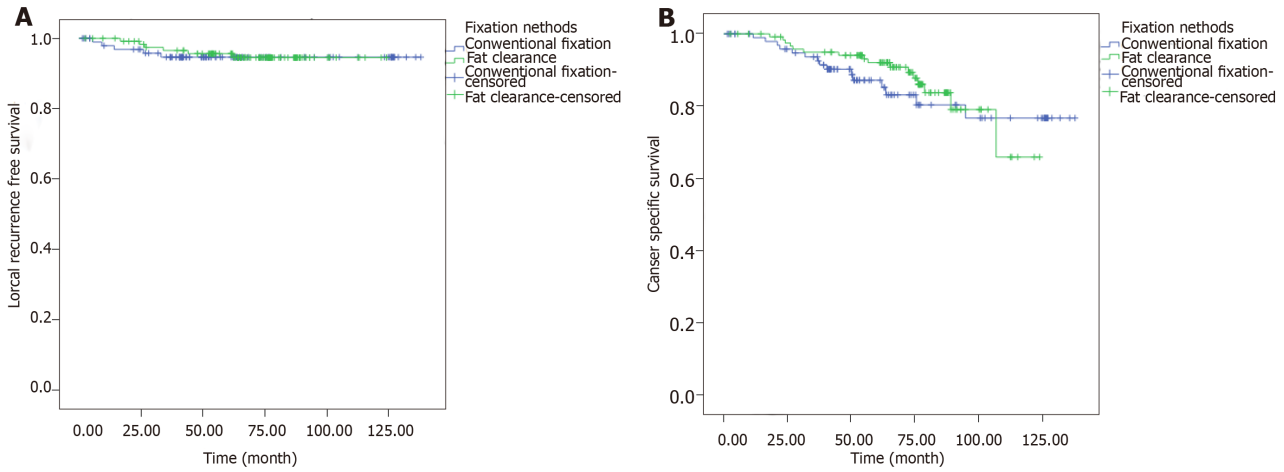

Figure 3 Kaplan–Meier curves. A: The Kaplan–Meier curve of local recurrence-free survival (LRFS): the estimated 5-year LRFS rates were 95.7% and 94.6% in fat clearance (FC) and conventional fixation (CF) groups, respectively (P = 0.819); B: The Kaplan–Meier curve of cancer-specific survival (CSS): the estimated 5-year CSS rates were 92.0% and 87.2% in FC and CF groups, respectively (P = 0.482).

ARTICLE HIGHLIGHTS

Research background

For accurate tumor staging, it is recommended to obtain at least 12 lymph nodes (LNs) by international guidelines (such as the National Comprehensive Cancer Network and European Society for Medical Oncology guidelines). However, the number of LN decreases after neoadjuvant chemoradiation, leading to the hypothesis that enhanced LN yield would bring survival benefit.

Research motivation

Different methods have been implemented, trying to increase LN harvest. In this study, we employed the fat-clearance technique for LN yielding. So far, this study provided convincing evidence with big numbers of cases and long-term follow-up.

Research objectives

This study aimed to evaluate the efficacy of fat-clearance technique in terms of LN retrieval and potential prognostic values.

Research methods

This study employed the fat-clearance technique, which was demonstrated to be effective with a high sensitivity.

Research results

The conclusion of this study confirms the fact that for patients without LN metastasis, higher yield of LN might be only a time-consuming procedure, rather than prognostic approach.

Research conclusions

In rectal cancer patients undergoing neoadvjuant chemoradiation without LN metastasis, the pursuit for more LN harvest is not beneficial. Fat-clearance technique might not be useful for pN0 patients. Decreased number of LN in rectal cancer patients with neoadjuvant chemoradiation might be of nature, with no necessity to increase retrieval in pN0 patients. In pN0 rectal cancer patients with neoadjuvant conformal radiotherapy (CRT), additional LN retrieval might be useless. The 12 LN rule might not be essential for accurate staging. The fat-clearance technique utilized in this paper is a new method. The increased number of LNs did not bring in longer survival and was not associated with survival benefit. The pursuit for higher number of LNs retrieved might be of no use, therefore, to prolong patients' survival, new strategy of treatment might be useful.

Research perspectives

The 12 LN rule might not work in patients with neoadjuvant CRT. Lymph node positivity or positive LNs might be more important in terms of prognostic value. Methods for tracing the positive LN might be the best way for the research in the future.

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ORIGINAL ARTICLE

Observational Study

Acylcarnitine: Useful biomarker for early diagnosis of hepatocellular carcinoma in non-steatohepatitis patients

Hiroaki Takaya, Tadashi Namisaki, Mitsuteru Kitade, Naotaka Shimozato, Kosuke Kaji, Yuki Tsuji, Keisuke Nakanishi, Ryuichi Noguchi, Yukihisa Fujinaga, Yasuhiko Sawada, Soichiro Saikawa, Shinya Sato, Hideto Kawaratani, Kei Moriya, Takemi Akahane, Hitoshi Yoshiji

ORCID number: Hiroaki Takaya (0000-0002-4990-7573); Tadashi Namisaki (0000-0002-3158-5318); Mitsuteru Kitade (0000-0001-7592-7589); Naotaka Shimozato (0000-0002-7558-4165); Kosuke Kaji (0000-0002-1822-6759); Yuki Tsuji (0000-0001-7625-4208); Keisuke Nakanishi (0000-0002-6330-6205); Ryuichi Noguchi (0000-0002-4559-7600); Yukihisa Fujinaga (0000-0003-2402-574X); Yasuhiko Sawada (0000-0002-4875-5360); Soichiro Saikawa (0000-0002-3440-050X); Shinya Sato (0000-0003-3049-3443); Hideto Kawaratani (0000-0002-4361-0592); Kei Moriya (0000-0002-2878-8296); Takemi Akahane (0000-0002-6675-0475); Hitoshi Yoshiji (0000-0002-5243-8544).

Author contributions: Takaya H, Kitade M, Shimozato N, Kaji K, Tsuji Y, Nakanishi K, Noguchi R, Fujinaga Y, Sawada Y, Saikawa S, Sato S, Kawaratani H, Moriya K and Akahane T performed data analysis; Takaya H, Namisaki T and Yoshiji H contributed to the writing of the manuscript.

Institutional review board statement: Informed consent for the use of resected tissue was obtained from all patients, and the study protocol was approved by the Ethics Committee of Nara Medical University.

Informed consent statement: All study participants or their legal

Hiroaki Takaya, Tadashi Namisaki, Mitsuteru Kitade, Naotaka Shimozato, Kosuke Kaji, Yuki Tsuji, Keisuke Nakanishi, Ryuichi Noguchi, Yukihisa Fujinaga, Yasuhiko Sawada, Soichiro Saikawa, Shinya Sato, Hideto Kawaratani, Kei Moriya, Takemi Akahane, Hitoshi Yoshiji, Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara 6348522, Japan

Corresponding author: Hiroaki Takaya, MD, PhD, Assistant Professor, Third Department of Internal Medicine, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 6348522, Japan.
htky@naramed-u.ac.jp
Telephone: +81-744-223051
Fax: +81-744-247122

Abstract

BACKGROUND

Early diagnosis of hepatocellular carcinoma (HCC) is necessary to improve the prognosis of patients. However, the currently available tumor biomarkers are insufficient for the early detection of HCC. Acylcarnitine is essential in fatty acid metabolic pathways. A recent study reported that a high level of acylcarnitine may serve as a useful biomarker for the early diagnosis of HCC in steatohepatitis (SH) patients. In contrast, another study reported that the level of acetylcarnitine (AC2) - one of the acylcarnitine species - in non-SH patients with HCC was decreased *vs* that reported in those without HCC.

AIM

To investigate the usefulness of acylcarnitine as a biomarker for the early diagnosis of HCC in non-SH patients.

METHODS

Thirty-three non-SH patients (14 with HCC and 19 without HCC) were enrolled in this study. Blood samples were obtained from patients at the time of admission. The levels of acylcarnitine and AC2 in the serum were determined through tandem mass spectrometry. The levels of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) were determined by enzyme-linked immunosorbent assay. Univariate and multivariate analyses were used to determine early diagnostic factors of HCC.

RESULTS

The level of acylcarnitine was significantly lower in non-SH patients with HCC *vs*

those without HCC ($P < 0.05$). In contrast, the level of lens culinaris agglutinin-reactive fraction of α-fetoprotein (AFP) - AFP-L3% - was significantly higher in non-SH patients with HCC *vs* those without HCC ($P < 0.05$). However, the levels of total carnitine, free carnitine, AFP, des-γ-carboxy prothrombin, VEGF, and VEGFR-2 were not different between patients with and without HCC. The multivariate analysis showed that a low level of acylcarnitine was the only independent factor for the early diagnosis of HCC. The patients with a low level of AC2 had a significantly higher level of VEGF *vs* those with a high level of AC2 ($P < 0.05$).

CONCLUSION

The metabolic pathways of fatty acids may differ between SH HCC and non-SH HCC. Further studies are warranted to investigate these differences.

Key words: Acylcarnitine; Acetylcarnitine; Biomarker; Hepatocellular carcinoma; Angiogenesis; Carnitine palmitoyltransferase 1; Oxidative stress

Core tip: There is an urgent clinical need for the early diagnosis of hepatocellular carcinoma (HCC) in cirrhotic patients to improve prognosis. A recent study reported that a high level of acylcarnitine may be a useful biomarker for the early diagnosis of HCC in steatohepatitis (SH) patients. However, the level of acylcarnitine was significantly lower in non-SH patients with HCC than in those without HCC. Multivariate analysis showed that a low level of acylcarnitine was the only independent early diagnostic biomarker for non-SH HCC. Thus, the fatty acid metabolic pathways in SH HCC and non-SH HCC patients may be different.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the types of cancer with the highest mortality rates worldwide[1,2]. Early diagnosis of HCC is necessary to improve the prognosis of patients. Recently, imaging diagnosis has been used for the detection of HCC at an early stage. However, this approach is limited by the high cost and side effects associated with the use of the contrast medium. Although numerous biomarkers, including α-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), and lens culinaris agglutinin-reactive fraction of AFP (AFP-L3%) have been developed, they are not useful in the early diagnosis of HCC[3].

Carnitine is a water-soluble compound and an essential nutrient required in fatty acid metabolic pathways such as β-oxidation[4,5]. In humans, approximately 98% of the carnitine is present in the liver, skeletal muscle, heart, and kidneys[4,5]. In the plasma, carnitine is present as free carnitine or acylcarnitine[4,5]. Acyl-coenzyme A (CoA) synthetase catalyzes the conversion of fatty acids and CoA into acyl-CoA. Acyl-CoA is converted to acylcarnitine by carnitine palmitoyltransferase 1 (CPT1) in the outer mitochondrial membrane, while acylcarnitine is converted back to acyl-CoA by CPT2 in the inner mitochondrial membrane[4,5]. This process is followed by β-oxidation. Therefore, CPT1 and CPT2 are involved in the metabolic pathways of fatty acids in the carnitine cycle. Following the downregulation of CPT1, the level of acylcarnitine is decreased. The inverse is observed following the downregulation of CPT2.

A recent study reported that the level of acylcarnitine in steatohepatitis (SH) patients with HCC was increased compared with that reported in SH patients without HCC[6]. In addition, CPT2 was downregulated in SH patients with HCC[6,7]. Hence, a high level of acylcarnitine may serve as a useful biomarker for the early diagnosis of HCC in SH patients. Consequently, a high level of acylcarnitine has been linked to the

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development of HCC in SH patients. In contrast, another study reported that the level of acetylcarnitine (AC2) - one of the acylcarnitine species - was decreased in non-SH patients with HCC *vs* those without HCC, the level of AC2 was associated with tumor stage, and the expression of AC2 in HCC tissue was decreased according to tumor stage[8]. In addition, CPT1 was shown to be downregulated in non-SH patients with HCC[8,9]. Therefore, a low level of AC2 has been associated with the development of HCC. Furthermore, a recent study reported that AC2 significantly downregulated the expression of vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2), C-X-C motif chemokine 12 (CXCL12) and C-X-C chemokine receptor 4 (CXCR4) in human umbilical vein endothelial cells (HUVECs)[10]. Thus, it was suggested that AC2 possesses anti-angiogenic properties through the VEGF and CXCL12 pathways.

Based on this evidence, it was hypothesized that the fatty acid metabolic pathways in the carnitine cycle may differ between SH HCC and non-SH HCC. Therefore, in the present study, we investigated the relationship between acylcarnitine and non-SH HCC, and assessed the usefulness of acylcarnitine as an early diagnostic biomarker for HCC in non-SH patients.

MATERIALS AND METHODS

Patients

The levels of acylcarnitine in the serum were evaluated in 40 cirrhotic patients (20 with HCC and 20 without HCC) admitted to Nara medical university from April to November 2016. We excluded patients with alcoholic SH and non-alcoholic SH. Eventually, a total of 33 non-SH patients (14 with HCC and 19 without HCC) were enrolled in this study. The diagnosis of liver cirrhosis was based on physical findings, laboratory tests, and histological criteria, according to the evidence-based clinical practice guidelines for liver cirrhosis established in 2015[11] by The Japan Society of Gastroenterology. All patients underwent blood examination, including for AFP, DCP, and/or AFP-L3%, every 3-4 mo. Moreover, they underwent ultrasound examination, dynamic computed tomography, and/or dynamic magnetic resonance imaging every 4-6 mo. The surveillance, diagnosis, and treatment of HCC was performed in accordance with the clinical practice guidelines for HCC established in 2013[12] by The Japan Society of Hepatology. After diagnosis of HCC, all HCC patients received radiofrequency ablation. None of the patients had infection, ascites, hepatic encephalopathy, uncontrolled gastroesophageal varices, or kidney disease. All patients provided written informed consent prior to their participation in this study.

Measurement of acylcarnitine levels

The levels of free carnitine and acylcarnitine in the serum were determined through tandem mass spectrometry[13] conducted at Sekisui Medical Co., Ltd. (Tokyo, Japan).

Measurement of VEGF and VEGFR-2 levels

The levels of VEGF were determined using commercially available immunoassay kits from RayBiotech, Inc. (Norcross, Georgia, United States), while the levels of VEGFR-2 were determined using immunoassay kits from R and D Systems, Inc. (Minneapolis, Minnesota, United States). The detection limit for the level of VEGF was < 10 pg/mL, while that for the level of VEGFR-2 was < 11.4 pg/mL.

Statistical analysis

Differences between the groups were analyzed using the Mann-Whitney *U* test. Correlations were calculated using the Spearman rank test. Categorical data were analyzed using the Fisher's exact test. Univariate and multivariate analyses were performed to identify early diagnostic factors of HCC. A logistic regression analysis with stepwise selection of variables was applied to determine independent early diagnostic factors of HCC. The data are expressed as median (interquartile range). A two-tailed $P < 0.05$ denoted statistical significance. Analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0). Specifically, EZR is a modified version of R commander (version 1.6-3), including statistical functions that are frequently used in biostatistics[14].

RESULTS

Clinical characteristics of the patients

The characteristics of the patients are shown in Table 1. The median age of the

patients was 73 years (range: 66-77 years). The study population included 20 males and 13 females. Twenty patients had hepatitis B, nine had hepatitis C, and four had autoimmune hepatitis. The median maximum tumor size and median total tumor volume were 1.2 cm (range: 1.1-1.3 cm) and 6.3 cm^3 (range: 5.0-14.2 cm^3), respectively. The levels of AFP, DCP, AFP-L3%, VEGF, and VEGFR-2 in the serum were 4.7 ng/mL (range: 2.5-10.0 ng/mL), 20.0 mAU/mL (range: 17.0-29.3 mAU/mL), 0.5% (range: 0.5-3.7%), 50.1 pg/mL (range: 33.4-91.5 pg/mL), and 6537.2 pg/mL (range: 5687.9-7622.9 pg/mL), respectively.

Differences in carnitine, tumor markers, and angiogenic factors between non-SH patients with and without HCC

The level of acylcarnitine (Figure 1H) was significantly lower in non-SH patients with HCC compared with that reported in non-SH patients without HCC ($P < 0.05$). In addition, the level of AFP-L3% (Figure 1C) was significantly higher in non-SH patients with HCC than that observed in non-SH patients without HCC ($P < 0.05$). However, the levels of AFP, DCP, VEGF, VEGFR-2, total carnitine, and free carnitine (Figures 1A, B, D, E, F, and G, respectively) were not different between the patients with HCC and those without. Acylcarnitine was directly correlated with albumin (r = 0.494, $P < 0.05$). However, acylcarnitine was not correlated with tumor makers, including AFP-L3%.

Advantage of acylcarnitine as an early diagnostic biomarker of HCC

The univariate analysis showed that acylcarnitine and AFP-L3% was associated with the early diagnosis of HCC (Table 2). We performed a univariate analysis using acylcarnitine, AFP-L3%, DCP, VEGF and VEGFR-2 to identify early diagnostic factors of HCC. These factors demonstrated a $P < 0.2$ in the univariate analysis. Notably, the multivariate analysis identified acylcarnitine as a useful early diagnostic biomarker of HCC (Table 2). Receiver operating characteristic (ROC) analysis revealed that the cutoff value was 5.088, the specificity was 89.5%, the sensitivity was 92.9%, and the area under the curve (AUC) was 0.925 (Figure 2).

Acylcarnitine profiles between non-SH patients with and without HCC

The levels of AC2, hexanoylcarnitine (AC6), octanoylcarnitine (AC8), decanoylcarnitine (AC10), dodecanoylcarnitine (AC12), myristoleylcarnitine (AC14:1), and octadecanoylcarnitine (AC18) were significantly lower in non-SH patients with HCC compared with those reported in non-SH patients without HCC (all $P < 0.05$) (Table 3). However, the levels of propionylcarnitine (AC3), butyrylcarnitine (AC4), isovalerylcarnitine (AC5), glutarylcarnitine (AC5DC), 3-hydroxy isovalerylcarnitine (AC5OH), myristoylcarnitine (AC14), palmitoylcarnitine (AC16), 3-hydroxy palmitoylcarnitine (AC16OH), oleoylcarnitine (AC18:1), and 3-hydroxy octadecenoylcarnitine (AC18:1OH) were not different between the two groups of patients (Table 3). In addition, the levels of short-chain fatty acids (SCFAs) (*i.e.*, AC2, AC3, AC4, AC5, AC5DC, AC5OH, and AC6), medium-chain fatty acids (MCFAs) (*i.e.*, AC8 and AC10), and long-chain fatty acids (LCFAs) (*i.e.*, AC12, AC14, AC14:1, AC16, AC16OH, AC18, AC18:1, and AC18:1OH) were significantly lower in non-SH patients with HCC than those observed in non-SH patients without HCC (all $P < 0.05$) (Table 3). The patients were categorized into two groups, according to the ROC cutoff value for AC2 (low, < 3.18; and high, ≥ 3.18; Figure 3A). The patients with AC2 < 3.18 had a significantly higher level of VEGF *vs* those with AC2 ≥ 3.18 (Figure 3B). The patients with HCC were categorized into two groups according to the median cutoff value for total tumor volume (low, < 6.3 and high, ≥ 6.3). The HCC patients with a total tumor volume of ≥ 6.3 had a significantly higher VEGF/AC2 ratio compared with those with a total tumor volume of < 6.3 (Figure 3C). These results indicated that AC2 may be associated with VEGF and HCC progression in non-SH patients.

