ISSN 1948-5204 (online)

World Journal of *Gastrointestinal Oncology*

World J Gastrointest Oncol 2022 September 15; 14(9): 1604-1891

Published by Baishideng Publishing Group Inc

World Journal of Gastrointestinal Oncology

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The *WJGO* is now abstracted and indexed in PubMed, PubMed Central, Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Scopus, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJGO* as 3.404; IF without journal self cites: 3.357; 5-year IF: 3.250; Journal Citation Indicator: 0.53; Ranking: 162 among 245 journals in oncology; Quartile category: Q3; Ranking: 59 among 93 journals in gastroenterology and hepatology; and Quartile category: Q3. The *WJGO*'s CiteScore for 2021 is 3.6 and Scopus CiteScore rank 2021: Gastroenterology is 72/149; Oncology is 203/360.

RESPONSIBLE EDITORS FOR THIS ISSUE

Production Editor: *Ying-Yi Yuan*; **Production Department Director:** *Xiang Li*; **Editorial Office Director:** *Jia-Ru Fan*.

NAME OF JOURNAL
World Journal of Gastrointestinal Oncology

ISSN
ISSN 1948-5204 (online)

LAUNCH DATE
February 15, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Monjur Ahmed, Florin Burada

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-5204/editorialboard.htm

PUBLICATION DATE
September 15, 2022

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World J Gastrointest Oncol 2022 September 15; 14(9): 1604-1621

DOI: 10.4251/wjgo.v14.i9.1604

ISSN 1948-5204 (online)

REVIEW

Advances in postoperative adjuvant therapy for primary liver cancer

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kanda T, Japan; Oon C, Malaysia

Received: December 29, 2021
Peer-review started: December 29, 2021
First decision: April 16, 2022
Revised: May 13, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: September 15, 2022

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Abstract

Hepatocellular carcinoma (HCC) is a highly heterogeneous, invasive, and conventional chemotherapy-insensitive tumor with unique biological characteristics. The main methods for the radical treatment of HCC are surgical resection or liver transplantation. However, recurrence rates are as high as 50% and 70% at 3 and 5 years after liver resection, respectively, and even in Milan-eligible recipients, the recurrence rate is approximately 20% at 5 years after liver transplantation. Therefore, reducing the postoperative recurrence rate is key to improving the overall outcome of liver cancer. This review discusses the risk factors for recurrence in patients with HCC radical surgical resection and adjuvant treatment options that may reduce the risk of recurrence and improve overall survival, including local adjuvant therapy (*e.g.*, transcatheter arterial chemoembolization), adjuvant systemic therapy (*e.g.*, molecular targeted agents and immunotherapy), and other adjuvant therapies (*e.g.*, antiviral and herbal therapy). Finally, potential research directions that may change the paradigm of adjuvant therapy for HCC are analyzed.

Key Words: Adjuvant therapy; Liver cancer; Immunotherapy; Chemotherapy; Targeted therapy

Core Tip: This review discusses the risk factors for recurrence in patients with hepatocellular carcinoma (HCC) radical surgical resection and adjuvant treatment options that may reduce the risk of recurrence and improve overall survival, including local adjuvant therapy (*e.g.*, transcatheter arterial chemoembolization), adjuvant systemic therapy (*e.g.*, molecular targeted agents), and other adjuvant therapies (*e.g.*, antiviral and herbal therapy). Finally, potential research directions that may change the paradigm of adjuvant therapy for HCC are analyzed.

Citation: Zeng ZM, Mo N, Zeng J, Ma FC, Jiang YF, Huang HS, Liao XW, Zhu GZ, Ma J, Peng T. Advances in postoperative adjuvant therapy for primary liver cancer. *World J Gastrointest Oncol* 2022; 14(9): 1604-1621
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1604.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1604

INTRODUCTION

Primary liver cancer (PLC) is one of the most common malignancies worldwide. According to the Global Cancer Data (GLOBOCAN) 2020, the annual number of new cases of liver cancer reached 905677 worldwide, ranking seventh in malignant tumors, whereas the annual number of deaths caused by PLC is 830180, ranking second in malignant tumors[1]. Approximately 50% of the cases of global liver cancer occur in China, and data released by the National Cancer Center in 2021 showed that liver cancer has become the fourth most common malignant tumor in China, and its mortality rate ranks second, with a ratio of incidence to mortality rates reaching 1:0.8[2], which seriously threatens the life and health of the population. The predominant histological type of PLC is hepatocellular carcinoma (HCC), which accounts for approximately 85% to 90% of cases. HCC often occurs in the setting of chronic liver disease with or without cirrhosis, and the most common etiologies are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcohol intake, and aflatoxin exposure. Growing evidence suggests that nonalcoholic fatty liver disease especially nonalcoholic steatohepatitis-related cirrhosis is associated with the development of HCC and represents an increasingly common risk factor for HCC in Western countries[3-6]. Cirrhosis is a crucial risk factor for HCC, and long-term follow-up studies have found that approximately 1% to 8% of patients with cirrhosis develop HCC each year[7]. As a result, HCC treatment faces two simultaneous challenges: the malignancy itself and the underlying liver disease, which not only increases the difficulty of the treatment but also increases the risk of tumor recurrence or new cancer. The main curative methods for the long-term survival of patients with HCC include surgical resection, liver transplantation, and radiofrequency ablation. However, the lack of liver transplant donors, the high cost of the procedure, and the small scope of radiofrequency ablation have limited their clinical application. Therefore, the current radical treatment for HCC is mainly hepatectomy. However, the 5-year recurrence rate after hepatectomy in patients with HCC eligible for surgical resection is as high as 70%[8,9], and even if they receive liver transplantation, the 5-year recurrence rate in recipients who meet the Milan criteria can reach approximately 20%[10]. HCC recurrence seriously affects the long-term outcome and quality of life of patients after surgery. Therefore, reducing the postoperative recurrence rate is the key to improving the overall outcome of HCC[11].