DISCUSSION

Our present study reported that acylcarnitine may serve as a useful early diagnostic biomarker for non-SH HCC. A recent study reported that the level of AC2 was decreased in non-SH patients with HCC *vs* those without HCC. In addition, AC2 was associated with the tumor-node-metastasis stage of HCC in non-SH patients[8]. Acylcarnitine may be associated with the development and progression of HCC. Furthermore, angiogenesis plays an important role in the development and progression of HCC that were related to VEGF and VEGFR-2 because the VEGF and VEGFR-2 levels of patients were increased with the development and progression of HCC[15-18]. A previous study reported that AC2 suppresses the synthesis of VEGF and VEGFR-2 in HUVECs[10]. In addition, adhesion to the extracellular matrix, migration,

Table 1 Baseline characteristics of non-steatohepatitis patients without and with hepatocellular carcinoma

Variable	Total (*n* = 33)	Patients without HCC (*n* = 19)	Patients with HCC (*n* = 14)	*P* value
Age (yr)	73 (66–77)	73 (68–77)	72 (66–78)	NS
Sex (male/female)	20/13	9/10	11/3	NS
HBV/HCV/AIH	20/9/4	11/5/3	9/4/1	NS
Albumin (g/dL)	4.2 (3.8-4.5)	4.4 (4.3-4.6)	4.0 (3.8-4.1)	< 0.05
Total bilirubin (mg/dL)	0.8 (0.7-1.2)	0.8 (0.8-1.2)	0.9 (0.6-1.2)	NS
Aspartate aminotransferase (IU/L)	29 (25-38)	29 (26-34)	28 (24-4)	NS
Alanine aminotransferase (IU/L)	24 (17-36)	21 (17-34)	27 (19-38)	NS
Alkaline phosphatase (IU/L)	314 (233-442)	314 (232-451)	343 (2252-432)	NS
γ-glutamyl transpeptidase (IU/L)	34 (24-45)	34 (24-43)	38 (24-63)	NS
Prothrombin time (%)	83 (75-90)	81 (77-86)	88 (68-96)	NS
Child–Pugh score	5.0 (5-6)	5.0 (5-5)	5.0 (5-6)	NS
Platelet count (× 10^4/μL)	12.9 (9.2-15.1)	11.9 (9.1-14.5)	13.6 (9.9-16.2)	NS
Maximum tumor size (cm)			1.2 (1.1-1.3)	
Total tumor volume (cm^3)			6.3 (5.0-14.2)	
UICC TNM stage (stage 1/stage 2/stage 3)			1/9/4	

Data are expressed as the median (interquartile range). *P*-values represent comparisons between non-steatohepatitis patients with and without hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis; UICC: Union for International Cancer Control; TNM: Tumor-node-metastasis.

and invasion are key steps in the neovascularization of cancer. AC2 suppresses these processes in HUVECs through inhibition of CXCL12 and CXCR4. Notably, CXCL12 and CXCR4 induce angiogenesis[10,19] and the tumor escapes from immune surveillance[19]. Therefore, based on this evidence, AC2 may suppress the development and progression of HCC through CXCL12 and CXCR4, as well as VEGF and VEGFR-2. Further studies are warranted to investigate the relationship between AC2 and angiogenic factors such as CXCL12 and CXCR4.

HCC is a type of cancer induced by inflammation. Inflammation leads to oxidative stress, causing genomic damage and promoting hepatocarcinogenesis[20,21]. Hence, oxidative stress plays an important role in the development and progression of HCC. A recent study reported that the expression of CPT1 in the carnitine cycle was downregulated by oxidative stress (*i.e.*, CPT1 inactivated by H_2O_2*in vitro*)[22]. Therefore, the level of acylcarnitine may decrease in non-SH patients with HCC through oxidative stress as a consequence of CPT1 downregulation in the carnitine cycle.

Our present and previous findings reported that the level of acylcarnitine was decreased in non-SH patients with HCC *vs* that detected in non-SH patients without HCC[8]. However, in SH patients with HCC, the level of acylcarnitine was increased compare with that reported in SH patients without HCC[6]. A recent study reported that the expression of CTP2 was downregulated in SH patients with HCC through suppression of peroxisome proliferator-activated receptor-α (PPAR-α), that is related to the development and progression of SH and HCC[6,23]. Furthermore, the downregulation of CPT2 induces activation of c-Jun N-terminal kinase, while AC18:1 - one of the LCFAs- promotes the activation of signal transducer and activator of transcription 3 (STAT3)[6]. The activation of c-Jun N-terminal kinase, activation of STAT3, and suppression of PPAR-α induce the development and progression of HCC. In contrast, a previous study reported that the levels of AC18:1 and AC16 - one of the LCFAs - were decreased in non-SH patients with HCC *vs* those measured in non-SH patients without HCC[24]. Moreover, the study showed that LCFAs suppress the growth of various types of cancer (*e.g.*, breast, prostate, *etc.*) *in vivo*[25,26]. In addition, our present study demonstrated that the levels of LCFAs in non-SH patients with HCC were decreased. Our present findings further show that the levels of MCFAs and SCFAs were decreased in non-SH patients with HCC compared with those observed in non-SH patients without HCC. Of note, a previous study reported that MCFAs and SCFAs suppress the growth of various tumors (*e.g.*, colorectal, skin, breast, *etc.*) *in vitro* through downregulation of the c-Myc, Hippo-Yap pathway and/or Mitogen-Activated Protein Kinase signaling[27-29] that induce the development and progression of HCC. In other words, the metabolic pathways of fatty acids in the carnitine cycle may differ between SH HCC and non-SH HCC. Further investigation is required to determine these differences.

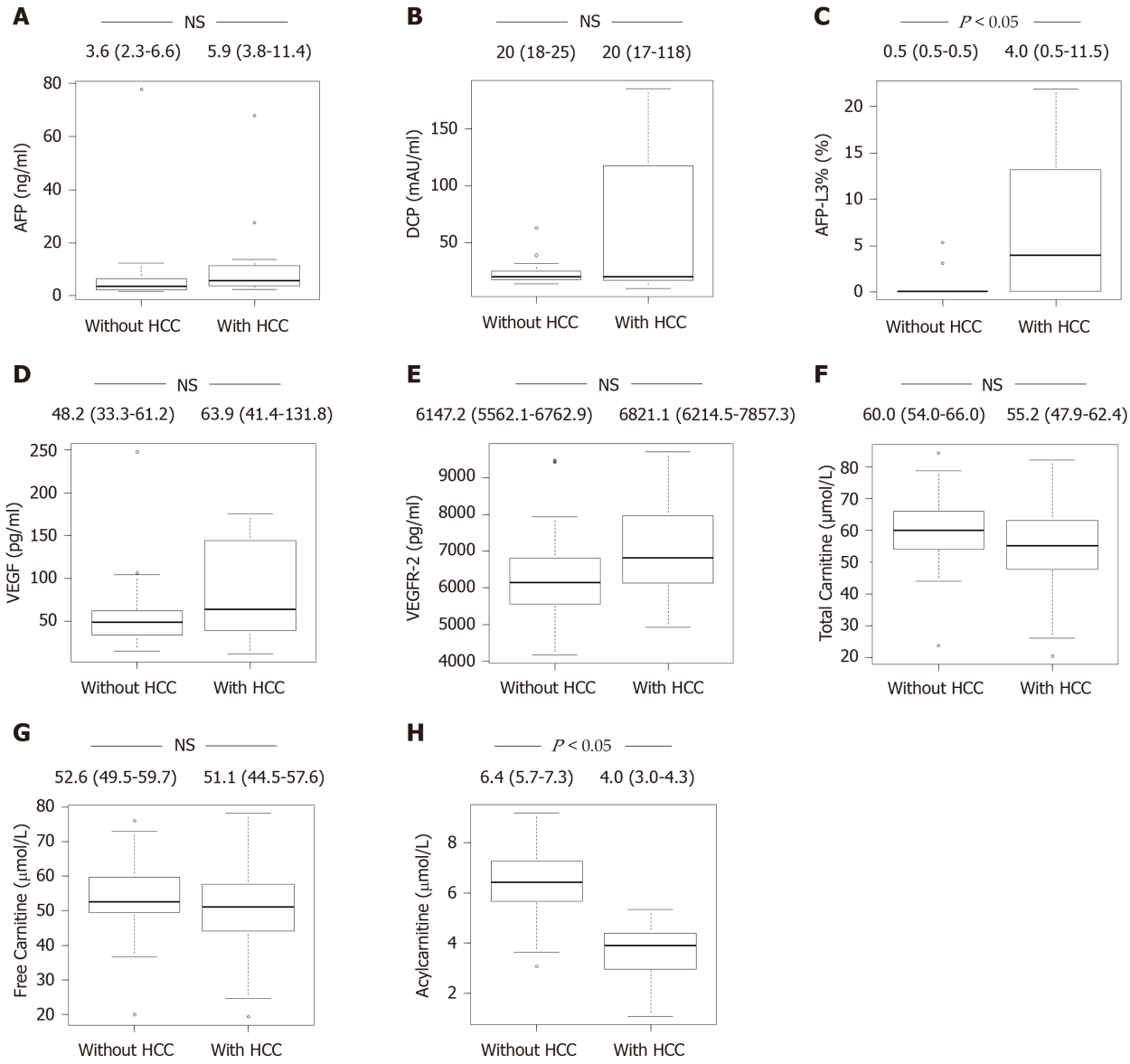

Figure 1 Differences in carnitine, tumor markers, and angiogenic factors between non-steatohepatitis patients with and without hepatocellular carcinoma. A: Level of AFP; B: Level of DCP; C: Level of AFP-L3%; D: Level of VEGF; E: Level of VEGFR-2; F: Level of total carnitine; G: Level of free carnitine; H: Level of acylcarnitine. The level of acylcarnitine (H) was significantly lower in non-SH patients with HCC *vs* that observed in non-SH patients without HCC ($P < 0.05$). In addition, the level of AFP-L3% (C) was significantly higher in non-SH patients with HCC compared with that reported in non-SH patients without HCC ($P < 0.05$). However, the levels of AFP, DCP, VEGF, VEGFR-2, total carnitine, and free carnitine (A, B, D, E, F, and G) were not different between the two groups of patients. SH: Steatohepatitis; HCC: Hepatocellular carcinoma; AFP: α-Fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: Lens culinaris agglutinin-reactive α-fetoprotein; VEGF: Vascular endothelial growth factor; VEGFR-2: VEGF receptor-2.

Several biomarkers[30], including AFP, DCP, and AFP-L3%, have been used for the early diagnosis of HCC. However, these examinations are associated with high cost and limited practicality in a clinical setting. In our present study, it was reported that acylcarnitine is a more useful early diagnostic biomarker of non-SH HCC compared with AFP, DCP, and AFP-L3%.

Notably, the present study was characterized by limitations. These were the small sample size and absence of pathophysiological data. Cirrhotic patients with HCC occasionally develop renal dysfunction. Moreover, a previous study reported that the level of acylcarnitine is decreased in patients with renal dysfunction compared with that measured in patients without renal dysfunction[31]. Therefore, treating physicians should note that renal dysfunction may affect the value of acylcarnitine, when the latter is used as a biomarker for the early diagnosis of HCC. In addition, tumor markers are typically used for the diagnosis and anti-tumor effect of treatment. Thus far, it has not been clarified whether acylcarnitine is a useful biomarker for the effectiveness of treatment in non-SH HCC, and future studies should address this.

In conclusion, a low level of acylcarnitine is an independent early diagnostic

Table 2 Diagnostic accuracy of biomarkers for the early diagnosis of hepatocellular carcinoma

Variable	OR (95%CI)	*P* value
Univariate analysis		
AFP > 10 ng/mL	1.67 (0.332-8.37)	0.535
DCP > 40 mAU/mL	8.00 (0.776-82.5)	0.0806
AFP-L3% > 5%	1.35 (0.999-1.84)	0.0221
VEGF > 60pg/mL	2.67 (0.630-1.3)	0.183
VEGFR-2 > 6500 pg/mL	2.83 (0.666-12.0)	0.159
Total carnitine (per 1 μmol/L increase)	0.979 (0.933-1.03)	0.380
Free carnitine (per 1 μmol/L increase)	0.991 (0.943-1.04)	0.710
Acylcarnitine (per 1 μmol/L increase)	0.0865 (0.0158-0.475)	0.0049
Multivariate analysis		
Acylcarnitine (per 1 μmol/L increase)	0.0941 (0.00137-0.646)	0.0162

HCC: Hepatocellular carcinoma; AFP: α-Fetoprotein; DCP: Des-γ-carboxy prothrombin; AFP-L3%: Lens culinaris agglutinin-reactive α-fetoprotein; VEGF: Vascular endothelial growth factor; VEGFR-2: VEGF receptor-2; CI: Confidence interval; OR: Odds ratio.

biomarker for non-SH HCC. Moreover, the level of AC2 is associated with that of VEGF. Based on these findings, we anticipate that the development of new diagnostic approaches for HCC may involve acylcarnitine.

Table 3 Profiles of acylcarnitine in non-steatohepatitis patients without and with hepatocellular carcinoma

Variable	Patients without HCC (*n* = 19)	Patients with HCC (*n* = 14)	*P* value
Acetylcarnitine (AC2)	3.96 (3.33–4.92)	2.06 (1.335–2.255)	< 0.05
Propionylcarnitine (AC3)	0.339 (0.2875–0.4115)	0.3375 (< 0.24–0.473)	NS
Butyrylcarnitine (AC4)	< 0.1 (< 0.1–0.1)	0.0795 (< 0.1–0.196)	NS
Isovalerylcarnitine (AC5)	< 0.06 (< 0.06–0.1085)	< 0.06 (< 0.06–0.0787)	NS
Glutarylcarnitine (AC5DC)	< 0.05 (< 0.05–0.05)	< 0.05 (< 0.05–0.05)	NS
3-hydroxy isovalerylcarnitine (AC5OH)	< 0.1 (< 0.1–0.1)	< 0.1 (< 0.1–0.1)	NS
Hexanoylcarnitine (AC6)	0.0531 (< 0.05–0.0596)	< 0.05 (< 0.05–0.05)	< 0.05
Octanoylcarnitine (AC8)	0.176 (0.1425–0.2375)	0.0871 (0.0622–0.11025)	< 0.05
Decanoylcarnitine (AC10)	0.335 (0.256–0.432)	0.1315 (0.099675–0.171)	< 0.05
Dodecanolycarnitine (AC12)	0.105 (0.0849–0.1335)	0.02725 (0–0.06195)	< 0.05
Myristoylcarnitine (AC14)	< 0.06 (< 0.06–0.06)	< 0.06 (< 0.06–0.06)	NS
Myristoleylcarnitine (AC14:1)	0.185 (0.134–0.2325)	0.07825 (0.06305–0.09855)	< 0.05
Palmitoylcarnitine (AC16)	0.133 (0.1255–0.1485)	0.116 (0.10425–0.1310)	NS
3-hydroxy palmitoylcarnitine (AC16OH)	< 0.03 (< 0.03–0.03)	< 0.03 (< 0.0 –0.03)	NS
Octadecanoylcarnitine (AC18)	0.0429 (0.03245–0.04775)	0.03315 (0.006625–0.039525	< 0.05
Oleoylcarnitine (AC18:1)	0.871 (0.6825–1.0350)	0.721 (0.61275–0.83775)	NS
3-hydroxy octadecenoylcarnitine (AC18:1OH)	< 0.025 (< 0.025–0.02615)	< 0.025 (< 0.025–0.025)	NS
Short-chain fatty acids (AC2–AC6)	4.3362 (3.83995–5.561750)	2.4316 (1.86085–2.994125)	< 0.05
Medium-chain fatty acids (AC8 + AC10)	0.50600 (0.40700–0.69100)	0.21385 (0.16545–0.28075)	< 0.05
Long-chain fatty acid (AC12–AC18:1OH)	1.3668 (1.106550–1.5871)	0.9425 (0.836625–1.1087)	< 0.05

Data are expressed as the median (interquartile range). *P*-values represent comparisons between non-steatohepatitis patients with and without hepatocellular carcinoma. HCC: Hepatocellular carcinoma.

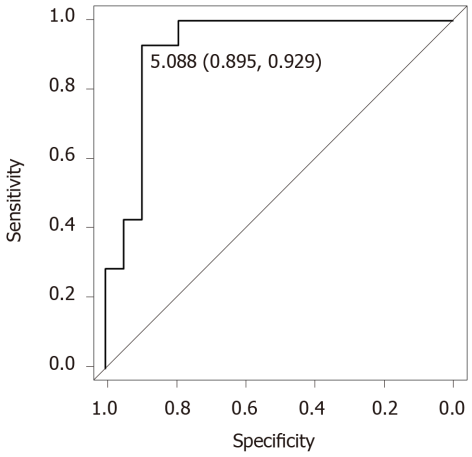

Figure 2 Diagnostic accuracy of acylcarnitine for the early diagnosis of hepatocellular carcinoma in non-steatohepatitis patients. ROC analysis of acylcarnitine revealed that the cutoff value was 5.088, the specificity was 89.5%, the sensitivity was 92.9%, and the AUC was 0.925.

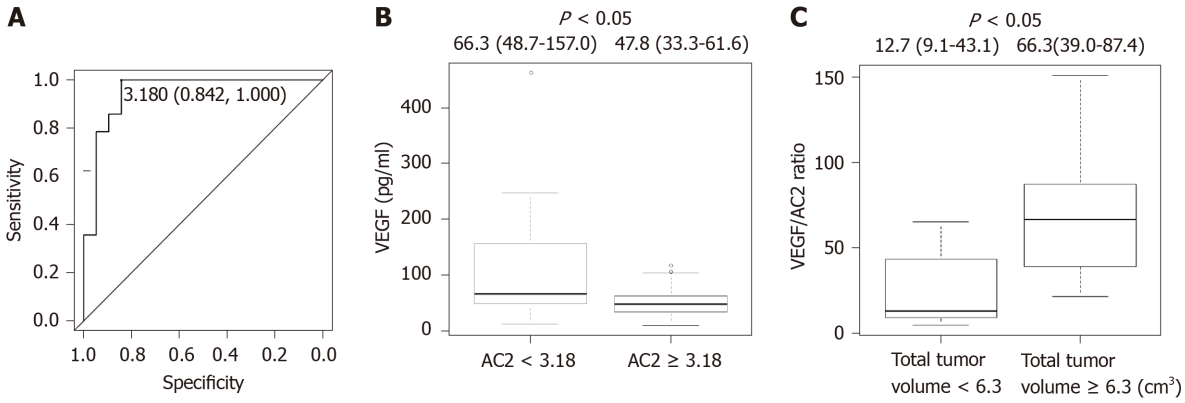

Figure 3 Acetylcarnitine is associated with vascular endothelial growth factor and hepatocellular carcinoma progression in non-steatohepatitis patients. A: ROC analysis of acetylcarnitine (AC2) for the early diagnosis of hepatocellular carcinoma (HCC) in non-steatohepatitis patients revealed that the cutoff value was 3.18, the specificity was 84.2%, the sensitivity was 100%, and the AUC was 0.925; B: The patients were categorized into two groups according to the ROC cutoff vale for AC2 (low, < 3.18; and high, ≥ 3.18). The patients with AC2 < 3.18 had a significantly higher level of vascular endothelial growth factor (VEGF) compared with those with AC2 ≥ 3.18. The patients with HCC were categorized into two groups according to the median cutoff value for total tumor volume (low, < 6.3 and high, ≥ 6.3); C: The HCC patients with a total tumor volume of ≥ 6.3 had a significantly higher VEGF/AC2 ratio compared with those with a total tumor volume of < 6.3. AC2: acetylcarnitine; VEGF: vascular endothelial growth factor.

ARTICLE HIGHLIGHTS

Research background

Although numerous biomarkers, including α-fetoprotein (AFP), des-γ-carboxy prothrombin, and AFP-L3%, have been developed for early diagnosis of hepatocellular carcinoma (HCC), they are not useful in the early diagnosis of HCC.

Research motivation

The fatty acid metabolic pathways in the carnitine cycle may differ between steatohepatitis (SH) HCC and non-SH HCC.

Research objectives

This study aimed to investigate the usefulness of acylcarnitine as a biomarker for the early diagnosis of HCC in non-SH patients.

Research methods

Thirty-three non-SH patients (14 with HCC and 19 without HCC) were enrolled in this study. Blood samples were obtained from patients at the time of admission. The levels of acylcarnitine and acetylcarnitine in the serum were determined using tandem mass spectrometry. Univariate and multivariate analyses were used to determine early diagnostic factors of HCC.

Research results

The level of acylcarnitine was significantly lower in non-SH patients with HCC compared with those without HCC ($P < 0.05$). The multivariate analysis showed that a low level of acylcarnitine was the only independent factor for the early diagnosis of HCC.

Research conclusions

A low level of acylcarnitine is an independent early diagnostic biomarker for non-SH HCC. Moreover, the level of acetylcarnitine is associated with that of VEGF.

Research perspectives

We anticipate that the development of new diagnostic approaches for HCC may involve acylcarnitine.

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META-ANALYSIS

Prognostic and pathological impact of tumor budding in gastric cancer: A systematic review and meta-analysis

Yi-Xian Guo, Zi-Zhen Zhang, Gang Zhao, En-Hao Zhao

ORCID number: Yi-Xian Guo (0000-0002-3067-6031); Zi-Zhen Zhang (0000-0003-4152-4364); Gang Zhao (0000-0002-9999-9402); En-Hao Zhao (0000-0003-1112-0043).

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Yi-Xian Guo, Zi-Zhen Zhang, Gang Zhao, En-Hao Zhao, Department of Gastrointestinal Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200120, China

Corresponding author: En-Hao Zhao, PhD, Associate Professor, Department of Gastrointestinal Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 160 Pujian Road, Shanghai 200120, China. microzhaoenhao@hotmail.com
Telephone: +86-21-68383731
Fax: +86-21-68383731

Abstract

BACKGROUND

Tumor budding, is a promising prognostic hallmark in many cancers, and can help us better assess the degree of malignancy in gastric cancer (GC) and in colorectal cancer. In the past few years, several articles on the relationship between tumor budding and GC have been published, but different results have been observed. As the relationship between tumor budding and GC remains controversial, we integrated the data from 7 eligible studies to conduct a systematic review and meta-analysis.

AIM

To systematically evaluate the prognostic and pathological impact of tumor budding in GC.

METHODS

Literature searches were conducted in the PubMed, Cochrane Library, EMBASE and Web of Science databases, and 7 cohort studies involving 2178 patients met our criteria and included in the analysis. The patients were divided into those with high-grade tumor budding and those with low-grade tumor budding, and the cut-off values for tumor budding varied across the included studies. The hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated to estimate the impact of tumor budding on overall survival (OS) in GC patients. The odds ratios (ORs) with 95%CIs were used to determine the correlation between tumor budding and pathological parameters (tumor stage, tumor differentiation, lymphovascular invasion, lymph node metastasis) of GC.

RESULTS

Seven studies involving 2178 patients were included in the meta-analysis. The combined ORs suggested that high-grade tumor budding was significantly associated with tumor stage (OR = 6.63, 95%CI: 4.01-10.98, $P < 0.01$), tumor

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differentiation (OR = 3.74, 95%CI: 2.68-5.22, $P < 0.01$), lymphovascular invasion (OR = 7.85, 95%CI: 5.04-12.21, $P < 0.01$), and lymph node metastasis (OR = 5.75, 95%CI: 3.20-10.32, $P < 0.01$). Moreover, high-grade tumor budding predicted a poor 5-year OS (HR = 1.79, 95%CI: 1.53-2.05, $P < 0.01$) in patients with GC and an adverse 5-year OS (HR = 1.93, 95%CI: 1.45-2.42, $P < 0.01$) in patients with intestinal-type GC.

CONCLUSION
High-grade tumor budding suggested a poor prognosis in patients with GC or intestinal-type GC.

Key words: Tumor budding; Gastric cancer; Intestinal-type gastric cancer; Epithelial-mesenchymal transition

Core tip: Tumor budding is known to be a specific pathological marker in the diagnosis of colorectal cancer and squamous cell carcinoma. However, the prognostic value of tumor budding in patients with gastric cancer (GC) has not been extensively studied and remains controversial. This is the first meta-analysis to evaluate the prognostic value of tumor budding in GC. The findings suggest that tumor budding is closely related to tumor stage, tumor differentiation, lymphovascular invasion and lymph node metastasis in GC.

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INTRODUCTION

Gastric cancer (GC), including cardia and noncardia GC, is a highly malignant cancer worldwide with over 1000000 new cases in 2018 and an estimated 783000 deaths (equating to 1 in every 12 deaths globally), making it the fifth most frequently diagnosed cancer and the third leading cause of cancer death[1]. Despite the use of multidisciplinary treatments, the 5-year survival rate for GC patients is reported to be 20%-40%[2].

Currently, the TNM staging system is considered the most robust system to predict the prognosis of patients with GC. According to the American Joint Committee on Cancer criteria, pathological staging of GC includes: depth of tumor stage (T), number of lymph nodes involved (N), and presence of distant metastasis (M)[3,4].

However, due to the pursuit of individualized diagnosis and medical treatment, the outcome parameters for patients with GC remain inadequate and inaccurate. In the future, the stratification of GC will depend on biochemical, morphological, molecular biological and treatment-related parameters to improve accuracy.

Thus, it is imperative to find available markers to precisely estimate the pathological diagnosis and prognosis of GC. One such marker is tumor budding, defined as the presence of single cancer cells or small clusters of fewer than five cells at the invasive front[5-7], and has been officially recognized by the Union for International Cancer Control as an additional prognostic factor in colorectal cancers. Moreover, tumor budding has recently been included in the guidelines for colorectal cancer screening and diagnosis in Europe[8] and Japan[9], highlighting the increased use of this parameter in clinical practice.

Importantly, tumor budding has been reported to be a promising prognostic hallmark in many other cancers[10-13], including GC[14,15]. However, the prognostic value of tumor budding in GC has not been fully clarified. Therefore, the purpose of this study was to explore the relationship between tumor budding and 5-year overall survival (OS) in patients with GC as well as the clinicopathological parameters.

MATERIALS AND METHODS

Search protocol

We systematically retrieved all studies that evaluated the relationship between tumor budding and the outcome of patients with GC using the PubMed, EMBASE, Cochrane Library and Web of Science databases. The search terms were as follows: "tumor budding", "tumour budding", "tumor-cell dissociation", "gastric cancer", "gastric carcinoma", "gastric neoplasm", "stomach cancer" and "prognosis", "prognostic" and "survival". The reference lists of all eligible studies were also assessed manually.

Inclusion and exclusion criteria

Studies were included if they met the following inclusion criteria: (1) The study demonstrated a relationship between tumor budding and OS or pathological features of GC; (2) Sufficient information was provided to estimate the hazard ratios (HRs) and odds ratios (ORs); and (3) Only English language literature was included.

The following articles were excluded: (1) Reviews, conference proceedings, abstracts, expert opinions, and case reports; (2) Studies with no available data on tumor budding in GC; (3) Overlapping studies; and (4) Nonhuman studies.

Data extraction

Two authors (Guo YX and Zhang ZZ) independently extracted information using a standardized form. The following characteristics were retrieved: First author's name, year of publication, country of patients' origin, the number of patients, staining methods, cut-off points for tumor budding, survival data and pathological data. If the survival data were not presented in the article, we obtained the data using Kaplan–Mhigeier curves according to Parmar *et al*[16]. The quality of each study was tested using the Newcastle-Ottawa quality assessment scale.

Statistical analysis

All statistical analysis was carried out using STATA 15.0 software. The impact of tumor budding on OS was quantitatively evaluated by HRs and their 95% confidence intervals (CIs). The most common method was used to obtain the HR and 95%CI directly from the paper or calculate them using the parameters provided in the manuscript. Otherwise, we extracted results from the Kaplan-Meier curves with Engauge Digitizer according to the methods reported by Parmar *et al*[16].

We extracted and combined data on tumor budding and several pathological characteristics, including tumor stage (I-II/III-IV), tumor differentiation (well/moderate and poor), lymphatic metastasis (absent/present), and lymphovascular invasion (absent/present), related to GC in each study. For these data, the Mantel-Haenszel ORs with their 95%CIs were calculated and combined to provide the effective value.

X^2 and I^2 tests were used to measure heterogeneity between each article. $P < 0.05$ was considered statistically significant, and $I^2 < 50\%$ indicated no heterogeneity between studies. If there was no heterogeneity ($I^2 < 50\%$), a fixed-effects model was used. Otherwise, a random-effects model was applied ($I^2 > 50\%$). Subgroup analysis was used to determine the source of heterogeneity.

Statistical significance is expressed as $P < 0.05$ or < 0.01 ($P > 0.05$ are denoted).

RESULTS

Selected literature and study characteristics

The preliminarily selected literature included 234 articles from the PubMed, EMBASE, Cochrane Library and Web of Science databases. After checking the titles and abstracts, irrelevant studies were excluded, and 19 potential studies were evaluated by intensive reading. As a result, 12 of these studies were excluded for the following reasons: the data could not be extracted from the study, non-English literature, and non-clinical trials. The search method for the studies included in this meta-analysis is presented in Figure 1. Finally, seven studies were selected for this analysis. The studies were conducted in seven countries (China, Japan, Turkey, Germany, Finland, the United States and the United Kingdom) and were published between 1992 and 2019. Six studies were on GC, and one study was related to gastroesophageal junction cancer. The main characteristics of the eligible studies are shown in Table 1. The HRs data from 3 studies were extracted from the original univariate analysis directly, while the data from the other 2 studies were estimated from survival curves. Evaluation by the Newcastle-Ottawa quality assessment scale showed that 6 (85.7%) of the studies had quality scores > 5, indicating that the included studies were of good

quality.

Correlation between tumor budding and clinicopathological features

We evaluated the correlation between tumor budding and depth of tumor stage, tumor differentiation status, lymph vascular invasion and lymph node metastasis of GC.

For tumor stage, 5 studies (1423 patients) were qualified for the meta-analysis and there was statistically significant association between high-grade tumor budding and tumor stage (OR = 6.63, 95%CI: 4.01-10.98, $P < 0.01$) (Figure 2). The test for heterogeneity was significant using the random-effects model ($I^2 = 60.5\%$, $P = 0.038$) (Figure 2). Furthermore, when the subgroups were stratified by the type of GC, the heterogeneity of studies with intestinal-type GC ($I^2 = 0.0\%$, $P = 0.531$) (Figure 2) was effectively eliminated, and heterogeneity of the studies with all-type GC ($I^2 = 54.5\%$, $P = 0.111$) (Figure 2) was decreased.

For tumor differentiation, 4 studies (980 patients) were qualified for the meta-analysis and there was statistically significant association between high-grade tumor budding and undifferentiated tumor status (OR = 3.74, 95%CI: 2.68-5.22, $P < 0.01$) (Figure 3). The test for heterogeneity was not significant using the fixed-effects model ($I^2 = 39.8\%$, $P = 0.173$) (Figure 3).

For lymph vascular invasion, 3 studies (545 patients) were qualified for the meta-analysis and there was statistically significant association between high-grade tumor budding and lymph vascular invasion (OR = 7.85, 95%CI: 5.04-12.21, $P < 0.01$) (Figure 4). The test for heterogeneity was not significant using the fixed-effects model ($I^2 = 0\%$, $P = 0.483$) (Figure 4).

For lymph node metastasis, 5 studies (966 patients) were qualified for the meta-analysis and there was statistically significant association between high-grade tumor budding and lymph node metastasis (OR = 5.75, 95%CI: 3.20-10.32, $P < 0.01$) (Figure 5). The test for heterogeneity was significant using random-effects model ($I^2 = 66.1\%$, $P = 0.019$) (Figure 5). Furthermore, when the subgroups were stratified by patient number, the heterogeneity of the studies with > 200 patients ($I^2 = 0.0\%$, $P = 0.573$) (Figure 5) and the studies with < 200 patients ($I^2 = 0.0\%$, $P = 0.346$) (Figure 5) was totally eliminated.

Correlation between tumor budding and 5-year OS

The 5-year OS was extracted from 5 studies (1833 patients) and analysis of the synthesized data with the fixed-effects model ($I^2 = 0.0\%$, $P = 0.549$) (Figure 6) revealed that high-grade tumor budding was associated with a poor 5-year OS (HR = 1.79, 95%CI: 1.53-2.05, $P < 0.01$) (Figure 6). Subsequently, 2 studies (572 patients) on intestinal-type GC also revealed that high-grade tumor budding was associated with an adverse 5-year OS (HR = 1.93, 95%CI: 1.45-2.42, $P < 0.01$) (Figure 7) and no significant heterogeneity was detected ($I^2 = 0.0\%$, $P = 0.929$) (Figure 7).

DISCUSSION

Tumor invasion - metastasis is a complex process that allows cancer cells to escape the major mass of the primary tumor and settle in distant organs or tissues[22]. Loss of cell cohesion is a crucial step in the process of cancer invasion, and metastasis is regarded as the most fatal event during cancer progression[23]. From a pathological point of view, tumor budding is a phenomenon encountered in various cancers in which a primary tumor sends a number of finger-like projections to adjacent stroma, some of which eventually detach from the main tumor mass as small cell clusters. It is generally accepted that tumor budding is the histological basis for invasion and metastasis[24].

Our meta-analysis integrated the data from 7 eligible studies involving 2178 patients with GC, and evaluated the role of tumor budding in GC, for the first time. Clinicopathological parameter analysis showed that high-grade tumor budding was correlated with an adverse grade of tumor differentiation, tumor invasion, lymph vascular invasion and lymph node metastasis. In addition, high-grade tumor budding was a statistically significant predictor of poor OS in patients with GC. We also observed the same results in intestinal-type GC, demonstrating that tumor budding may also have a prognostic role in intestinal-type GC. These factors are traditionally unfavorable predictors in patients with GC.

The combination of different types of GC was a disadvantage in the studies that evaluated tumor budding in GC. Niko Kemi indicated that there was no statistically significant relationship between tumor budding and OS in diffuse-type gastric adenocarcinoma[15]. Therefore, assessment of tumor budding in diffuse-type gastric adenocarcinoma is not recommended. Our study demonstrated that tumor budding

Table 1 Main characteristics of the included studies

Author	Year	Country	Cases	Cancer	Stage	Staining	Cut off	Microscopic magnification	Survival analysis	Newcastle-Ottawa score
Gabbert *et al*[14]	1992	Germany	445	GC	I-IV	HE	5 buds	NA	OS	7
Brown *et al*[17]	2010	UK	356	EGJA	I-IV	HE	5 buds	NA	OS	7
Tanaka *et al*[18]	2014	Japan	320	GC	I-IV	HE	Median	× 400	OS	8
Gulluoglu *et al*[19]	2015	Turkey	126	GC	I	HE	5 buds	× 400	NA	4
Che *et al*[20]	2017	China	296	GC	I-IV	HE	5 buds	× 400	OS	8
Olsen *et al*[21]	2017	USA	52	GC	I-IV	HE	Median	× 200	PFS	6
Kemi *et al*[15]	2019	Finland	583	GC	I-IV	HE	10 buds	× 400	OS	8

UK: United Kingdom; USA: United States; HE: Hematoxylin eosin stain; NA: Not applied; OS: Overall survival; PFS: Progression free survival

was closely related to OS and tumor stage in patients with intestinal-type GC. Compared to other cancers, intestinal-type GC has a histopathological morphology similar to colorectal cancer[25]. In colorectal cancer, tumor budding has been proved to be an independent prognostic factor and has been included in European and Japanese guidelines[8,9]. A detailed investigation of the relationship between tumor budding and intestinal-type GC is required. The relationship between different types (Lauren classification) of GC and tumor budding may be different. The current study did not include a clear classification of GC, and this may have contributed to inaccurate results. In the future, separate analyses should be conducted on the relationship between tumor budding and different types of (Lauren classification) GC in order to better evaluate the impact of tumor budding on the prognosis of GC.

Tumor budding is considered to be the first step in cancer metastasis, as budding cells are thought to migrate through the extracellular matrix, invade lymph vascular structures and form metastatic tumor colonies in lymph nodes and at distant sites[26], and our results proved this point of view. The initiation of tumor budding is based on the epithelial-mesenchymal transition (EMT) process[26]. The relationship between tumor budding and EMT has been studied in many cancers, including colorectal cancer[27], esophageal cancer[28], pancreatic cancer[29,30], tongue squamous cell carcinoma[31], head and neck cancer[32], lung cancer[33], and breast cancer[12]. However, most EMT processes in tumor budding are incomplete, which suggests that tumor budding undergoes partial EMT. Thus, tumor budding has been proposed to be "EMT-like"[24]. In 2014, Tanaka *et al*[18] showed that higher TrkB expression in tumor budding was observed at the tumor invasive front. TrkB is closely related to EMT and was demonstrated in colon cancer and head and neck squamous cell carcinoma[34,35], which promoted the EMT process and induced chemotherapy resistance. In the future, in-depth research should be conducted on this aspect, in order to determine the relationship between tumor budding and EMT, and the molecular mechanism underlying this relationship.