RISK FACTORS AFFECTING RECURRENCE OF LIVER CANCER AFTER SURGERY

It is currently accepted that HCC recurrence may originate from intrahepatic metastases or from *de novo* development of tumors. The clinical pattern of postoperative recurrence is usually divided into early and distant recurrences. Early recurrence refers to the one that occurs within 2 years after the initial treatment and is of monocentric origin (also called monoclonal origin), *i.e.* tumors arising from occult micrometastases of the primary tumor or residual microscopic cancer foci *in situ* at the site of postoperative resection[12]. These recurrences, which are usually associated with invasive tumor characteristics, are considered true recurrences accounting for approximately 70% or more of the total. In contrast, the distant recurrence is defined as the one that appears 2 years after the initial treatment and is multicentric in occurrence (also known as polyclonal in origin), *i.e. de novo* tumors induced by the oncogenic microenvironment of the diseased liver associated with hepatic inflammation or cirrhosis [13]. Studies have shown that independent risk factors associated with early recurrence are mainly related to the initial characteristics of the tumor and surgical variables, including large tumor size (> 5 cm in diameter), multiple nodes (two or more tumor nodes), macrovascular/microvascular invasion, non-anatomic liver resection, satellite nodes, cut margins < 1 cm, high preoperative HBV-DNA load and serum alpha fetoprotein (AFP) > 400 μg/L[14-16]. Studies have shown that in addition to high viral load and progression of cirrhosis, factors such as the tumor size, microvascular invasion, and no/irregular

postoperative antiviral therapy are also associated with distant recurrence[14-17]. Factors affecting the recurrence of liver cancer after liver transplantation mainly include preoperative factors, such as the selection criteria for the recipients of liver transplants (Milan criteria, University of California San Francisco criteria that exceeds Milan criteria, Hangzhou criteria that exceeds Milan criteria and introduces biological characteristics); preoperative descending therapy and biomarkers; and intraoperative factors such as surgical operation, bleeding volume, time of ischemia of the donor liver, postoperative immunosuppressive regimen, and systemic treatment regimen in three areas[18].

It is not difficult to find a recurrence of HCC after surgery in relation to the tumor biology, medical history, and viral infection. Therefore, individualized adjuvant treatment strategies based on risk factors for recurrence should be the most effective ones. At this stage, there is no accepted postoperative adjuvant treatment option for HCC, but recent clinical studies have provided new approaches to improve the prognosis of the disease. This article reviews the current research on postoperative adjuvant therapy for HCC and discusses possible directions for future adjuvant therapy research.

ADJUNCTIVE LOCAL TREATMENT

Postoperative adjuvant transcatheter arterial chemoembolization

The blood supply to normal liver tissue is 20%-25% from the hepatic artery and 70%-75% from the portal vein, whereas 95%-99% of the blood supply to HCC tissue originates from the hepatic artery. Transcatheter arterial chemoembolization (TACE) is a mixture of an embolic agent and chemotherapeutic drugs injected precisely into the lesion through the branch of tumor blood supply artery to achieve embolization of the tumor neovascularization, induce ischemia, hypoxia, and necrosis of the tumor tissue, and achieve the purpose of killing the tumor through the cytotoxic effect of chemotherapeutic drugs. TACE is widely used for locally progressive HCC that is not suitable for surgical resection or liver transplantation. However, the results available are inconsistent in their conclusions regarding the benefits of adjuvant TACE therapy after hepatectomy. The conclusions of several successive Asian randomized controlled trials (RCTs) starting in 1994, support postoperative adjuvant TACE therapy to reduce recurrence rates and/or improve overall survival (OS) in patients at moderate to high risk of recurrence; in addition, the therapy is well tolerated by patients[19-23]. These results were confirmed by two recently published RCTs. Wang *et al*[24] reported a randomized, open-label, single-center phase III RCT that included 280 patients with HBV-related HCC at moderate to high risk of recurrence (single tumor diameter > 5 cm without large vessel invasion, single tumor with large vessel invasion, or 2-3 tumors), in which patients were randomly assigned to either TACE or observation groups after radical hepatectomy. Patients in the TACE group had a significantly lower recurrence rate and significantly longer recurrence-free survival (RFS) and OS compared to those of the observation group[24]. In another randomized, open-label, single-center phase III study including 250 cases Wei *et al*[25] randomly assigned 1:1 patients with HCC and tumor diameter > 5 cm with microvascular invasion (MVI) to either adjuvant TACE or non-adjuvant treatment groups. The results showed a median disease-free survival (DFS) of 17.45 mo in the TACE group compared with 9.27 mo in the control group (hazard ratio [HR] = 0.70; P = 0.020)[25]. Qi *et al*[26] reported a prospective clinical study in which 200 patients with postoperative pathologically MVI-positive HCC were divided into adjuvant TACE and control groups. The results showed that TACE improved the prognosis of the disease, especially in patients with tumors > 5 cm in diameter or multinodular tumors. Several large single-center retrospective studies[27-31] found that postoperative adjuvant TACE therapy prolonged OS and DFS/RFS in patients with high-risk recurrence factors such as MVI positivity, tumor diameter > 5 cm, poorly differentiated pairs, and multiple tumors. Concerning safety, adjuvant TACE treatment was generally well tolerated, although it increased the incidence of adverse events.

In patients at low risk of recurrence, a retrospective study[32] including 180 patients with hepatectomized HCC reported that the median progression-free survival of patients treated with TACE after surgery was 52.0 mo compared to 11.1 mo in the surgery-only group, and the median OS of 90.7 mo in the TACE group was significantly longer than that of 54.4 mo in the surgery-only group, suggesting that prophylactic interventions are equally effective in reducing recurrence in patients at low risk of recurrence, and that the results of this study may be related to the rigorous screening of TACE-treated patients. In addition, a meta-analysis and systematic review of randomized studies of the adjuvant TACE therapy suggested that patients with low-risk recurrent HCC do not seem to benefit from the adjuvant therapy[33]. However, patients with high-risk recurrence of HCC (including tumor diameter > 5 cm, combined vascular invasion, multiple tumors or satellite lesions, and the presence of residual lesions) undergo hepatic resection followed by hepatic artery intervention as adjuvant therapy based on standardized antiviral and hepatoprotective therapy, which may reduce the postoperative recurrence rate and improve DFS/RFS and OS[34,35]. Huang *et al*[36] developed a scoring system based on data from 1150 patients with HCC who underwent hepatectomy between 2002 and 2008 to test the efficacy of the TACE adjuvant therapy. This system uses multivariate analysis to identify tumor diameter, multiple tumors, presence of MVI, incomplete tumor envelope, and surgical margins as independent risk factors for OS. The weighted sum method was used to develop the scoring system to predict OS: MVI (present