In this meta-analysis, no heterogeneity was observed, except in the studies on tumor budding associated with lymph node metastasis and tumor stage. To identify the source of heterogeneity of the association between tumor budding and tumor stage, we found that the included patients were all diagnosed with intestinal-type GC, whereas other authors chose to include patients with all types of GC. There was no significant heterogeneity in the correlation between tumor budding and tumor stage when the patients were divided into two groups (group 1: intestinal-type GC, group 2: all-types of GC). The study by Tanaka *et al*[18] was excluded from group 2, and no significant heterogeneity was observed in group 2. The study by Tanaka *et al*[18] did not include undifferentiated tumor samples, which may have contributed to the significant heterogeneity observed. Small sample size may also have contributed to the heterogeneity observed in the correlation between tumor budding and lymph node metastasis, as no significant heterogeneity was found when the number of patients was extended to 200.

Our meta-analysis has a few limitations. First, although the patients were divided into those with high-grade tumor budding and those with low-grade tumor budding, the stratification may change depending on the cut-off values, as the cut-off values for tumor budding varied across the included studies due to differences in the study populations and experimental methods. Second, some HRs and their corresponding 95%CIs were extracted from the survival curves. However, these data might be less reliable than those directly obtained from survival data. Third, a potential language

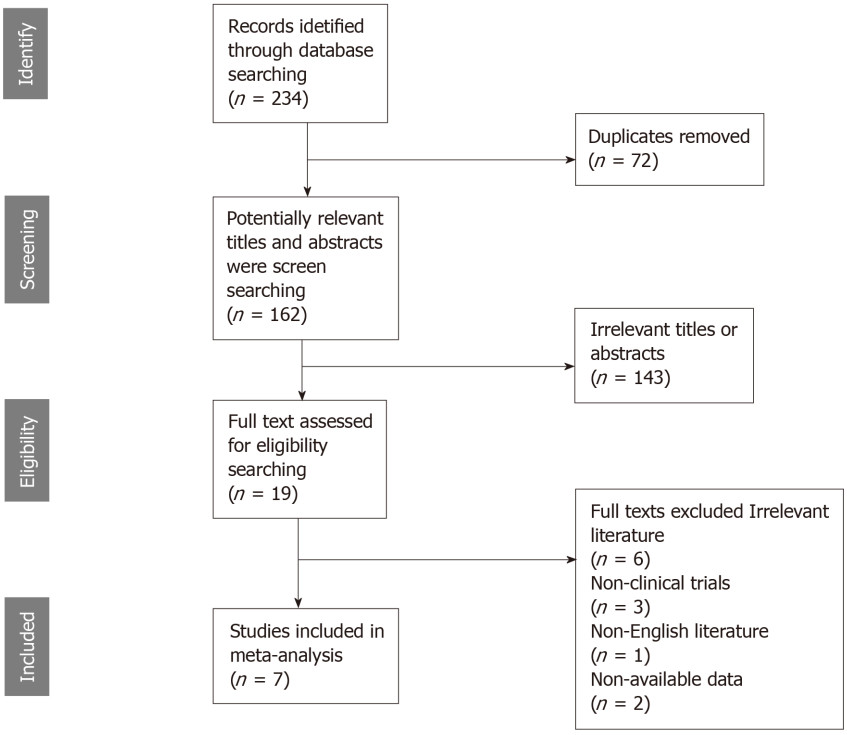

Figure 1 Flow diagram of study enrollment.

bias exists in this meta-analysis, as non-English publications were excluded. Finally, publication bias was not tested due to the small number of included studies in the evaluation of tumor budding and prognosis of GC, which may have induced potential bias.

In conclusion, studies included in this meta-analysis came from seven countries and had large sample sizes. The results of this study showed that high-grade tumor budding was related to a poor 5-year OS and aggressive clinicopathological features in patients with GC. Tumor budding may be a unique predictive marker and the method used to detect tumor budding is simple, reproducible and inexpensive. Furthermore, we strongly advocate further studies on larger preoperative GC biopsies and different types of GC to confirm these results.

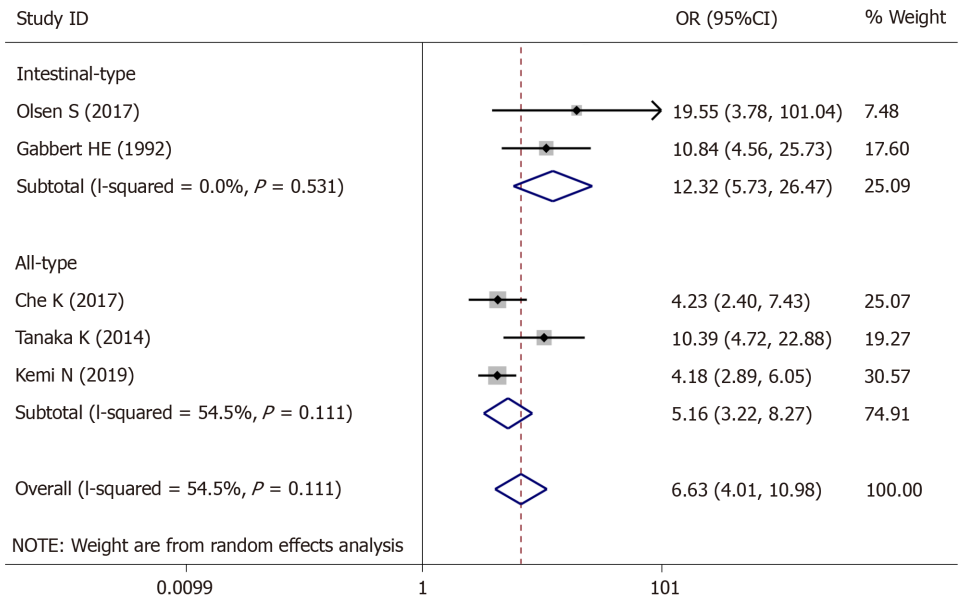

Figure 2 Pooled analysis of the association between tumor budding and tumor stage in patients with gastric cancer.

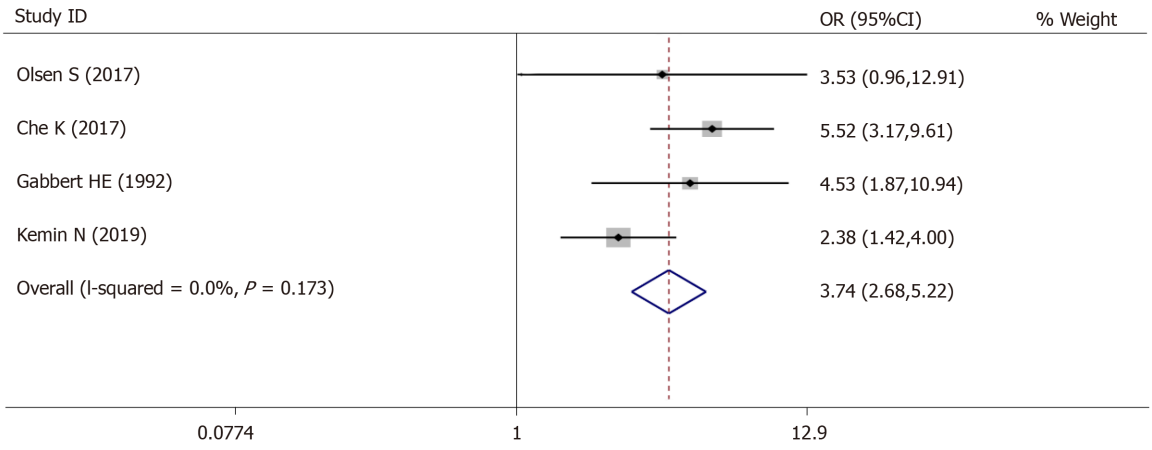

Figure 3 Pooled analysis of the association between tumor budding and undifferentiated tumor status in patients with gastric cancer.

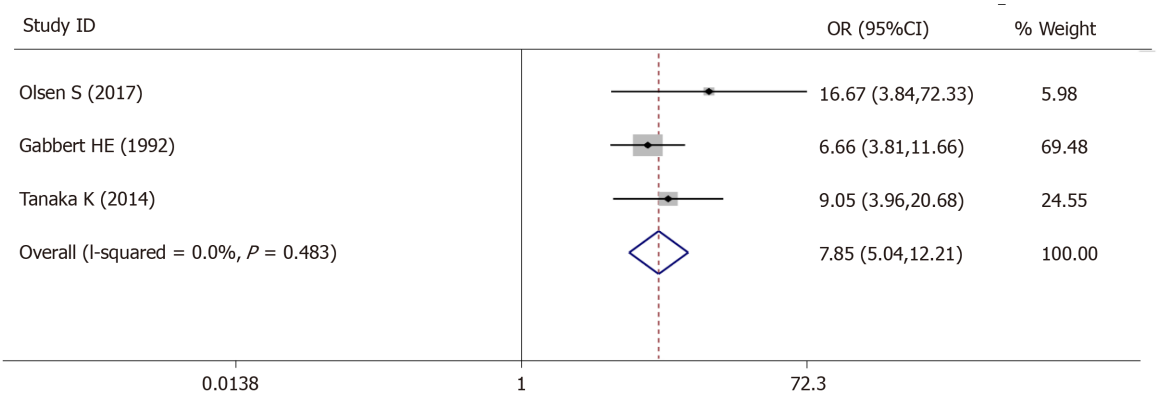

Figure 4 Pooled analysis of the association between tumor budding and lymph vascular invasion in patients with gastric cancer.

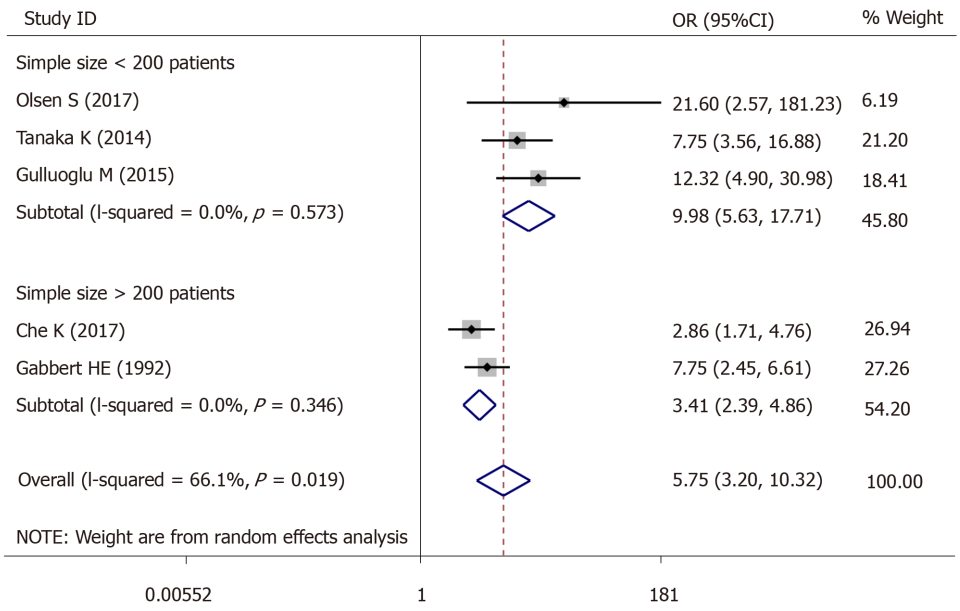

Figure 5 Pooled analysis of the association between tumor budding and lymph node metastasis in patients with gastric cancer.

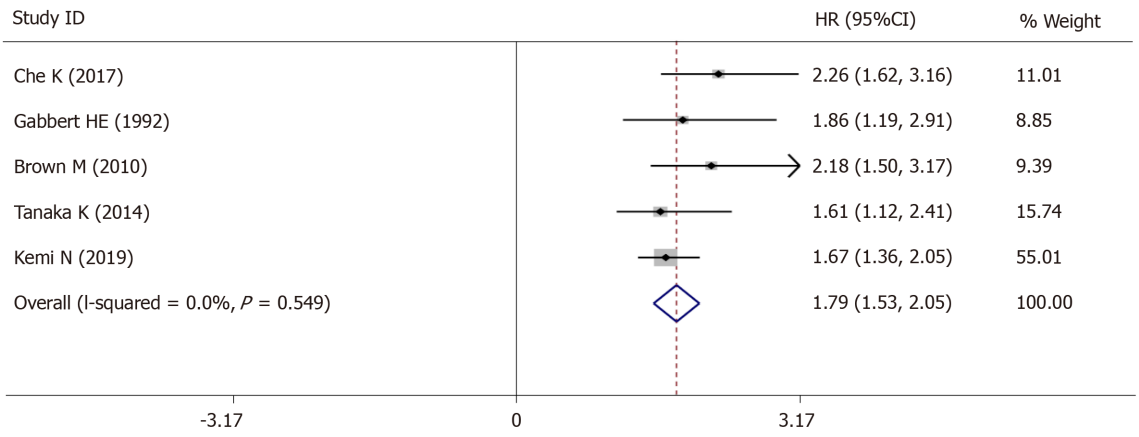

Figure 6 Pooled analysis of the association between tumor budding and overall survival in patients with gastric cancer.

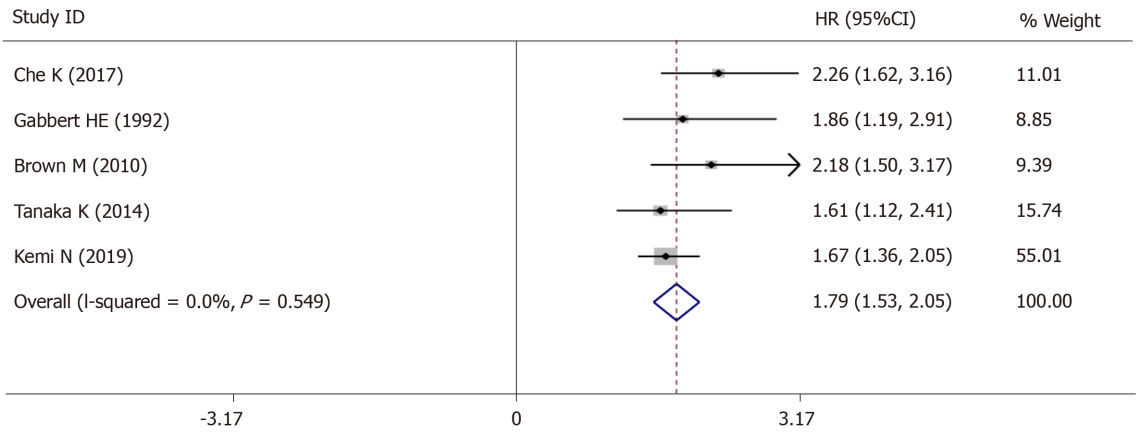

Figure 7 Pooled analysis of the association between tumor budding and overall survival in patients with intestinal-type gastric cancer.

ARTICLE HIGHLIGHTS

Research motivation

Our results demonstrated that high-grade tumor budding was related to poor 5-year overall survival (OS) in patients with gastric cancer (GC). Tumor budding may be a new prognostic indicator in GC.

Research objectives

This meta-analysis was carried out to clarify the prognostic and pathological impact of tumor budding in patients with GC.

Research methods

The PubMed, EMBASE, Web of Science, and the Cochrane Library databases were searched. The data were extracted, and statistical analysis was conducted using STATA 15.0 software to assess the clinicopathological features and OS related to tumor budding in patients with GC. The odds ratios (ORs) were presented for dichotomous variables with 95% confidence intervals (CIs), and the HR was presented for time-to-event variables with 95%CIs.

Research results

Our meta-analysis suggested that high-grade tumor budding was significantly associated with tumor stage (OR = 6.63, 95%CI: 4.01-10.98, $P < 0.01$), undifferentiated tumor status (OR = 3.74, 95%CI: 2.68-5.22, $P < 0.01$), lymphovascular invasion (OR = 7.85, 95%CI: 5.04-12.21, $P < 0.01$), and lymph node metastasis (OR = 5.75, 95%CI: 3.20-10.32, $P < 0.01$). Moreover, high-grade budding predicted poor 5-year OS (HR = 1.79, 95%CI: 1.53-2.05, $P < 0.01$) in patients with GC and poor 5-year OS (HR = 1.93, 95%CI: 1.45-2.42, $P < 0.01$) in patients with intestinal-type GC.

Research conclusions

This research is the first to demonstrate that high-grade tumor budding is related to poor 5-year OS and aggressive clinicopathological features in patients with GC.

Research perspectives

In this meta-analysis, the close relationship between poor prognosis in GC and tumor budding was demonstrated, and it was found that intestinal-type GC is more closely related to tumor budding, and related research on diffuse GC is lacking. In the future, we will study the relationship between diffuse GC and tumor budding using our own sample library, and determine the underlying mechanism of the relationship between tumor budding and poor prognosis.

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META-ANALYSIS

Abnormally expressed circular RNAs as novel non-invasive biomarkers for hepatocellular carcinoma: A meta-analysis

Ya-Lin Jiang, Meng-Meng Shang, Shi-Zhen Dong, Yong-Chao Chang

ORCID number: Ya-Lin Jiang (0000-0002-8628-2285); Meng-Meng Shang (0000-0002-1678-8969); Shi-Zhen Dong (0000-0003-2167-957X); Yong-Chao Chang (0000-0002-5149-5424).

Author contributions: Jiang YL acquired, analyzed, and interpreted the data and drafted, revised, and finally approved the article; Shang MM and Dong SZ acquired, analyzed, and interpreted the data and finally approved the article; Chang YC conceived and designed the study and critically revised and finally approved the article.

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Ya-Lin Jiang, Meng-Meng Shang, Shi-Zhen Dong, Yong-Chao Chang, Department of Clinical Laboratory, The First Affiliated Hospital and College of Clinical Medicine of Henan University of Science and Technology, Luoyang 471003, Henan Province, China

Meng-Meng Shang, Department of Clinical Laboratory, The Family Planning Guidance Station of Luanchuan County, Luoyang 471500, Henan Province, China

Corresponding author: Yong-Chao Chang, MD, PhD, Professor, Department of Clinical Laboratory, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, No. 24, Jinghua Road, Jianxi District, Luoyang 471003, Henan Province, China. zero6666888@163.com
Telephone: +86-379-64830723
Fax: +86-379-64830723

Abstract

BACKGROUND

Circular RNAs (circRNAs) are a newly discovered class of endogenous non-coding RNAs that may have roles in cancer genesis and development. In the recent literature, dysregulated circRNAs have been extensively investigated in hepatocellular carcinoma (HCC). Whether or not circRNAs are of clinical value for the management of HCC has not been characterized.

AIM

To meta-analyze the diagnostic and prognostic value of abnormally expressed circRNAs in HCC.

METHODS

Eligible studies were sourced from PubMed, EMBASE, and CNKI online databases. Data on patients' clinical characteristics, including diagnostic efficacy and overall survival, were extracted. The diagnostic and prognostic parameters were respectively synthesized using the bivariate meta-analysis model and multivariate Cox hazard regression analysis based on Stata 12.0. The trim and fill method was adopted to assess the possible effects from publication bias.

RESULTS

A total of 21 eligible studies were included. The pooled sensitivity, specificity, and area under the curve of abnormally expressed circRNAs in distinguishing HCC from non-cancer controls were 0.78 (95%CI: 0.69–0.85), 0.80 (95%CI: 0.74–0.86), and 0.86, respectively. Survival analyses showed that the down-regulated circRNA expression signature correlated perfectly with HCC survival

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[hazard ratio (HR) = 0.42, 95%CI: 0.19–0.91, P = 0.028; I^2 = 92.7%, P = 0.000], whereas the HCC cases with high circRNA levels had significantly poorer prognoses than those of patients with low circRNA levels (HR = 2.22, 95%CI: 1.50–3.30, P = 0.000; I^2 = 91%, P = 0.000). Moreover, abnormally expressed circRNAs were intimately associated with tumor size, differentiation grade, microvascular invasion, metastasis, TNM stage, and serum alpha fetal protein level in patients with HCC. Stratified analysis based on sample type, control source, and expression status also yielded robust results.

CONCLUSION
Abnormally expressed circRNA signatures show immense potential as novel non-invasive biomarker(s) for HCC diagnosis and prognosis.

Key words: CircRNA; Hepatocellular carcinoma; Diagnosis; Prognosis; Meta-analysis

Core tip: Current studies investigating the clinical significance of circular RNAs (circRNAs) in hepatocellular carcinoma (HCC) were conducted using single-center and small-scale design, and the findings were controversial. We collected and analyzed the up-to-date clinical data on the significance of circRNAs in the diagnosis and prognosis of HCC. The results indicated that circRNAs may be novel indicators for the prognosis and diagnosis of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC), one of the most common cancers of the digestive system, remains one of the leading causes of cancer deaths worldwide[1]. In China, the incidence of HCC was shown to have increased remarkably over the past decades, which has resulted in great health and economic burdens[2]. Although the technological advances for HCC treatment in recent years have vastly improved the clinical outcomes of patients with HCC, the 5-year survival rate is very low[3]. Particularly for patients with advanced HCC, the median survival time was shown to be only 3–9 mo[4]. The sensitivity and specificity of the currently used blood biomarkers such as alpha fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II (PIVKA-II) are not satisfactory for HCC detection[5]. For prognosis monitoring, no biomarkers were well developed. Therefore, it is necessary to investigate novel effective biomarkers for HCC.

Non-coding RNAs play important roles in cancer biology, providing potential targets for cancer intervention. As a new class of endogenous noncoding RNAs, circular RNAs (circRNAs) are a series of functional non-coding transcripts generated from the backsplicing of exons, introns, or both[6]. Unlike linear RNAs, circRNAs form covalently closed continuous loop structures, characterized by stability, abundance, and specific expression in different tissues and cells during development[6,7]. CircRNAs act as key regulators in a wide range of biological processes, including the initiation and progression of several types of cancer[8,9]. CircRNAs are aberrantly expressed in cancer tissues, especially in HCC, suggesting that these molecules could be novel biomarkers for HCC diagnosis and prognosis[10-30]. Whether or not circRNAs are of clinical value for the diagnosis of HCC must be clarified. Herein, we conducted this meta-analysis, aiming to assess the diagnostic and prognostic utility of circRNA expression signature in HCC.

MATERIALS AND METHODS

Study selection

The international online databases including PubMed, EMBASE, EBSCO, Biomed central, and CNKI were searched for eligible studies indexed until May 1, 2018. The searching items were: ("liver cancer" or "liver neoplasms [MeSH Terms]" or "hepatocellular carcinomas [MeSH Terms]") and ("circular RNA [MeSH Terms]" or "circRNA" or "hsa circ") and ("prognosis" or "prognoses [MeSH Terms]" or "prognostic factors [MeSH Terms]" "HR" or "hazard ratio" or "overall survival" or "OS" or "survival [MeSH Terms]" or "disease-free survival" or "DFS" or "EFS" or "event-free survival" or "progression-free survival" or "PFS") or ("diagnosis [MeSH Terms]" or "diagnoses [MeSH Terms]" or "sensitivity and specificity [MeSH Terms]" or "ROC" or "ROC curve [MeSH Terms]" or "AUC"). The attached reference list of literature was also manually searched to increase the search sensitivity.

Selection criteria

Studies were selected in compliance with the following criteria: (A) Studies were limited to those which evaluated the diagnostic or prognostic or clinicopathological features of circRNA(s) in HCC patients; (B) the true positive, false positive, false negative, and true negative values for diagnosis, or estimated hazard ratio (HR) values with 95%CIs for survival, were either available among studies or could be extracted indirectly; (C) cases were definitely diagnosed with pathological evidence; and (D) the specimens were obtained prior to any radiotherapy or chemotherapy treatments. Irrelevant papers were excluded according to the following criteria: (A) Studies with insufficient data to form the 2 × 2 table for diagnosis, or the HRs with 95%CIs for survival were unavailable; (B) Studies were rated as low quality; and (C) Basic studies, reviews, meta-analyses, comments, letters or case reports, *etc*. were also excluded.

Data extraction

The baseline contents were collected independently by two trained authors. The information covered included data such as the name of the first author, year of publication, study design, ethnicity, sample size, pathologic data of the study population, circRNA signature, test methods, sensitivity, specificity, cut-off value setting, HR values with 95%CIs for survival, follow-up time, *etc*. Any disagreements which appeared during data summarization were resolved by group consensus, or the articles' authors were reached out to.

Study quality grading

Study quality for diagnostic articles was evaluated by the Quality Assessment for Studies of Diagnostic Accuracy II checklist[31]. The tool comprises two domains including "risk of bias" and "applicability concerns", containing seven questions regarding patient selection, index tests, reference standards, flow, and timing. The answer of risk for bias could be rated as "no" (0 points), "yes" (1 point), or "unclear" (0 points). The study quality for the case-control study was judged in line with the Newcastle-Ottawa Quality Assessment Scale (NOS) checklist[32], in which the assessment focuses on a total of eight items categorized in terms of study selection, comparability, and outcome, with a maximum judgment score of "9". An answer of "yes" receives a score of "1"; otherwise, no sores were awarded.

Statistical analysis

Statistical analyses were conducted based on the Stata 12.0 program (Stata Corporation, College Station, TX, United States). Heterogeneity among studies was assessed using chi-square and I^2 tests. Either $P < 0.05$ in the chi-square test or $I^2 > 50\%$ was regarded as significant heterogeneity. The diagnostic parameters were synthesized using the bivariate meta-analysis model, and HRs with 95%CIs were combined using multivariate Cox hazard regression analysis. A random-effects model was chosen when significant heterogeneity appeared in the pooled effect size. Sensitivity analysis was performed to trace the underlying outlier studies included in the pooled effects. The bias due to publication was detected by Deek's funnel plot and Begg's and Egger's tests, and $P < 0.05$ was set to indicate statistically significant differences. If publication bias appeared, the trim and fill method was adopted to assess the possible effects of bias on the overall pooled effects[33].

RESULTS

Study enrollment

Figure 1 presents the flowchart of the literature search procedure. Searching PubMed,

EMBASE, EBSCO, Biomed central, and CNKI databases, as well as other sources, resulted in an initial inclusion of 236 records after duplicates were removed. Two authors independently screened the titles and abstracts of the 236 publications, and 187 records were excluded because their study contents were unrelated to cirRNAs in HCC. The remaining articles were intensively evaluated for the full-text contents, and 28 of them were evaluated as review articles, or basic studies with irrelevant data, or relevant articles with insufficient information, which therefore were all eliminated. In the final stage, only 21 studies, including 8 publications for diagnosis[11-13,19-21,25,29], 11 for prognosis[10,11,14-18,22,26,28,30], and 14 for clinicopathological feature[11-13,15,17,18,22-24,26-30], were included in the quality assessment and quantitative synthesis.

Characteristics of included studies

Study characteristics are shown in Tables 1 and 2. All of the included studies were identified as case-control studies, in which eight studies with 712 HCC cases and 788 controls assessed the diagnostic performance of circRNAs in HCC, and eleven studies including 2719 cohorts focused on the evaluation of the prognostic value of cirNRAs. All HCC cases were reliably diagnosed based on histopathological methods. The control sources included chronic hepatitis, liver cirrhosis, para-tumorous tissues[11,20,21,25], and non-cancer/healthy individuals[19,29]. Amplification of circRNAs was enabled by using the qPCR test, and *GAPDH* or *β-actin* was used for normalization. The circRNA signatures for diagnosis included hsa_circ_0003570, circZKSCAN1, hsa_circ_0005075, Hsa_circ_0001649, hsa_circ_0091582, hsa_circ_0128298, hsa_circ_0004018, hsa_circ_0001445, and circRNAs panel sets. CircRNA profiles for prognosis contained hsa_circ_0001649, circ-ITCH, circMTO1, cSMARCA5, circC3P1, hsa_circRNA_100338, hsa_circ_0064428, circRNA101368, hsa_circ_0103809, and circ-ZEB1.33.

Methodological quality assessment

The quality and bias of all diagnostic studies were independently appraised by two authors in accordance with the QUADAS-II criteria, whereby studies were assessed for patient selection, index test, reference standard, flow, and timing[31]. As reported in Figure 2, all included eight publications for diagnosis were judged as low risk for applicability concerns, and three studies were assessed with bias in patient selection, or index test, or reference standard, and received rated QUADAS scores equal to three points. Evaluation of the quality of all case-control studies was enabled by applying the NOS checklist[32]. As shown in Table 3, all the included prognostic studies received rated NOS scores higher or equal to six, and thus they were all included in the final synthesis.

Investigations of heterogeneity

In the overall diagnostic meta-analysis, the chi-square and I^2 tests revealed significant substantial heterogeneity among pooled effects (Q = 49.403, df = 2.00, P = 0.000; I^2 = 95.95, 95%CI: 92.85–99.05). In line with the diagnostic effects, clear heterogeneity was also observed in the pooled prognostic effects for both the elevated (P = 0.000; I^2 = 91%) and down-regulated circRNA profiles (P = 0.000; I^2 = 92.7%). Thus, all weights were synthesized using a random-effects model.

Overall diagnostic performance

The summary receiver operating characteristic curve was employed to assess the diagnostic efficacy of circRNA profiles in distinguishing HCC from non-tumorous controls. The pooled sensitivity (Figure 3A), specificity (Figure 3B), positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR) (Figure 3C), and area under the curve (AUC) (Figure 3D) were estimated to be 0.78 (95%CI: 0.69–0.85), 0.80 (95%CI: 0.74–0.86), 3.97 (95%CI: 2.85–5.54), 0.27 (95%CI: 0.19–0.39), 14.59 (95%CI: 7.83–27.21), and 0.86, respectively.

Prognostic value

We found distinct prognostic value of the abnormally expressed circRNA signature in HCC, wherein the signature covered up-regulated circRNAs and was negatively correlated with the overall survival (OS) of patients with HCC (HR = 2.22, 95%CI: 1.50–3.30, P = 0.000) (Figure 4A), hinting that these circRNAs could be considered as independent prognostic biomarkers in HCC. Meanwhile, the significantly higher survival time (OS) was found in HCC patients with down-regulated circRNA profile (HR = 0.42, 95%CI: 0.19–0.91, P = 0.028) (Figure 4B), suggesting that circRNAs with decreased expression status were more prone to act as tumor suppressor genes in HCC.

Clinicopathological association

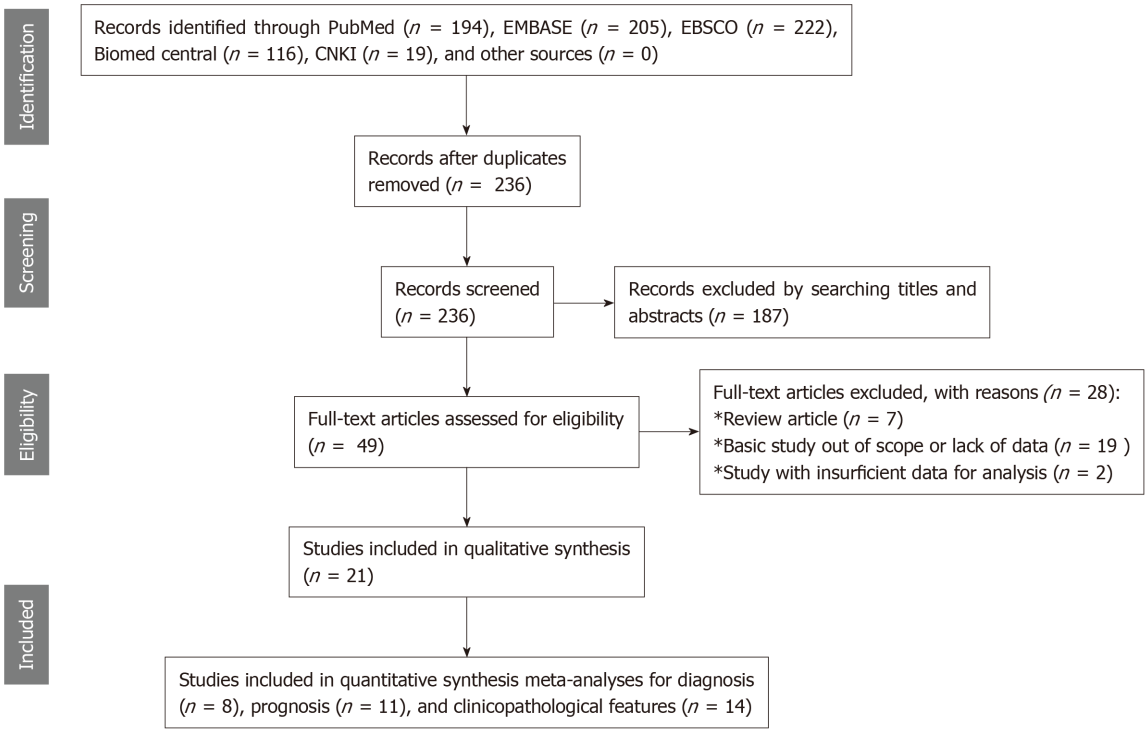

Figure 1 Study flow diagram for the diagnostic and prognostic meta-analyses.

Analysis of the association between circRNA expression and clinicopathological factors in HCC also yielded robust results. As shown in Table 4, significant associations were observed between the circRNA expression and alcoholism (pooled $P = 0.0323$), tumor size (pooled $P = 0.00012$), differentiation grade (pooled $P = 0.000$), microvascular invasion (pooled $P = 0.003744$), TNM stage (pooled $P = 0.000$), metastasis (pooled $P = 0.000$), and serum AFP level (pooled $P = 0.0115$).

Stratified analysis

The stratified analysis depending on sample type revealed that the tissue-based circRNA testing had higher diagnostic efficacy in confirming HCC than plasma-based analysis (AUC: 0.88 *vs* 0.72; DOR: 15.17 *vs* 8.93). Different effects were also observed in differentially expressed circRNAs, wherein up-regulated circRNA profile yielded a better diagnostic performance than down-regulated circRNAs (AUC: 0.97 *vs* 0.81; DOR: 11.48 *vs* 8.75). Moreover, the analysis grouped by control type showed that circRNA profiles could differentiate chronic hepatitis or cirrhosis from HCC, with an AUC of 0.84, sensitivity of 0.77, and specificity of 0.76; additionally, the circRNA expression signature was able to distinguish adjacent non-cancerous liver tissues from HCC samples, with an AUC of 0.73 and specificity of 0.75 (Table 5).

Sensitivity analysis

Sensitivity analysis was performed in both the diagnostic and prognostic effect sizes. As exemplified by Figure 4, one study[30] was identified as the outlier in the pooled prognostic effects of down-regulated circRNAs in HCC. After elimination of the outlier data and re-analysis of the effect, the I^2 dropped from 92.3% to 90%, indicating that included heterogeneous studies were a substantial cause of study heterogeneity. No outliers were detected in other pooled effects (Figure 5).

Publication bias

Publication bias was judged using different methods for different pooled effects. As shown in Figure 6A, no clear publication bias was detected in the combined diagnostic effects (Deek's funnel plot, $P = 0.446$), nor in the analysis of down-regulated circRNA profile (Egger's test, $P = 0.606$, Figure 6B). Nevertheless, the funnel plot showed evidence of publication bias in the effects of up-regulated circRNA profile (Egger's test, $P = 0.001$, Figure 6C), and the trim and fill method was applied to trace the possible impacts from bias[33]. As indicated in Figure 6D, the filled funnel plots identified five imputed studies, but the effect was slightly altered before and after adjustment (variance = 0.187, $P = 0.005$ *vs* variance = 0.287, $P = 0.000$).

Table 1 Characteristics of the included studies for diagnosis and clinicopathologic features

Study	Ethnicity	Patient size	Control size	Control source	Sample type	CircRNA Name	Expression status	Method	Cut-Off value	Control gene	AUC	QUADAS score
Fu *et al*[12], 2017	Chinese	107	107	CH & LC	Tissue	hsa_circ_0003570	Decreased	qRT-PCR	12.24	*GAPDH*	0.70	4
Yao *et al*[25], 2017	Chinese	102	102	Adjacent non-cancerous liver tissue	Tissue	circZKSCAN1	Decreased	qRT-PCR	Unclear	*GAPDH*	0.834	4
Shang *et al*[21], 2016	Chinese	30	30	Adjacent nontumorous tissue	Tissue	hsa_circ_0005075	Increased	qRT-PCR	0.000586	*GAPDH*	0.94	6
Qin *et al*[20], 2016	Chinese	89	89	Paired adjacent liver tissues	Tissue	Hsa_circ_0001649	Decreased	qRT-PCR	0.00079	*β*-actin	0.63	6
Chen *et al*[11], 2018	Chinese	30	30	Para-tumorous tissues	Tissue	hsa_circ_0091582	Increased	qRT-PCR	Unclear	*GAPDH*	0.679	5
Chen *et al*[11], 2018	Chinese	30	30	Para-tumorous tissues	Tissue	hsa_circ_0128298	Increased	qRT-PCR	Unclear	*GAPDH*	0.664	5
Chen *et al*[11], 2018	Chinese	48	48	Para-tumorous tissues	Tissue	hsa_circ_0128298	Increased	qRT-PCR	Unclear	*GAPDH*	0.668	5
Huang *et al*[17], 2017	Chinese	102	129	Para-tumorous and CH tissues	Tissue	hsa_circ_0004018	Decreased	qRT-PCR	0.531	*GAPDH*	0.848	5
Zhang *et al*[28],2018	Chinese	104	52	Healthy control	Plasma	hsa_circ_0001445	Decreased	qRT-PCR	Unclear	*GAPDH*	0.862	5
Zhang *et al*[29], 2018	Chinese	104	57	LC	Plasma	hsa_circ_0001445	Decreased	qRT-PCR	Unclear	*GAPDH*	0.672	5
Zhang *et al*[29], 2018	Chinese	104	44	CH	Plasma	hsa_circ_0001445	Decreased	qRT-PCR	Unclear	*GAPDH*	0.764	5
Han *et al*[16], 2017	Chinese	80	80	Non-cancer tissue	Tissue	CircRNA pattern	/	qRT-PCR	Unclear	Unclear	0.988	3
Han *et al*[16], 2017	Chinese	20	20	Non-cancer tissue	Tissue	CircRNA pattern	/	qRT-PCR	Unclear	Unclear	0.976	3

AUC: Area under the curve; circRNA: Circular RNA; CH: Chronic hepatitis; LC: Liver cirrhosis; qRT-PCR: Quantitative real-time PCR; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase; QUADAS: Quality Assessment for Studies of Diagnostic Accuracy.