= 3, absent = 0) + envelope (incomplete = 2, complete = 0) + tumor diameter (< 5 cm = 4, 3-5 cm = 2, ≤ 3 cm = 0) + number of tumors (multiple = 1, single = 0) + surgical margin (≤ 1 cm = 1, > 1 cm = 0). Patients were divided into three prognostic subgroups based on scores of 0-5, 6-9, and 10, with better, intermediate, and worse survival outcomes, respectively. Moreover, through validation with data from 379 surgical patients between 2008 and 2010, the results showed that the adjuvant TACE treatment improves OS in patients with a score ≥ 10 and observation groups with 1-, 3-, and 5-year OS rates of 63.9%, 22.6%, and 9.0% *vs* 33.8%, 5.6%, and 2.8%, respectively (P = 0.001), suggesting that this scoring system has good discriminatory validity for screening the population for adjuvant TACE therapy[36]. In summary, adjuvant TACE is safe and effective in Asian patients with HCC at high risk of recurrence and may be an effective treatment to prevent tumor recurrence and metastasis after surgical resection of early to mid-stage HCC. However, there are different reports on the population, treatment protocol, timing, and course of adjuvant TACE that deserve in-depth clinical exploration.

Postoperative adjuvant hepatic artery or portal vein infusion chemotherapy

Hepatic arterial infusion chemotherapy (HAIC) and portal vein infusion chemotherapy (PVC) are considered to have higher drug concentration and lower systemic toxicity than those of the standard systemic chemotherapy. HAIC and PVC have been reported less frequently in the postoperative adjuvant treatment of HCC. The results of a retrospective study including 85 patients in China showed that the 5-year RFS was significantly better in the postoperative adjuvant HAIC group (5-fluorouracil, oxaliplatin, and mitomycin combination regimen) than in the non-chemotherapy group[37]. In addition, for patients with HCC with combined portal vein tumor thrombosis (PVTT), a retrospective study showed that the median time to recurrence (TTR) and OS were significantly longer in the postoperative adjuvant PVC group (n = 67) than in the control group, and the cumulative recurrence rate was significantly lower in the PVC group compared to that of the control group[38]. Hamada *et al*[39] reported that DFS and OS were higher in patients with HCC with combined portal infiltration treated with adjuvant HAIC than those in patients without HAIC. For patients with multiple tumors combined with MVI, Hsiao *et al*[40] reported higher OS in the HAIC group than that in the surgery alone group. A meta-analysis based on 11 retrospective cohort studies showed that adjuvant HAIC after surgical resection improved OS and DFS compared to surgical treatment alone[41]. Li *et al*[42] reported a prospective, open-label, phase III, randomized controlled trial that included 127 patients and the results showed that postoperative transarterial infusion chemotherapy (FOLFOX regimen) as adjuvant therapy in patients with HCC with MVI prolonged OS and DFS compared to those of the postoperative observation group. However, more patients need to be included in prospective randomized controlled clinical trials and long-term follow-up to confirm this result.

Postoperative adjuvant radiation therapy

Postoperative adjuvant external radiation therapy: Radiation therapy (RT) is an important tool in oncology treatment, and there is limited information about postoperative radiotherapy as an adjuvant treatment after surgical resection of HCC. Studies have shown that three-dimensional conformal RT may have some application in the anti-recurrence of HCC after surgery. For central HCC, it is often difficult to obtain adequate resection margins. A prospective randomized study enrolling 119 patients with centrally located HCC who underwent narrow margin hepatectomy found that adjuvant radiotherapy for centrally located HCC did not improve RFS and OS; subgroup analysis showed that RFS was significantly longer in the adjuvant radiotherapy group than in the control group in the subgroup of patients with small HCC (< 5 cm)[43]. Another prospective randomized controlled study provided an update of 10-year real world evidence exploring the feasibility and efficacy of adjuvant radiotherapy after narrow margin hepatectomy (< 1 cm) for central HCC. The results showed no significant difference in RFS between the adjuvant radiotherapy and control groups, while RFS was significantly longer in patients with small HCC (5 cm) and OS was significantly improved in patients with small HCC compared to those of the control group at 2 to 5 years after treatment[44]. By contrast, Wang *et al*[45] showed that in patients with HCC with close to large vessels, postoperative adjuvant radiotherapy led to better OS and DFS in patients with narrow margins (< 1 cm) than those in the non-radiotherapy group. A single-arm prospective phase II trial enrolled 76 eligible patients who underwent narrow margin resection and received adjuvant radiotherapy, and showed a 3-year OS and DFS of 88.2% and 68.1%, respectively, and a 5-year OS and DFS of 72.2% and 51.6%, respectively. Intrahepatic recurrence is the predominant form, with no marginal recurrence observed[46]. In patients with positive MVI, the study showed that the postoperative adjuvant radiotherapy group had significantly better RFS and OS than those of the TACE and unadjuvanted groups in patients with HCC combined with MVI [47]. A study of patients with MVI combined with narrow margin HCC showed that postoperative radiotherapy was significantly superior to controls, regardless of the degree of MVI staging[48]. Sun *et al* [49] reported an RCT in which the postoperative radiotherapy significantly prolonged DFS and OS in patients with combined PVTT HCC, with 1-, 2-, and 3-year DFS rates (radiotherapy group: 86.2%, 70.5%, and 63.4%; control group: 46.4%, 36.1%, and 36.1%; P = 0.006) and OS rates (radiotherapy group: 96.6%, 80.7%, and 80.7%; control group: 79.7%, 58.3%, and 50.0%; P = 0.004), which were significantly higher than those in the observation group. Therefore, intensity-modulated radiotherapy after hepatectomy in patients with narrow margins, combined MVI, or PVTT may be a favorable treatment approach.

Postoperative adjuvant internal radiation therapy: Currently, the commonly used routes for internal radiation therapy include the hepatic artery infusion and local modality particle implantation. Lau *et al* [50] first proposed the use of intra-arterial iodine-131 (131I)-labeled iodine oil after hepatectomy as adjuvant therapy for HCC, and in this prospective randomized trial, DFS and OS were significantly better in patients with postoperative intra-arterial infusion of 131I-iodine oil than in patients with hepatectomy alone. An RCT included 43 patients with radical resection of HCC, 21 of whom received postoperative iodine-131 particulate hepatic artery infusion and 22 did not receive the treatment, and showed that intra-arterial adjuvant 131I-iodine oil significantly improved long-term DFS and OS for up to 7 years[51]. Subsequently, several non-randomized studies also confirmed that adjuvant 131I-iodine oil after HCC resection improved DFS and OS after hepatectomy[52-54]. However, a multicenter RCT involving 103 patients showed that the adjuvant 131I-iodine oil treatment did not improve RFS and OS [55]. Another retrospective study with the largest sample to date showed no significant survival improvement with the 131I-iodine oil adjuvant therapy[56]. The results of the meta-analysis showed that intra-arterial instillation of 131I-iodine oil after hepatectomy significantly reduced the risk of HCC recurrence and improved DFS and OS[57,58], but it still needs to be confirmed by multicenter large sample RCTs. A recent multicenter RCT included 156 patients with HCC with positive HAb18G/CD147 antigen expression in HCC tissues who underwent radical resection and showed that the hepatic artery infusion of iodine-131-labeled HAb18G/CD147 monoclonal antibody (methotrexate monoclonal antibody) significantly improved 5-year RFS in patients with cluster of differentiation 147-expressing tumors after hepatectomy and is well tolerated by patients; subgroup analysis showed that the main effective targets were high-risk recurrent patients with MVI-positive, tumor diameter > 5 cm, poorly differentiated tumors, and incomplete tumor envelope[59]. In addition, the intraoperative implantation of iodine-125 particles in the hepatectomy wound has been performed in some units in China, and the RCT showed that 125I brachytherapy significantly prolonged TTR and OS in patients with HCC who underwent radical resection[60].

ADJUNCTIVE SYSTEM THERAPY

Postoperative adjuvant targeted therapy

Sorafenib monotherapy is used as a standard treatment option for advanced HCC, but its effectiveness in postoperative adjuvant therapy has been unsatisfactory. The STORM trial, a randomized, double-blind, placebo-controlled phase III clinical study of sorafenib as adjuvant therapy for patients with HCC, enrolled 1114 patients treated with surgical resection or local ablation for limited HCC. Patients were randomly assigned to sorafenib treatment or placebo groups[61], which showed no statistical difference in RFS between the two groups (33.3 *vs* 33.7 mo; $P = 0.26$). Conversely, sorafenib treatment increases adverse effects. The failure of the STORM study may be due to a deficiency in effectively selecting patients at high risk of recurrence. A meta-analysis of data from five studies with 296 participants[62] reported results consistent with the STORM trial. However, several retrospective studies have shown the efficacy of the adjuvant therapy with sorafenib after hepatectomy to prevent recurrence and metastasis in patients with HCC with high-risk recurrence factors. In a phase II clinical trial of 31 patients with HCC with high-risk recurrence factors after radical resection, 14 patients who received sorafenib adjuvant had a longer time to recurrence (21.45 mo ± 1.98 mo in the sorafenib group *vs* 13.44 mo ± 2.66 mo in the control group; $P = 0.006$), and the recurrence rate was significantly lower in the sorafenib-treated than in the control group (29.4% *vs* 70.7%; $P = 0.032$)[63]. Li *et al*[64] showed that patients treated with sorafenib within 30 d after surgery had 7 mo longer tumor-free survival than those treated with surgery only, with safe and manageable side effects. A retrospective analysis found that treatment with adjuvant sorafenib is beneficial for patients with postoperative high-risk recurrence HCC. Wang *et al*[65] retrospectively collected data from 209 patients with intermediate to advanced HCC at high risk of recurrence after hepatectomy at 15 study centers in China and showed that the 1-year survival rate was significantly higher in the sorafenib group than in the control group. Another retrospective study including 728 patients with HCC after R0 resection but with MVI-positive surgical specimens showed that for patients with HCC with combined MVI, patients in the adjuvant sorafenib group had significantly better OS and RFS than those of the surgery alone group[66]. Several novel targeted therapeutics have been successful in phase III studies in advanced HCC, including first-line treatment with lenvatinib, second-line treatment with regorafenib, ramucirumab (for AFP > 400 ng/mL HCC), and cabozantinib. There has been some progress in the adjuvant treatment with novel targeted drugs. A single-center, open-label, single-arm, phase II study of apatinib for postoperative adjuvant treatment of HCC combined with PVTT showed that patients with HCC after radical hepatectomy have 1-year RFS 36.1%, 1-year OS 93.3%, median RFS, 7.6 mo; therefore, the results obtained were better than previous historical ones in terms of the median RFS[64]. Moreover, apatinib is tolerated by most of the patients, which is significant for patients with HCC in combination with PVTT. The American Society for Clinical Oncology reported in 2020 the interim results from a multicenter, prospective cohort study of 90 patients with HCC at high risk of recurrence after surgery, treated with lenvatinib combined with

TACE for the adjuvant treatment, and showed that the median DFS was significantly longer in the lenvatinib combined with TACE group than that in the TACE alone group (12.0 mo *vs* 8.0 mo, HR 0.5; P = 0.0359)[67]. These results showed the effectiveness of new targeted drugs, such as apatinib and lenvatinib, in reducing the risk of recurrence after HCC surgery, and that a combination therapy may be a more optimal treatment modality.