DISCUSSION

HCC is among the most frequent causes of cancer death in digestive system tumors[1-3]. There are many investigated biomarkers for HCC, such as AFP, PIVKA-II, and the ratio of lens culinaris agglutinin-reactive alpha-fetoprotein to total AFP (AFP-L3/AFP)[34]. However, these biomarkers retain several limitations on their overall diagnostic efficacies[5,34]. In this respect, ideal noninvasive biomarkers are urgently needed to reinforce HCC detection. Circular RNAs (circRNAs), which are a group of covalently closed circular non-coding RNAs, have been recently identified as key regulators in cell development and function in HCC[35]. Accumulating investigations have shown that a large number of circRNAs are dysregulated in HCC[10-30], giving rise to the differential expression status and association in tumor diagnosis and prognosis. In the present study, we conducted diagnostic and prognostic meta-analyses and assessed the clinical significance of circRNA expression profiles in HCC.

A recently published meta-analysis showed that circRNAs are promising diagnostic biomarkers for tumors[36]. In our diagnostic meta-analysis, a total of eight studies were included, covering 712 HCC cases. The combined ROC curve showed

Table 2 Characteristics of the included studies for prognosis and clinicopathologic features

Study	Locale	Patient size	TNM stage (I/II/III/IV)	Sample type	CircRNA signature	Expression status	Survival indicator	Follow-up time	HR and 95%CI extraction	*P*-value (survival)	NOS scores
Cai *et al*[10], 2018	China	78	Unclear	Tissue	hsa_circ_0103809	Increased	OS	Unclear	Indirectly	0.001	6
Zhong *et al*[30], 2018	China	47	7, 15, 16, 9	Tissue	circC3P1	Decreased	OS	Unclear	Indirectly	0.030	6
Li *et al*[18], 2018	China	51	I–II: 24, III–IV: 27	Tissue	circRNA101368	Increased	OS	Unclear	Directly	0.001, 0.033	7
Weng *et al*[22], 2018	China	120	I–III: 60, 14, 46	Tissue	hsa_circ_0064428	Increased	OS	Unclear	Indirectly	0.033	7
Chen *et al*[11], 2018	China	78	Unclear	Tissue	hsa_circ_0128298	Increased	OS	Median: 37 months	Directly	0.009, 0.014	8
Gong *et al*[14], 2018	China	64	12, 22, 17, 13	Tissue	circ-ZEB1.33	Increased	OS	Unclear	Indirectly	0.015, 0.019	7
Yu et *al*[26], 2018	China	208	I: 62, II–III: 101	Tissue	cSMARCA5	Decreased	OS	Unclear	Directly	0.001, 0.021	7
Huang *et al*[17], 2017	China	80	I–II: 43, III–IV: 37	Tissue	hsa_circRNA_100338	Increased	OS	5 years	Indirectly	<0.01	8
Han *et al*[16], 2017	China	116	Unclear	Tissue	circMTO1	Decreased	OS	Unclear	Indirectly	0.0023	7
Guo *et al*[15], 2017	China	1800	Unclear	Tissue	circ-ITCH	Decreased	OS	Unclear	Directly	<0.001	6
Zhang *et al*[27], 2018	China	77	I–II: 34, III–IV: 43	Tissue	hsa_circ_0001649	Decreased	OS	Unclear	Directly	0.015, 0.011	6
Xu *et al*[23], 2018	China	76	I–II: 23, III–IV: 53	Tissue	hsa_circ_0001649	Decreased	/	/	/	/	/
Zhang *et al*[28], 2018	China	86	Early: 38, Late: 48	Tissue	circsMaD2	Decreased	/	/	/	/	/

circRNA: Circular RNA; OS: Overall survival; HR: Hazard ratio; NOS: Newcastle-Ottawa Scale; TNM: Tumor-node-metastasis.

that the circRNA expression profile had favorable sensitivity (0.78), specificity (0.80), and AUC (0.86) values in confirming HCC. Moreover, the respective PLR and NLR values were 3.97 and 0.27, which means that the circRNA signature achieved a ratio of nearly 4 between the true positive and false positive rates, and the probability of HCC patients that tested negative for circRNAs versus the probability of cases that tested positive had a ratio of 0.27. Importantly, the pooled DOR, a key parameter used in meta-analyses of diagnostic test accuracy studies, was estimated to be 14.59 and suggested the powerful capability of circRNA signatures in discriminating HCC from non-cancer cases. These encouraging findings suggest that circRNA expression signatures could be considered as important potential biomarkers for the diagnosis of patients with HCC.

An increasing number of single studies have documented the prognostic value of circRNAs in HCC[10,11,14,16-18,22,26,28,30]. In our pooled analysis, we found that circRNAs with different expression statuses in HCC displayed distinct prognostic features. The down-regulated circRNA profile (hsa_circ_0001649, circ-ITCH, circMTO1, cSMARCA5, and circC3P1) was closely associated with favorable OS in patients with HCC, whereas the up-regulated circRNA signature (hsa_circRNA_100338, hsa_circ_0064428, circRNA101368, hsa_circ_0103809, and circ-ZEB1.33) was related to worse OS time in HCC. A newly published study has reviewed the oncogenic (tumor suppression) roles of single circRNAs in HCC[37], further evidencing our findings. These encouraging results showed that circRNA expression signatures may be developed as potential indicator(s) for predicting the OS of HCC patients. The clinicopathological value of the circRNA expression profile also manifested robust results; circRNAs were found to be markedly associated with alcoholism, tumor size, differentiation grade, microvascular invasion, TNM stage, metastasis, and serum AFP level, suggesting that abnormally expressed circRNAs are likely to be implicated in tumor progression in HCC as well.

Our study still retains many limitations. The overriding problem is the substantial heterogeneity which appeared among studies. The sensitivity analysis identified one study[30] as the outlier in the pooled prognostic effects of down-regulated circRNAs in HCC. Our analysis further confirmed the impact of heterogeneous studies on the

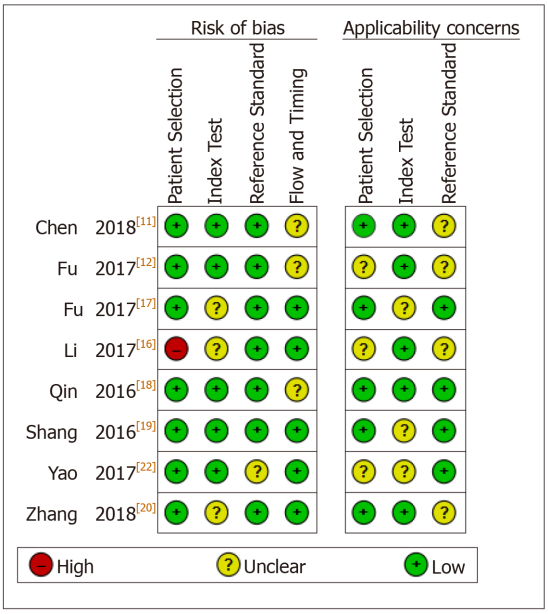

Figure 2 Study quality regarding the risk of bias and applicability concerns as assessed by the QUADAS II tool.

generation of heterogeneity among combined effects. Additionally, biases from publications appeared in one of our pooled prognostic analyses. Nevertheless, our further assessment using a nonparametric trim and fill procedure confirmed that the combined accuracy is not an artifact of unpublished negative studies. Consequently, the accuracy of all the pooled effects was shown to be relatively reliable.

In summary, our study shows evidence that abnormally expressed circRNAs may play a critical role in HCC progression and could serve as diagnostic and prognostic biomarkers for cases of HCC. Future in-depth research is required to further evaluate the utilities of single or panel circRNA(s) for HCC diagnosis and prognosis.

Table 3 Study quality and bias in the retrospective cohort studies assessed *via* the Newcastle-Ottawa Scale checklist

Study	Cohort selection				Comparability	Outcome ascertainment		
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cases and controls on the basis of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts
Cai *et al*[10], 2018	1	1	1	1	1	1	0	0
Zhong *et al*[30], 2018	1	1	1	1	1	1	0	0
Li *et al*[18], 2018	1	1	1	1	2	1	0	0
Weng *et al*[22], 2018	1	1	1	1	2	1	0	0
Chen *et al*[11], 2018	1	1	1	1	1	1	1	1
Gong *et al*[14], 2018	1	1	1	1	2	1	0	0
Yu et *al*[26], 2018	1	1	1	1	2	1	0	0
Huang *et al*[17], 2017	1	1	1	1	1	1	1	1
Han *et al*[16], 2017	1	1	1	1	2	1	0	0
Guo *et al*[15], 2017	1	1	1	1	1	1	0	0
Zhang *et al*[27], 2018	1	1	1	1	1	1	0	0

Table 4 Associations between circular RNA expression and clinicopathological factors in patients with hepatocellular carcinoma

Class	Included studies	χ^2	Pooled *P*-value
Gender	18	36.426	0.4487
Age	18	32.517	0.635
Smoking (yes *vs* no)	5	8.597	0.5707
Alcoholism	5	19.684	0.0323
Tumor size	13	57.979	0.00012
Tumor number (single *vs* multiple)	7	14.3614	0.4231
Encapsulation (incomplete/complete)	3	3.8078	0.7026
Differentiation grade (well/moderate/poor)	11	66.9698	1.97×10^{-6}
Microvascular invasion	3	19.261	0.003744
TNM stage	13	76.1066	2.51×10^{-7}
HBsAg	7	14.4284	0.418306
Serum AFP	12	42.4249	0.0115
Metastasis	12	79.8852	6.35×10^{-8}
ALT	3	5.4896	0.4827
AST	4	12.3545	0.1361
GGT	3	14.3614	0.4231
Cirrhosis (yes/no)	5	5.8236	0.8298

ALT: Alanine transaminase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transpeptidase; AFP: Alpha fetoprotein; TNM: Tumor-node-metastasis; HbsAg: Hepatitis B surface antigen.

Table 5 Subgroup analysis conducted based on sample type, control type, and expression status among the diagnostic studies

Analysis	Included individual studies	Sensitivity 95%CI	Specificity 95%CI	PLR 95%CI	NLR 95%CI	DOR 95%CI	AUC	Heterogeneity
Sample type								
Tissue	10	0.73 (0.70–0.77)	0.82 (0.78–0.84)	4.03 (2.98–5.46)	0.29 (0.19–0.43)	15.17 (8.42–27.34)	0.88	I^2 = 73.9%, P = 0.0001
Plasma	3	0.79 (0.74–0.84)	0.65 (0.57–0.73)	2.33 (1.47–3.7)	0.28 (0.12–0.63)	8.93 (2.37–33.64)	0.72	I^2 = 87.3%, P = 0.0004
Expression status								
Up-regulated circRNAs	4	0.70 (0.62–0.78)	0.83 (0.76–0.89)	4.00 (2.71–5.91)	0.37 (0.28–0.50)	11.48 (5.90–22.33)	0.97	I^2 = 21.2%, P = 0.2832
Down-regulated circRNAs	7	0.74 (0.70–0.77)	0.75 (0.71–0.78)	2.75 (2.16–3.5)	0.33 (0.22–0.49)	8.75 (5.31–14.43)	0.81	I^2 = 70.4%, P = 0.0025
Control type								
Chronic hepatitis/cirrhosis *vs* HCC	3	0.77 (0.72–0.81)	0.76 (0.71–0.81)	3.08 (2.49–3.80)	0.32 (0.25–0.41)	10.89 (7.51–15.78)	0.84	I^2 = 0.0%, P = 0.5262
Adjacent non-cancerous liver tissue *vs* HCC	6	0.63 (0.57–0.68)	0.75 (0.69–0.81)	2.33 (1.44–3.75)	0.52 (0.40–0.69)	4.7 (3.12–7.08)	0.73	I^2 = 0.0%, P = 0.4953

AUC: Area under the curve, PLR: Positive likelihood ratio; NLR: Negative likelihood ratio; DOR: Diagnostic odds ratio; HCC: Hepatocellular carcinoma.

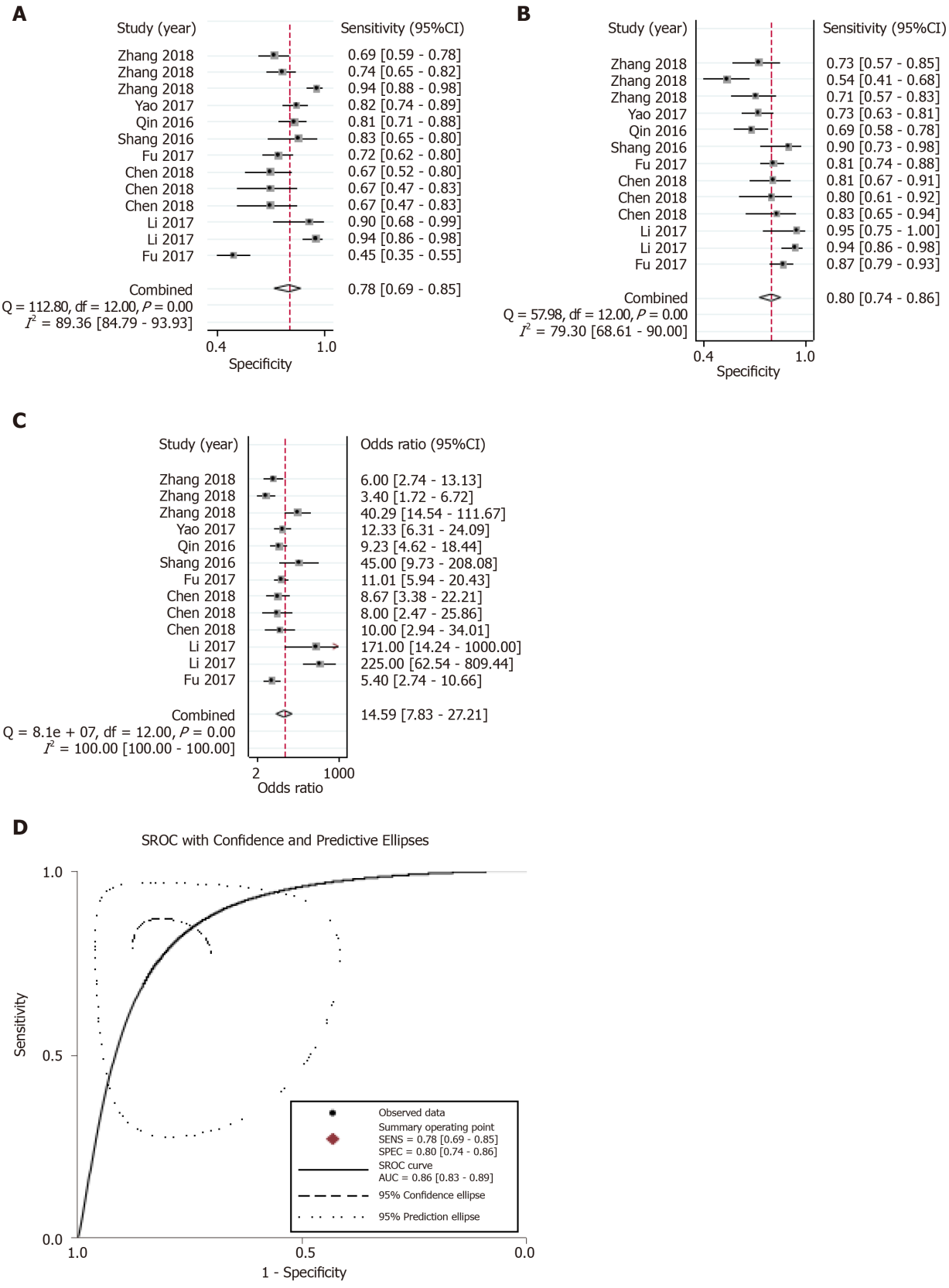

Figure 3 Overall diagnostic performance. A-D: Forest plots of the combined (A) sensitivity, (B) specificity, (C) diagnostic odds ratio, and (D) area under the curves among the eight diagnostic studies.

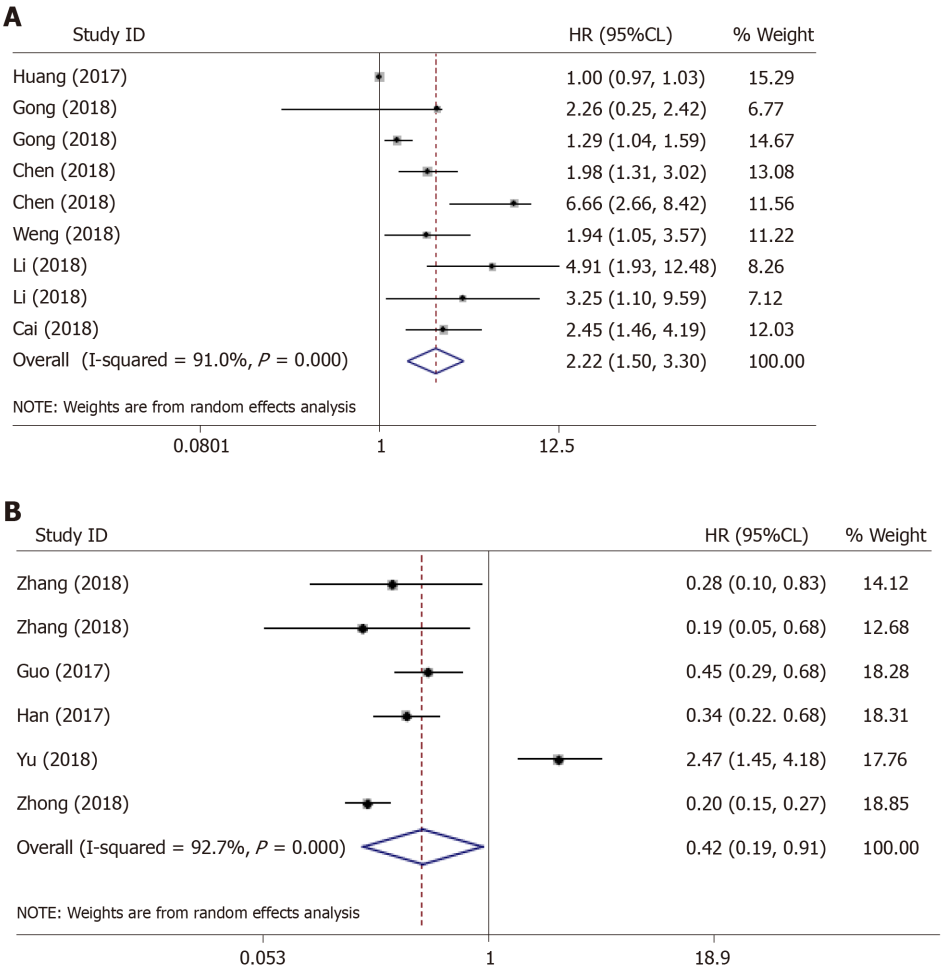

Figure 4 The outlier in the pooled prognostic effects of down-regulated circular RNAs in hepatocellular carcinoma. A and B: Forest plots of the combined hazard ratios with 95% confidence intervals for the (A) up-regulated and (B) down-regulated circular RNA profiles in predicting the overall survival of hepatocellular carcinoma patients.

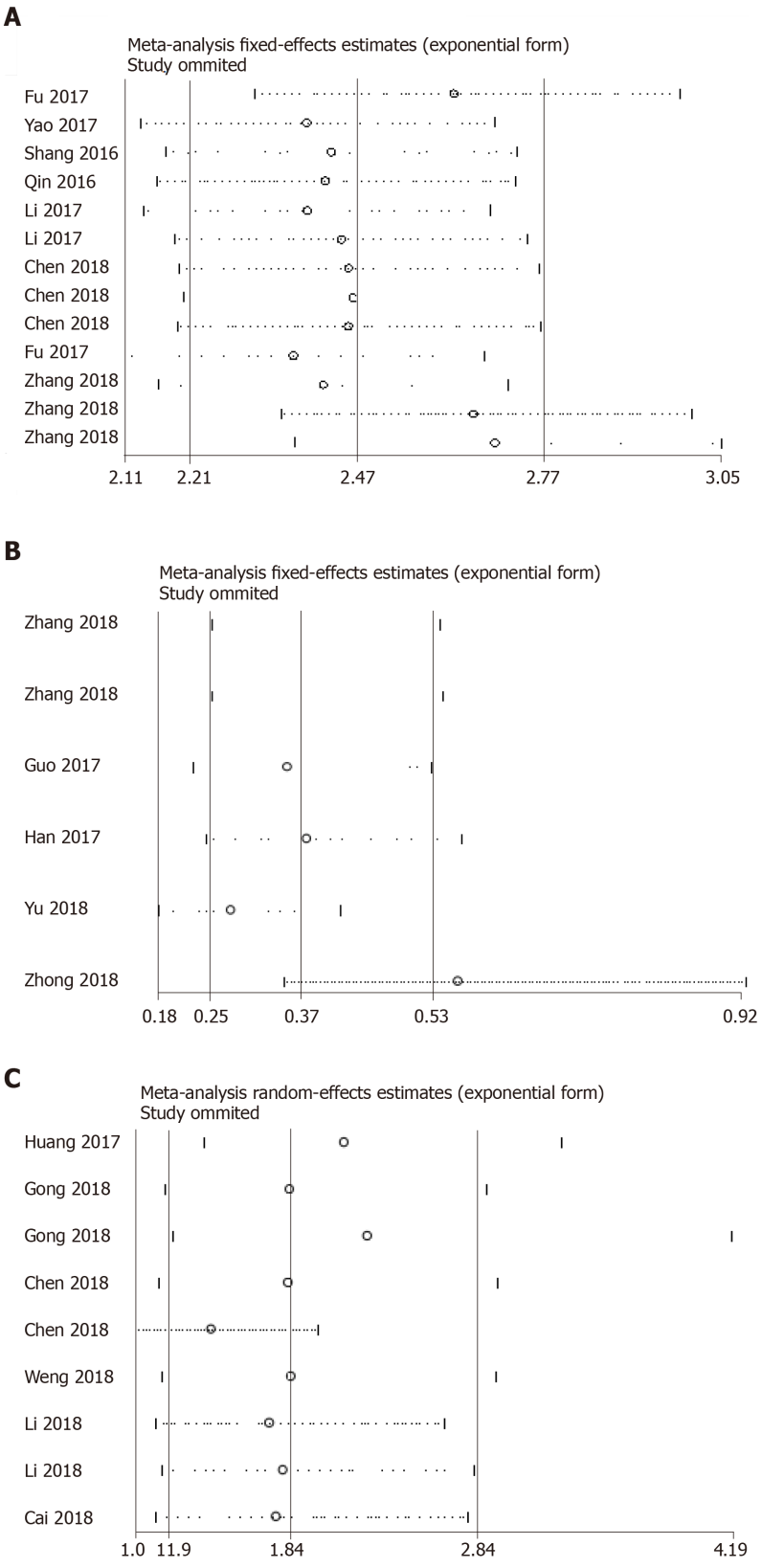

Figure 5 Sensitivity analysis of the outlier data. A: Diagnostic studies; B: Down-regulated; and C: Up-regulated circular RNA profiles in predicting the overall survival in hepatocellular carcinoma.

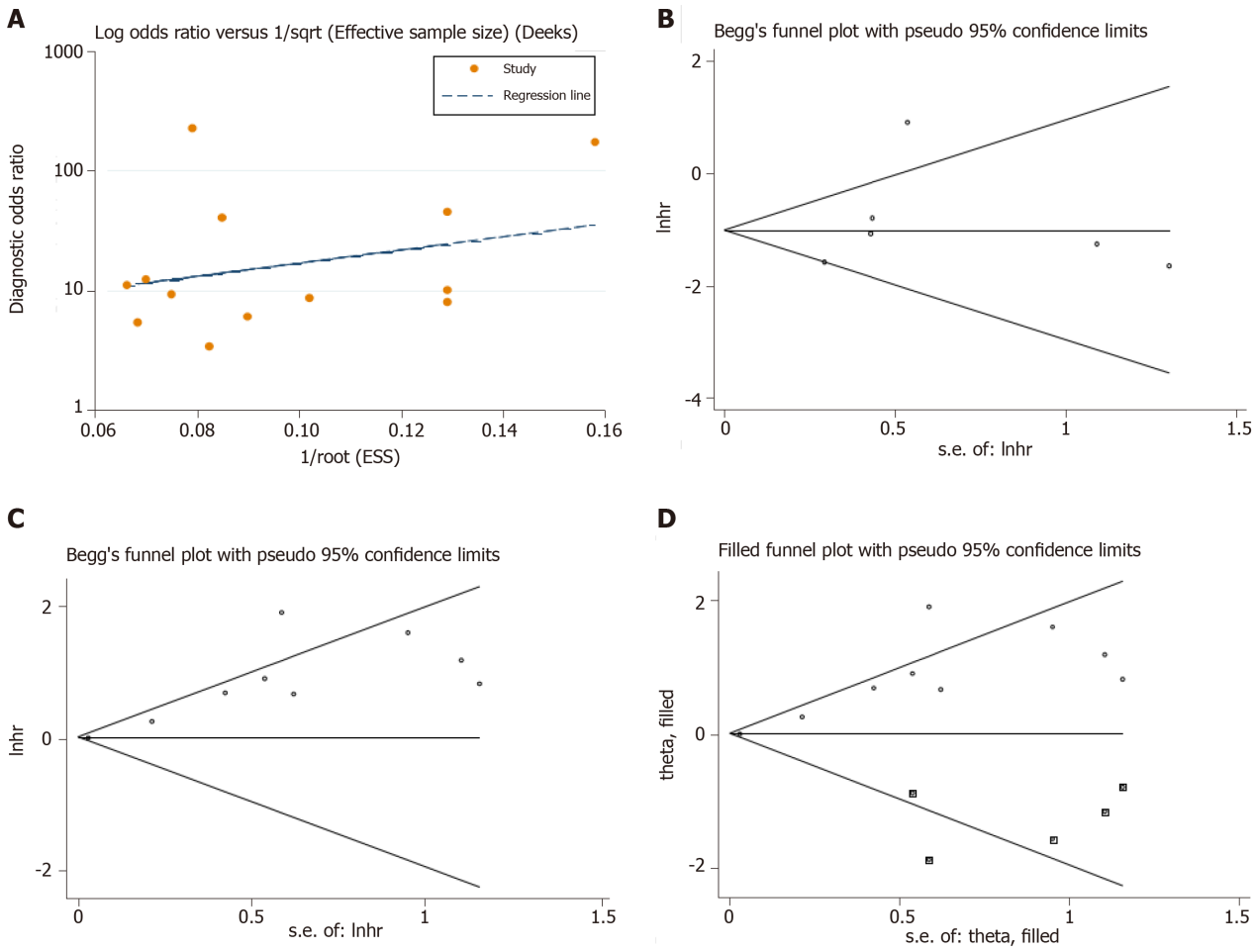

Figure 6 Publication bias judged by the Deek's funnel plot for the diagnostic meta-analysis. A-C: Begg's test for the down-regulated and up-regulated circular RNA (circRNA) signatures in predicting the overall survival in hepatocellular carcinoma; D: The trim and fill method performed to assess the possible effects of bias on the overall pooled effects of the up-regulated circRNA signature. The hollow circles in squares indicate the imputed studies.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. At present, reliable biomarkers for HCC are unavailable, so it is necessary to investigate novel effective ones. The application of circular RNAs (circRNAs) in numerous tumors has been drawing considerable attention. However, the clinical value of circRNAs in HCC has not been determined.

Research motivation

We sought to provide evidence on the potential clinical value of abnormal circRNAs in HCC from the perspective of evidence-based medicine.

Research objectives

This meta-analysis was designed to reveal the clinicopathological, prognostic, and diagnostic features of circRNAs in HCC.

Research methods

We searched for articles in PubMed, EMBASE, and CNKI databases before May 2019. Studies reporting on the clinicopathologic, diagnostic, or prognostic significance of circRNAs in HCC were eligible for inclusion. The meta-analysis was performed with Stata and Meta-DiSc software, and the study quality was assessed in accordance with the Quality Assessment of Diagnostic Accuracy Studies-2 Checklist and the Newcastle-Ottawa Scale. According to the heterogeneity of the studies, a fixed- or random-effects model was used for pooling analysis.

Research results

A total of 21 studies were eligible for the meta-analysis. The results showed that the abnormality in the expression of circRNAs was of good significance in the diagnostic determination of HCC. The down-regulation of circRNAs was negatively correlated with HCC prognosis, while the up-

regulated circRNAs were positively related to the overall survival. The circRNAs were significantly associated with poor clinicopathological features in patients with HCC.

Research conclusions

This meta-analysis suggested that circRNAs may be a promising biomarker for the determination of diagnosis and prognosis of HCC in clinical practice.

Research perspectives

The results of this meta-analysis may contribute to a better understanding of the potential clinical application of abnormal circRNAs in HCC.

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CASE REPORT

Gastric submucosa-invasive carcinoma associated with Epstein-Barr virus and endoscopic submucosal dissection: A case report

Yu Kobayashi, Takehito Kunogi, Hiroki Tanabe, Yuki Murakami, Takuya Iwama, Takahiro Sasaki, Keitaro Takahashi, Katsuyoshi Ando, Yoshiki Nomura, Nobuhiro Ueno, Shin Kashima, Kentaro Moriichi, Hidehiro Takei, Mikihiro Fujiya, Toshikatsu Okumura

ORCID number: Yu Kobayashi (0000-0002-6196-604X); Takehito Kunogi (0000-0001-7028-1256); Hiroki Tanabe (0000-0001-9029-5081); Yuki Murakami (0000-0002-7738-3578); Takuya Iwama (0000-0002-8641-8605); Takahiro Sasaki (0000-0002-0412-8760); Keitaro Takahashi (0000-0003-4188-0246); Katsuyoshi Ando (0000-0003-3500-5488); Yoshiki Nomura (0000-0002-2607-0029); Nobuhiro Ueno (0000-0001-5448-4766); Shin Kashima (0000-0001-8309-4281); Kentaro Moriichi (0000-0002-1298-3265); Hidehiro Takei (0000-0002-2095-670X); Mikihiro Fujiya (0000-0002-4321-7774); Toshikatsu Okumura (0000-0003-1810-007X).

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Yu Kobayashi, Takehito Kunogi, Hiroki Tanabe, Yuki Murakami, Takuya Iwama, Takahiro Sasaki, Keitaro Takahashi, Katsuyoshi Ando, Yoshiki Nomura, Nobuhiro Ueno, Shin Kashima, Kentaro Moriichi, Mikihiro Fujiya, Toshikatsu Okumura, Division of Gastroenterology and Hematology/Oncology Department of Medicine, Asahikawa Medical University, Asahikawa 078-8510, Japan

Hidehiro Takei, Pathological Department of Asahikawa Medical University Hospital, Asahikawa Medical University, Asahikawa 078-8510, Japan

Corresponding author: Hiroki Tanabe, MD, PhD, Doctor, Division of Gastroenterology and Hematology/Oncology Department of Medicine, Asahikawa Medical University, 2-1-1-1, Midorigaoka-Higashi, Asahikawa 078-8510, Japan. tant@asahikawa-med.ac.jp
Telephone: +81-166-682462
Fax: +81-166-682469

Abstract

BACKGROUND

Epstein-Barr virus (EBV)-associated carcinoma is a gastric cancer subtype with a morphology characterized by gastric carcinoma with lymphoid stroma (GCLS). Clinicopathological studies have indicated a better prognosis for GCLS than for common gastric carcinomas. Some previous cases of early gastric cancer associated with EBV had been diagnosed by endoscopic resection.

CASE SUMMARY

We present two GCLS cases subjected to endoscopic submucosal dissection (ESD) for a definitive diagnosis. A protruded gastric lesion was identified by routine endoscopic examination, but forceps biopsy showed no atypical cells before ESD. The resected specimen showed a poorly differentiated adenocarcinoma with lymphoid cells involving the mucosa and submucosa. The final diagnosis was submucosa-invasive poorly differentiated gastric adenocarcinoma. Accordingly, additional gastrectomy was recommended to obtain a complete cure. One patient underwent additional distal gastrectomy with lymph node dissection, but the other was refused because of cardiovascular complications. Both patients remained in remission for more than half a year. EBV positivity was determined by EBV-encoded RNA *in situ* hybridization. We also conducted a literature review of cases of early gastric cancer associated with EBV that had been diagnosed by ESD.

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CONCLUSION
Submucosa-invasive GCLS could be dissected using ESD, and EBV positivity should be subsequently assessed to determine whether or not any additional curative surgery is required. Further prospective investigations on the prevalence of lymph node metastasis in EBV-associated carcinoma should be performed to expand the indications for endoscopic resection.

Key words: Herpesvirus 4; Human; Stomach neoplasms; Gastric carcinoma with lymphoid stroma; Epstein-Barr virus-associated gastric carcinoma; Case report

Core tip: Two cases of Epstein-Barr virus (EBV)-associated gastric carcinoma were diagnosed by endoscopic submucosal dissection (ESD). Because of its low frequency of lymph node metastasis, EBV-associated carcinoma can be treated with ESD without additional surgery.

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INTRODUCTION

Epstein-Barr virus (EBV), also known as human herpesvirus 4, is associated with Burkitt lymphoma, nasopharyngeal carcinoma, and natural killer/T lymphoma. EBV is also positive in 80%-90% of cases of gastric carcinoma with lymphoid stroma (GCLS), which includes undifferentiated adenocarcinoma with intense lymphoid infiltration[1].

GCLS was first proposed as a separate entity in 1976[2], and lymphoepithelioma-like carcinoma associated with EBV was found in 1990[3,4]. The clinical features of EBV-associated gastric carcinoma include its location in the middle or upper stomach and a superficially depressed or submucosal tumor (SMT)-like appearance. Clinicopathological studies have indicated that EBV-positive carcinomas have a better prognosis and lower rate of lymph node metastasis than EBV-negative carcinomas[1,5,6]. In the Cancer Genome Atlas (TCGA) project, gastric cancers are divided into four subtypes, one of which is positive for EBV. EBV-positive gastric cancer has recently attracted much attention due to dramatic advances in drug therapies such as DNA methylation inhibitors and immune checkpoint inhibitors[7].

EBV-associated GCLS is defined as a poorly differentiated carcinoma admixed with marked subepithelial lymphoid cell infiltration. Because the carcinoma is covered with an overlying normal epithelium, an endoscopic biopsy sometimes fails to yield tissue specimens to be pathologically diagnosed as malignant. We performed an endoscopic mucosal dissection (ESD) in two GCLS cases to obtain a definitive diagnosis. EBV positivity in cancer cells was confirmed by EBV-encoded small RNA *in situ* hybridization. We also reviewed cases of EBV-associated submucosa-invasive GCLS that were subjected to ESD.

CASE PRESENTATION

Case 1

Chief complaint: A 72-year-old woman complaining of abdominal discomfort had been treated for chronic gastritis in our hospital.

History of present illness: She had a medical history of eradication of *Helicobacter pylori* without a family history of gastric malignancy.

Physical examination: There were no abnormal findings in physical examination, and

the serum chemistry and complete blood count were normal.

Imaging examinations: She underwent esophagogastroduodenoscopy at her routine check-up. A 20-mm protruding lesion with a central depression was noted in the middle gastric body (Figure 1A). Forceps biopsy showed no atypical cells. Computed tomography (CT) did not revealed gastric tumor or lymph node swelling. Four months later, an endoscopic re-examination revealed no significant difference in findings, with no atypical cells in the biopsy specimen, but subsequent endoscopic ultrasonography indicated a hypoechoic lesion that massively infiltrating the submucosa (Figure 1B and C). Due to a strong suspicion of gastric carcinoma, ESD was performed. The ESD specimen showed a poorly differentiated adenocarcinoma with accompanying prominent lymphoid tissues involving the mucosa and submucosa (Figure 2). Lymphatic invasion was observed, and EBV-encoded RNA (EBER) was detected by *in situ* hybridization.

Case 2

Chief complaint: A 73-year-old man was referred to our hospital due to an SMT in the lower gastric body, which had been followed at a city hospital for 4 years.

History of present illness: A follow-up endoscopic examination showed no apparent changes from previous evaluations. The SMT was further examined because the patient needed treatment for myocardial infarction and abdominal aortic aneurysm at a tertiary hospital.

Physical examination: There was no family history of malignancy. No physical finding in his abdomen was observed in our consultation room.

Laboratory examinations: His complete blood count was normal, and most of the blood parameters were within normal range, except for a slight decrease in the renal function test (estimated glomerular filtration rate, 48.5 mL/min).

Imaging examinations: Annual endoscopy revealed no significant change for the following 3 years, and no atypical cells were obtained by forceps biopsy. CT did not reveal gastric tumor or lymph node metastasis. However endoscopic ultrasonography demonstrated a multinodular hypoechoic lesion, measuring 1.5 cm in the greatest dimension, with submucosal involvement (Figure 3). GCLS was suspected, and ESD was performed for a definitive pathological diagnosis when the patients was 76 years old. The pathological diagnosis (Figure 4) was a carcinoma with lymphoid stroma, 19 x 16 mm, and infiltrating into the deep submucosa (SM2, 5200 μm). No lymphovascular invasion was detected. EBV was positive in cancer cells based on EBER *in situ* hybridization.