Liver transplantation is an effective curative tool for HCC. For patients beyond Milan criteria, the risk of recurrence after transplantation is significantly increased, and the need to receive adjuvant therapy with targeted drugs has not been supported by high-level medical evidence. Teng *et al*[68] reported a case-control study dividing 17 patients with beyond Milan criteria for HCC after liver transplantation into three groups: the adjuvant group (n = 5) was given adjuvant sorafenib starting within 6 wk postoperatively, the palliative group (n = 6) was given sorafenib after the development of recurrent metastases postoperatively, and the control group (n = 6) was not given sorafenib. The results showed that RFS at 6, 12, and 18 mo was better in the adjuvant group than in the palliative care and control groups (P = 0.034, 0.026, and 0.011, respectively), and OS at 24 mo of follow-up show the same trend (P = 0.031). Shetty *et al*[69] found a reduction in the overall recurrence rate of HCC in the adjuvant sorafenib treatment group (7 patients) compared to 12 historical control patients (29% *vs* 75%; P = 0.07). Huang *et al*[70] divided 30 patients with HCC after beyond Milan criteria liver transplantation into two groups of 15 patients each. The test group was given sorafenib orally and the control group was given capecitabine orally, and the drug was discontinued in both groups who did not show recurrence 18 mo after surgery. The results showed that the 1-year recurrence rate was significantly lower in the test group compared to the control group (53.3% *vs* 86.6%; P < 0.05) and the OS was significantly longer (28.3 ± 2.5 mo *vs* 17.9 ± 3.5 mo; P < 0.05). Han *et al*[71] retrospectively analyzed 23 patients at high risk of recurrence who underwent liver transplantation, including 14 in the adjuvant lenvatinib group and 9 in the control group, and showed that the median DFS in the adjuvant lenvatinib group was 291 [95% confidence interval (CI): 204-516] d, which was significantly longer than that in the control group of 182 (95%CI: 56-537) d (P = 0.04); the drug safety and patient tolerability were acceptable.

The aforementioned studies were all single-center, small-sample clinical explorations, and although the credibility of the results was limited, the survival benefit of the adjuvant therapy with targeted agents was observed in patients who received liver transplantation either by radical surgery or by beyond Milan criteria. Further confirmation is urgently needed in prospective, multicenter, randomized controlled phase III studies.

Postoperative adjuvant immunotherapy

The liver is a natural immune-tolerant organ, shielded from autoimmune damage and thus creating a microenvironment of autoimmune tolerance[72], but also favoring immune escape of HCC cells[73]. The current immunotherapy for HCC mainly includes tumor pericyte therapy as well as immune checkpoint inhibitor therapy.

Tumor relay cellular immunotherapy: Cytokine-induced killer cells have shown promising applications in the overt immunotherapy of HCC. An RCT[74] on the application of secondary immunotherapy after surgery for HCC showed that secondary immunotherapy reduced the risk of recurrence by 41% compared with that of the control group, and RFS and disease-specific survival were significantly better in the immunotherapy group than in the control group, but the difference in OS between the two groups was not statistically significant. A large phase III RCT[75] randomized 230 patients with HCC treated with surgical resection and ablation into an autologous cytokine-induced killer (CIK) cells infusion group and an observation group. The results showed that adjuvant immunotherapy not only extended the median RFS time from 30 to 44 mo but also reduced the overall risk of death and had mild toxic effects. A median follow-up of 68.5 mo showed a significant 33% reduction in the risk of recurrence or death in the immunization group (P = 0.009)[76]. A single-center, phase III, open-label RCT that included 200 patients with BCLC stage A or B HCC treated with radical hepatectomy showed that adjuvant cytokine-induced killer (CIK) therapy is safe and effective in prolonging the median TTR in patients with radical resected HCC, but does not improve patient DFS and OS[77]. A meta-analysis that included eight RCTs and two cohort studies containing 2120 patients showed that patients with HCC treated with adjuvant overt immunotherapy had significantly lower recurrence rates at 1, 3, and 5 years than those of the surgical treatment alone group[78]. However, another meta-analysis containing eight RCTs showed that CIK reduced the 1- and 3-year postoperative recurrence rates and increased OS from 1 to 5 years in patients with HCC but had no effect on the 5-year recurrence rate and 6-year OS[79]. Although several RCTs have demonstrated the efficacy of CIK cell immunotherapy in the adjuvant treatment of early-stage HCC, the results are not yet conclusive, and the value and the prospect of CIK therapy in the adjuvant treatment of HCC after radical treatment remains to be proven.

Immune checkpoint inhibitors: There is an increasing understanding of the immune microenvironment of liver tumors, and researchers have identified programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) upregulated tumor-infiltrating lymphocytes in HCC and HCC-associated Kupffer cells[80] as well as the emergence of PD-1 and PD-L1 inhibitors and their promising

results in the treatment of advanced liver cancer. These findings showed that there is an interest in adjuvant immunotherapy after resection of HCC. Several immune checkpoint inhibitors have been approved by the United States Food and Drug Administration for the systemic treatment of advanced HCC, and adjuvant therapy is often derived from the effective treatment of the advanced disease. As more immunotherapies are shown to be safe and effective for advanced disease, we speculate that these therapies could be successful in adjuvant therapy for the appropriate patients. Additional clinical studies have preliminarily validated the efficacy and safety of immune checkpoint inhibitors used in the perioperative period. Kudo *et al*[81] explored the efficacy and safety of the adjuvant nivolumab in the treatment of patients with HCC after radical resection or radiofrequency ablation in a multicenter, single-arm, phase II clinical study. A total of 55 patients with HCC at moderate-to-high risk of recurrence were included in the study. The results showed a 1-year RFS rate of 76.7%, a median RFS of 26 mo, and a safe and manageable grade 3-4 adverse event rate of 18.9%. Several clinical studies of the immune checkpoint inhibitor-related adjuvant therapy for postoperative HCC, such as CheckMate 9DX, KEYNOTE 937, and IMBrave050 (Table 1), are currently under evaluation, and their results are worthy of anticipation.

In addition, local combination systemic therapy is currently the trend in adjuvant therapy, such as an ongoing clinical, open-label, multicenter, single-arm observational study designed to explore the efficacy and safety of sequential tislelizumab adjuvant therapy with TACE in patients with high-risk recurrent HCC after surgery (NCT04981665).

Postoperative adjuvant chemotherapy

The basic principle of adjuvant chemotherapy is to remove tumor cells or microscopic tumor lesions circulating in the body. An RCT that included 160 patients with HCC treated with oral uracil-tegafur showed no difference in RFS and OS between the adjuvant chemotherapy and observation groups after hepatectomy. Conversely, the proportion of patients with late recurrence is significantly higher in the adjuvant chemotherapy group than in the control group[82]. In a randomized controlled trial of 60 patients after hepatectomy for HCC conducted in China, patients who received oral capecitabine postoperative adjuvant therapy have a reduced risk of tumor recurrence, but no significant improvement in 5-year survival after surgery[83]. A recently published prospective RCT[84] showed that postoperative oral cotrimoxazole adjuvant chemotherapy does not prolong recurrence-free and OS in patients with HCC compared with those with surgery alone. The role of systemic chemotherapy in patients after liver transplantation is currently inconclusive. Zhang *et al*[85] randomized 58 patients with HCC who underwent liver transplantation beyond Milan criteria into adjuvant chemotherapy and observation groups (29 patients in each group), and the chemotherapy group was given six cycles of chemotherapy with the FOLFOX regimen after transplantation. The results showed a significant increase in 1-year survival with adjuvant FOLFOX regimen chemotherapy compared with the control group (P = 0.043), a 24.1% increase in 6-mo tumor-free survival in the treatment group, and a significant decrease in the 6-mo recurrence rate (P = 0.036), but no significant difference in the 3-year recurrence rate (P = 0.102). Subsequently, Wang *et al*[86] divided 58 patients with HCC after beyond Milan criteria liver transplantation into two groups, in which 26 patients in the treatment group were given six cycles of OXA+5-Fu+CF adjuvant chemotherapy after surgery and 32 patients in the observation group were treated with graft surgery alone. The results showed that the 1-, 2-, and 3-year survival rates were 89.7%, 86.2%, and 78.8% in the adjuvant chemotherapy group, respectively, which was significantly higher than those in the observation group (64.5%, 61.1%, and 53.6% in the 1-, 2-, and 3-year survival rates, respectively). Another retrospective study that included 117 patients with beyond Milan criteria *in situ* liver transplantation for HCC showed 1-year survival rates of 87.5%, 84.2%, 81.6%, and 67.5% in the adjuvant gemcitabine group, conventional chemotherapy (adriamycin + 5-fluorouracil + cisplatin), oxaliplatin plus capecitabine, and best supportive care (BSC) groups, respectively, and 3-year survival rates of 48.1%, 25.9%, 31.6%, and 33.7%, respectively. Stratified analysis showed that the gemcitabine regimen and conventional chemotherapy significantly improved survival and DFS in patients with HCC who developed macrovascular invasion and/or microvascular invasion after liver transplantation compared to those of the BSC group[87]. Although earlier studies suggested that adjuvant systemic chemotherapy might be associated with reduced recurrence and prolonged RFS[88], the results failed to be validated. The reasons may be related to the relative lack of efficacy of cytotoxic chemotherapeutic for HCC drugs and the poor tolerance of chemotherapeutic drugs because of the combined hepatitis, liver fibrosis, and cirrhosis in patients with HCC. The failure of the adjuvant chemotherapy for HCC to achieve the same effect as for other solid tumors may be largely determined by the biological characteristics of the HCC and the underlying liver disease of the patients.

OTHER ADJUVANT TREATMENTS

Postoperative adjuvant antiviral therapy

Viral hepatitis is the main cause of HCC in China. Nearly 90% of the patients with HCC are associated

Table 1 Clinical studies of postoperative adjuvant therapy under investigation for hepatocellular carcinoma

NCT	Phase	Treatment option	Patient population	Expected group entry	Primary endpoint	Status
NCT03383458 (CheckMate 9DX)	III	Nivolumab	High-risk recurrent HCC after radical resection/ablation	530	RFS	Follow-up
NCT04233840	I/II	Nivolumab ± P1101	Post-radical resection of HBV-related HCC	72	Phase I: DLT, phase II: RFS	Recruiting
NCT03867084 (KEYNOTE-937)	III	Pembrolizumab	Imaging CR after surgical resection/local ablation	950	RFS, OS	Recruiting
NCT04639180	III	Camrelizumab + apatinib	High-risk recurrent HCC after surgical resection or ablation	674	RFS	Recruiting
NCT03839550	II	Camrelizumab + apatinib	High-risk recurrent HCC after radical surgery	200	RFS	Not yet recruited
NCT04102098 (IMbrave050)	III	Atezolizumab + bevacizumab	High-risk recurrent HCC after surgical resection/ablation	662	RFS	Recruiting
NCT04649489	-	Atezolizumab + bevacizumab	Post hepatectomy with portal vein carcinoma thrombosis HCC	198	TTF	Not yet recruited
NCT03847428 (EMERALD-2)	III	Durvalumab + bevacizumab	High-risk recurrent HCC after radical resection/ablation	888	RFS	Recruiting

CR: Complete response; DLT: Dose-limiting toxicity; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; OS: Overall survival; RFS: Recurrence-free survival; TTF: Time to treatment failure.