FINAL DIAGNOSIS

Case 1 and Case 2

Submucosa-invasive GCLS, EBV-positive. Both patients were recommended to undergo additional distal gastrectomy because there is a risk of lymph node metastasis when poorly differentiated gastric carcinoma is invading the submucosa.

TREATMENT

Case 1

The patient underwent distal gastrectomy, and no residual tumor or lymph node metastasis was observed in the resected specimen.

Case 2

The patient refused surgical operation because of his cardiovascular complications, and was hoping for careful medical observation without chemotherapy or radiotherapy.

OUTCOME AND FOLLOW-UP

Case 1

The patient remained without recurrence at the 14-month follow-up.

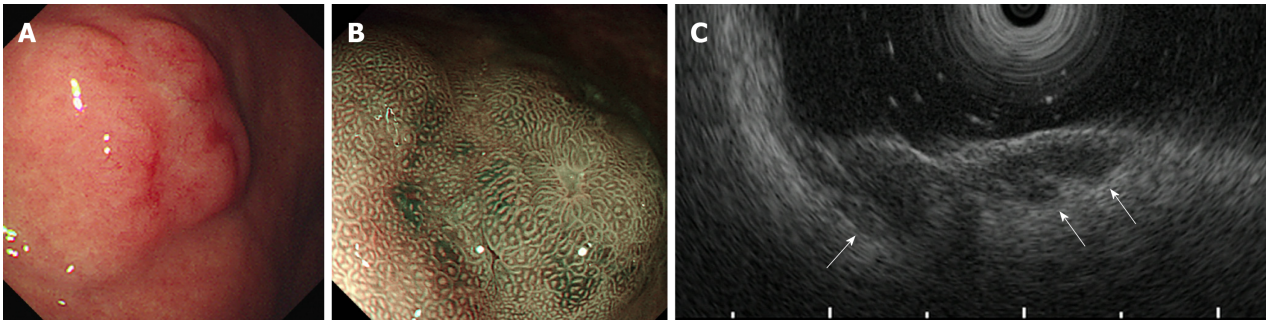

Figure 1 Endoscopic and ultrasonographic images of gastric carcinoma with lymphoid stroma (Case 1). A: A protruding lesion with central depression is covered with mucosa, the surface of which shows slight hyperemia; B: Magnifying narrow-band imaging reveals regular round and oval pits on the surface of the tumor; C: A 7.5-MHz endoscopic ultrasound image shows hypo-echoic lesions projecting into the third hyperechoic layer (white arrows).

Case 2

The patient underwent re-examination by gastroscopy, and no residual atypical cells were pathologically observed at 12 months after ESD. *Helicobacter pylori* was positive in the biopsy specimen taken from the gastric body, and eradication therapy was therefore performed.

DISCUSSION

Helicobacter pylori is the major cause of gastric cancer, and its eradication is recommended to decrease the risk of gastric cancer. An association between EBV and gastric cancer has also been suggested, as viral clonality is observed in proliferating cancer cells[8]. EBV-associated gastric cancer is characterized as poorly differentiated carcinoma with prominent lymphoid infiltration, clearly distinguishable from *Helicobacter pylori*-associated gastric cancer. As the infection rate with *Helicobacter pylori* is decreasing worldwide, trends in EBV-associated gastric cancer are now being monitored with interest by many researchers.

TCGA classifies gastric cancer into four subtypes: EBV-positive tumors, microsatellite unstable tumors, genetically stable tumors, and tumors with chromosomal instability[7]. The EBV-positive subtype shows DNA hypermethylation in host cell DNA due to methyltransferase 1 transcription induced by EBV latent membrane protein 2A. It displays amplification of programmed death-ligand 1 (PD-L1) and PD-L2, and the immune tolerance of the neoplasms may be associated with carcinogenesis. While patients with EBV-associated gastric cancer tend to have a good prognosis, the underlying molecular mechanism remains unclear[9,10].

Early GCLS has peculiar clinicopathological features, and its prognosis depends on the EBV infection status[11]. Early GCLS has also been analyzed in relation to lymph node metastasis, and EBV positivity is a predictive marker of a negative lymph node metastasis[12,13]. The risk of lymph node metastasis of mucosal GCLS and that of submucosal GCLS are 0 and 4.0%-10.6%, respectively. Limited to EBV-positive cases, intramucosal gastric cancer displays no lymph node metastasis, as reported by Japanese researchers (Tokunaga *et al*[14] and Murai *et al*[15]). Therefore, intramucosal EBV-positive gastric carcinomas could be treated by ESD rather than radical gastric surgery, because these cases are associated with a minimal risk of lymph node metastasis. The association between EBV positivity of submucosa-invasive GCLS and lymph node metastasis has not been previously described. A study in Korea reported that EBV positivity is a favorable risk factor for lymph node metastasis in submucosa-invasive gastric cancer, with a rate of metastasis of 4.7%[9]. The authors suggested that EBV positivity might be considered an additional criterion for the indication of endoscopic resection. We therefore reviewed cases of EBV-positive gastric cancer in which ESD was performed for diagnosis and treatment.

We conducted a database search of PubMed, Scopus and ScienceDirect using the following terms: ("gastric carcinoma" or "gastric cancer") and (EBV or "Epstein-Barr virus" or "human herpesvirus 4") and ("endoscopic submucosal dissection" or ESD). An search of reported references was also performed. A total of 9 cases were described with detailed clinical information in six reports[16-21]. Upon the addition of our 2 cases, 11 EBV-positive gastric submucosal cancer cases were ultimately collected by our database search (Table 1). This disease was found to be associated with male sex, proximal location, and depressed or SMT-like appearance. The diagnosis appeared to be difficult, as 5 cases (45.5%) were negative on pathological examination

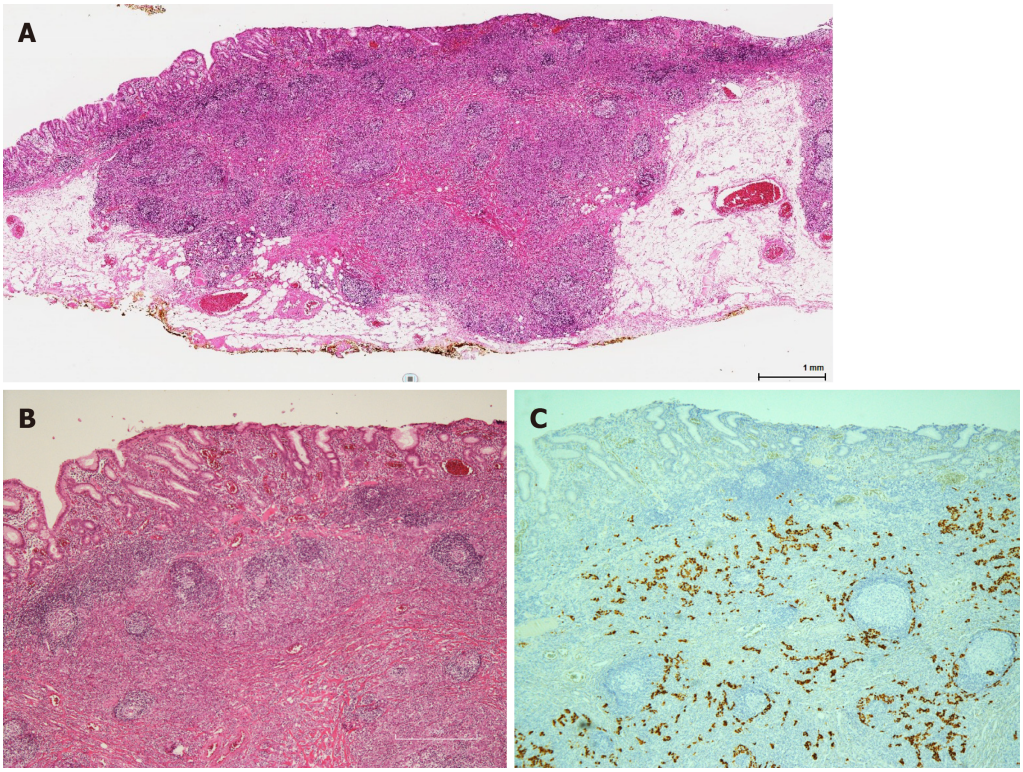

Figure 2 Findings on histological examination of the endoscopically resected specimen (Case 1). A: The low-magnification view shows gastric carcinoma with lymphoid stroma (GCLS) located in the mucosa to deep submucosa (hematoxylin and eosin: HE); B: A microscopic view of the GCLS reveals normal epithelium overlying the tumor (HE, × 100); C: Epstein-Barr virus-encoded RNA *in situ* hybridization shows intense staining in carcinoma cells (× 100). GCLS: Gastric carcinoma with lymphoid stroma; HE: Hematoxylin and eosin.

by forceps biopsy. Repeated biopsies failed to yield malignant cells in both of our cases. The depths of the tumor invasion were more than 1 mm into the submucosa from the deepest portion of the muscularis mucosae; however, the horizontal dimension was smaller than 2 cm in most cases. In the five cases that were subjected to subsequent radical gastrectomy with lymph node dissection, no lymph node metastasis was observed. Furthermore, neither local recurrence nor distant metastasis was reported throughout the follow-up periods. Therefore, submucosa-invasive cancer can be completely excised, allowing us to cure the patients with early gastric cancer. Excessive and unnecessary gastrectomy might therefore be avoided and replaced by minimally invasive endoscopic resection.

The European Society of Gastrointestinal Endoscopy guidelines strongly recommend endoscopic resection for the treatment of superficial gastric neoplastic lesions that possess a very low risk of lymph node metastasis[22]. According to the recommendations of Japanese gastric cancer treatment guidelines, "endoscopic resection is considered for tumors that have a very low possibility of lymph node metastasis and are suitable for *en-bloc* resection" as a general principle regarding the indications for endoscopic resection[23]. The absolute indication as a standard treatment is well-differentiated adenocarcinoma with no ulceration, T1a depth, and diameter < 2 cm. The expanded indication for undifferentiated-type adenocarcinoma is T1a depth, no ulceration, and diameter < 2 cm. Based on our review of the previous reports of GCLS and EBV-associated carcinomas, endoscopic resection may thus be acceptable for mucosal GCLS even if it is of the undifferentiated-type, regardless of its horizontal diameter. For cases of submucosa-invasive EBV-associated carcinoma, ESD can also be a diagnostic procedure unless a diagnosis of GCLS is confirmed by forceps biopsy.

CONCLUSION

Further prospective studies into whether or not endoscopic resection can cure EBV-associated carcinomas are required to expand the therapeutic indications for gastric cancer. Positivity for EBV will be a useful predictive marker of lymph node metastasis, which can help us determine the optimal treatment strategy.

Table 1 A summary of cases of Epstein-Barr virus-associated gastric cancer treated using endoscopic submucosal dissection

Study	Age (yr), Sex	Diameter (cm)	Lesions	Features	Biopsy diagnosis	ESD diagnosis	EBV	Depth	Additional surgery	Lymph node metastasis	Prognosis
Gromski *et al*[16], 2012	67, M	2 × 1.2	Lower body	Centrally depressed lesion	Chronic gastritis	Lymphoepithelioma-like gastric carcinoma	Positive	SM2	Not performed	ND	No recurrence for 12 M
Lee *et al*[17], 2012	43, M	NA	NA	Multiple elevated erosive lesions with mild central depressions	Adenocarcinoma	Lymphoepithelioma-like gastric carcinoma with lymphoid-rich stroma	Positive	SM2 (1538 μm)	Not performed	ND	No recurrence for 24 M
Matsumoto *et al*[18], 2013	58, M	NA	Upper body	Submucosal tumor associated with a slightly depressed lesion	Adenocarcinoma	Gastric carcinoma with lymphoid stroma	Positive	SM2	Formal resection	Negative	NA
Lee *et al*[19], 2014	63, M	2.0	High body	Elevated lesion that displayed surface hyperemia	Moderately differentiated adenocarcinoma	Lymphoepithelioma-like gastric carcinoma	Positive	SM2 (1800 μm)	Total gastrectomy	Negative	No recurrence for 48 M
	65, M	1.0	Low body	Slightly elevated	Moderately differentiated adenocarcinoma	Lymphoepithelioma-like gastric carcinoma	Positive	SM (1800 μm)	Not performed	ND	No recurrence for 32 M
	74, M	1.5	Low body	Slightly elevated lesion with central dimpling	A few markedly atypical cells	Lymphoepithelioma-like gastric carcinoma	Positive	SM2 (2500 μm)	Not performed	ND	No recurrence for 28 M
	84, M	1.5	Cardia	Large reddish and slightly depressed lesion	Moderately differentiated adenocarcinoma	Lymphoepithelioma-like gastric carcinoma	Positive	SM2 (2300 μm)	Not performed	ND	No recurrence for 27 M
Chen *et al*[20], 2016	50, M	2.5 × 2.5	Gastric body	A submucosal columnar lesion with surface erosion	Moderate chronic superficial gastritis	Lymphoepitheliomalike gastric carcinoma	Positive	SM	Total radical gastrectomy	Negative	No recurrence for 12 M
Kato *et al*[21], 2018	53, M	2.0	Middle body	Subepithelial lesion with center depressed	Benign gastric mucosa	Gastric cancer with lymphoid stroma	Positive	SM2	Distal gastrectomy	Negative	NA
Present cases	72, F	2.0	Middle body	Protruding lesions with central depression	No atypical cells	Gastric carcinoma with lymphoid stroma	Positive	SM2 (> 4000 μm)	Distal gastrectomy	Negative	No recurrence for 14 M
	76, M	1.5	Lower body	Submucosal tumor	No atypical cells	Gastric carcinoma with lymphoid stroma	Positive	SM2 (5200 μm)	Not performed	ND	No recurrence for 12 M

ESD: Endoscopic submucosal dissection; EBV: Epstein-Barr virus; SM: Submucosa; ND: Not determined; NA: Not available; M: Male.

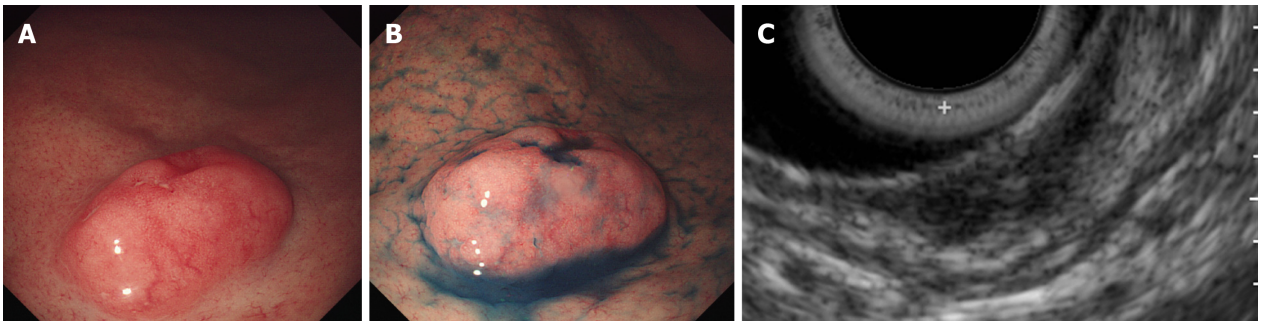

Figure 3 Endoscopic and ultrasonographic images of gastric carcinoma with lymphoid stroma (Case 2). A: Submucosal tumor-like lesion with dilated capillary vessels is found in the lower gastric body at the first endoscopic examination; B: Indigo carmine dye spraying emphasizes the protruded tumor with central depression; C: A 20 MHz endoscopic ultrasound image shows a multinodular hypoechoic mass located in the submucosal layer.

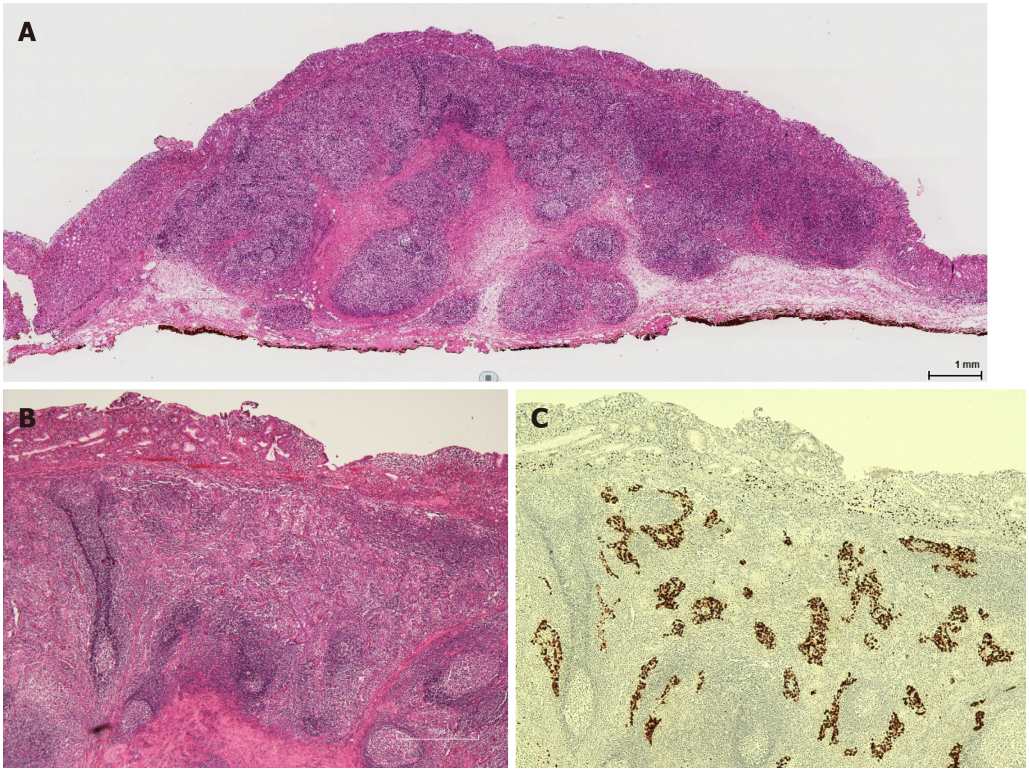

Figure 4 Findings on histological examination of the endoscopically resected specimen (Case 2). A: The low-magnification view shows gastric carcinoma with lymphoid stroma mainly located in the submucosal layer (hematoxylin and eosin: HE); B: A microscopic view reveals massive infiltration of lymphoid cells (HE, × 100); C: Epstein-Barr virus-encoded RNA *in situ* hybridization shows intensely positive staining in the nests of cancer cells (× 100). HE: Hematoxylin and eosin.

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