with chronic hepatitis B, and very few are associated with hepatitis C caused by the HCV. In patients with HBV-associated HCC, higher hepatitis B surface antigen levels[89] and viral load (serum HBV DNA >10^6 copies/mL) before and after surgery[90,91] are associated with an increased risk of recurrence after resection. In patients with HBV infection, antiviral therapy with nucleoside analogues significantly inhibits progression to cirrhosis and reduces the risk of HCC[92]. Two randomized trials[93,94] supported significantly higher OS and RFS in patients with HCC treated with postoperative adjuvant antiviral therapy. One of these studies[94] showed that the antiviral therapy is an independent prognostic factor for distant recurrence after HCC surgery (HR 0.348) but not for recurrence within 2 years after resection (HR 0.949). A meta-analysis that included 13 cohort studies on HBV-associated HCC and the two randomized controlled trials mentioned above (8060 patients in total) came to the same conclusion, with a significantly lower recurrence rate in patients receiving antiviral therapy [1-year recurrence rate relative risk (RR) 0.50, 3-year recurrence rate RR 0.70][95] and a significantly higher OS rate in the antiviral therapy group (5-year survival rate RR 1.40). HBV infection is a major risk factor for the development of HCC, which may occur even after HBsAg serum clearance. The guidelines recommend prompt and effective antiviral therapy for HBV-associated HCC if HBV replication is found to be active (HBV-DNA ≥ 1000 copies/mL or 2000 IU/mL). Even in those cases with low HBV-DNA quantification, if HBsAg (+) and/or HBcAb (+), the combination of antiviral drugs is recommended before and throughout antitumor therapy to avoid HBV reactivation[96,97]. The results suggest that IFN-based HCV antiviral therapy reduces recurrence rates and improves survival, but this regimen is no longer recommended for current HCV antiviral therapy. A retrospective multicenter cohort study enrolled a total of 797 patients with HCV-associated HCC who achieved complete remission with initial therapy over 4 years[98], of whom 383 patients were treated with direct antiviral agents (DAAs), and showed significantly lower mortality in the DAA-treated group compared with that of patients not treated with DAAs. This study provides evidence of the potential benefit of the DAA adjuvant therapy for HCV-associated HCC. Similar results were obtained in another small prospective analysis that included 163 consecutive patients with HCV-related cirrhosis and a diagnosis of early HCC treated with DAA after achieving complete remission on imaging by radical resection or ablation, compared with a historical cohort of 328 patients treated for early HCC but not with DAA[99], showing that the DAA treatment did not reduce HCC recurrence rates but significantly improved OS. Studies have shown that the use of DAA, either before or after hepatectomy, can improve the prognosis of the disease[100], but the optimal timing for anti-HCV therapy in relation to HCC treatment has yet to be determined. For HCV-associated HCC, the antiviral therapy has a protective effect on the liver function, and current Chinese Society of Clinical Oncology guidelines state that the antiviral therapy for HCV has entered the pan-genotypic era of direct antivirals, with a preference for interferon (IFN)-free pan-genotypic regimens.

Adjuvant traditional herbal medicine treatment

Traditional herbal medicine exhibits antitumor activity by inhibiting tumor cell growth, inducing apoptosis, inhibiting angiogenesis, and enhancing immune function[101,102]. Traditional herbal medicine (THM) has its own unique advantages in controlling the progression of patients with liver cancers, reducing recurrence, reducing symptoms and signs, improving survival quality, and prolonging survival. A cohort study based on Taiwanese population showed that the treatment with THM in patients with chronic hepatitis B significantly reduces the risk of HCC[103]. A retrospective study with a large sample size showed that a comprehensive THM treatment improved OS in patients with HCC[104]. THM may prevent disease recurrence and prolong survival by modulating immunity and altering the local microenvironment. To investigate the clinical efficacy of THM in preventing recurrence of small HCC after surgery, an open-label, prospective, multicenter RCT enrolling 364 patients was conducted in five centers in China. A total of 180 patients in the THM group were treated with intravenous cinobufagin and oral detoxification granules, and 184 patients in the TACE group were treated with a single course of TACE, and at a mean follow-up of 26.61 mo, THM was found to be superior to TACE in preventing recurrence of small HCC and prolonged OS[105]. Another randomized, controlled, national multicenter phase IV clinical study that included 1,044 patients with HCC showed that in patients with HCC in BCLC staging A and B, the administration of the modern herbal medicinal preparation Huaier granules after radical resection results in a significantly longer RFS and a significantly lower rate of extrahepatic recurrence[106]. Lei *et al*[107] retrospectively analyzed 53 patients with HCC who underwent liver transplantation and divided them into the Huaier-granule treatment and control groups, in which 28 patients received Huaier granules after surgery and 25 patients did not. The long-term predicted OS is similar between the two groups (P = 0.202). However, the tumor-free survival rate is higher in the Huaier-granule treatment group than that in the control group (P = 0.029). The predicted recurrence rates at 10 and 30 mo in the Huaier-granule treatment group were 17.9% and 35.7%, respectively, which were significantly lower than those in the control group (60% and 64%; P < 0.05). THM has shown some efficacy in the postoperative adjuvant treatment of HCC, but most of the regimens lack strong medical evidence, and their efficacy still needs to be confirmed through more prospective studies.

Adjuvant IFN

IFN is considered a promising adjuvant therapy after hepatic resection for hepatitis virus-induced HCC due to its antiviral, antiproliferative, antiangiogenic and immunomodulatory effects. Several randomized controlled trials, the majority of which were undertaken in Asian patients with HCC, have looked into the efficacy of postoperative IFNα[108-115] and IFNβ[116]. Ikeda *et al*[116] suggested adjuvant IFNβ administration lowered postoperative recurrence rate in patients with HCC after their hepatic resection or ablation. However, RCTs on curative effects of IFNα showed conflicting results. Mazzaferro *et al*[109] reported that IFNα2b induced a decrease on late recurrence rate in HCV-infected patients but showed no influence on overall prevention of tumor recurrence after surgery. Chen *et al*[113] indicated it made no contribution to postoperative recurrence reduction, while Lo *et al*[114] found that patients with pathological tumor-node-metastasis stage III and IVA tumors showed dramatically lower risk of recurrence compared to the untreated group. Numerous systematic reviews and meta-analyses including these RCTs and plentiful comparative studies revealed that additional IFN suppressed tumor recurrence and increased overall survival within certain time periods[117-128]. Notwithstanding, IFNα significantly reduced recurrence rate in patients with HCC caused by HCV but not by HBV, according to subgroup analysis[117,125,127].

Adjuvant vitamin K2 analogs and retinoids

As a crucial hydrophobic vitamin, vitamin K2 (VK2) shows substantial anti-angiogenic effects, induce cell cycle arrest, and inhibits the proliferation of HCC cells[129-131]. The effects of VK2 were explored in six RCTs[132-137] and a cohort trial[138] conducted in Japan, focusing on recurrence prevention and prolonging survival periods in patients with HCC following local ablative therapy or resection. The studies from Mizuta *et al*[132], Kakizaki *et al*[134] and Yoshiji *et al*[138] pointed out that VK2 or the combination utilization of VK2 and angiotensin-converting enzyme inhibitor was efficacious in reducing HCC recurrence. Other studies, on the other hand, reported no change in DFS between treated and untreated participants[133,135-137]. VK2 analogues showed no noticeable impact on OS after hepatic resection and ethanol ablation in all mentioned investigations, while it significantly reduced tumor recurrence rates at the second and third years, and improved 1-, 2-, and 3-year OS according to the findings of Zhong *et al*[139]. Current research results may be inconsistent regarding the curative effects of VK2 and its analogs for postoperative patients with HCC, so more investigations with larger sample size and longer observation period are in great need.

Adjuvant PI-88

In exploratory clinical studies of HCC therapy, phosphomannopentaose sulfate (PI-88), an efficient inhibitor of heparanase, exerted anti-recurrence and anti-metastasis effectiveness[140,141]. It was reported to inhibit the relapse in patients who have undergone hepatectomy through disrupting the

rapid growth of heparanase level after liver resection[142]. Liu *et al*[143] assessed the efficacy, safety and optimal dosage of PI-88 with a phase II/stage 1 RCT, concluding that 160 mg/d is acceptable and shows the potential to prolong time to recurrence. Additionally, in the observational follow-up study conducted by the same research group, they reported that PI-88 at 160 mg/d increased the recurrence-free rate and postponed the time to recurrence, despite both RFS and OS were not significantly improved[144].

CONCLUSION

This review summarizes several adjuvant therapies that may have anti-HCC recurrence efficacy, including TACE, targeted therapy, immunotherapy, and THM therapy. Although many adjuvant therapies other than the antiviral drug therapy have been reported to improve survival and/or reduce the risk of postoperative recurrence in patients after HCC surgery or liver transplantation, there is a lack of strong evidence-based support for other treatments, and there is no globally accepted adjuvant treatment option for postoperative HCC at this stage. Asian guidelines are usually more favorable than Western ones for postoperative adjuvant therapy for HCC. Differences in recommendations for adjuvant therapy between Asian and Western guidelines are not surprising, as differences in ethnicity, environment, and causative factors may influence the pathogenesis and survival of patients with liver cancer. In addition, larger tumors are usually removed through surgery in Asian countries, while surgical treatment is usually not considered in Western countries.

Due to the heterogeneity of tumors, the underlying liver disease, recurrence patterns in patients with HCC, and the presence of multiple risk factors in most patients with the disease, there is often a wide variation in the efficacy and tolerance of patients to the same treatment regimen. Therefore, it is important to identify the most effective postoperative adjuvant therapy for a specific subgroup of patients. The most frequently mutated genes in HCC patients are tumor protein p53, telomerase reverse transcriptase, and catenin beta 1, which mainly lead to the occurrence and development of HCC[145-147]. Many of these abnormalities may be pharmacologically tractable. However, biomarker-matched trials are still limited in this disease, and many of the genomic alterations in HCC remain challenging to target. Future research on adjuvant therapy after HCC surgery may focus on three points: first, the signaling pathways of HCC recurrent metastasis may be different from those of the primary tumor. More in-depth basic research is needed to elucidate the mechanisms of HCC at the level of signaling pathways or driver genes to find ways to contain tumor recurrence and metastasis. Second, patients with early and distant recurrences need to be identified and stratified for the risk of recurrence, and different treatment strategies need to be adopted for patients with liver cancer with different predicted timing of recurrence. Finally, appropriate postoperative adjuvant treatment modalities were explored based on specific preoperative subgroups of patients with HCC. Several studies have explored statistical models for predicting the risk of recurrence after HCC surgery[148,149], aiming to guide clinicians to estimate the risk of recurrence in individual patients. These findings will also help to design clinical trials of drugs aimed at reducing recurrence in subgroups with different recurrence risks. Combination therapies, such as targeted combined with immunotherapy and targeted combined with TACE therapies, have also been conducted in the field of advanced HCC in successive clinical studies and have initially shown good efficacy. Optimized postoperative adjuvant therapy should focus on improving the immune system and liver functions while removing residual tumor cells. For patients with a high risk of recurrence, optimizing a more individualized combination therapy model may be a breakthrough in the bottleneck of postoperative adjuvant therapy for HCC.

In conclusion, there is still a lack of perspective, phase III, multicenter, randomized controlled clinical studies with large samples to confirm the efficacy of particular adjuvant treatment after HCC surgery. Therefore, comprehensive treatments with multidisciplinary cooperation, more randomized controlled trials, and new therapies need to be promoted to explore treatment modalities to reduce the postoperative recurrence of HCC and improve patient survival.

FOOTNOTES

Author contributions: Zeng ZM and Peng T designed this study and wrote the manuscript; Zeng ZM, Mo N, Zeng J, Ma FC, Jiang YF, Huang HS, Liao XW, Zhu GZ, Ma J, and Peng T provided the research idea, conducted the literature search, selected the papers, and wrote and edited the final manuscript; Zeng ZM conducted the literature search and wrote the manuscript; Peng T supervised the writing; All authors read and approved the final manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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S-Editor: Gong ZM
L-Editor: Filipodia
P-Editor: Gong ZM

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1622-1636

DOI: 10.4251/wjgo.v14.i9.1622

ISSN 1948-5204 (online)

REVIEW

Immunotherapy for nonalcoholic fatty liver disease-related hepatocellular carcinoma: Lights and shadows

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Hatanaka T, Japan; Oura S, Japan

Received: March 18, 2022
Peer-review started: March 18, 2022
First decision: April 17, 2022
Revised: May 5, 2022
Accepted: July 5, 2022
Article in press: July 5, 2022
Published online: September 15, 2022

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Abstract

About one-fourth of adults globally suffer from nonalcoholic fatty liver disease (NAFLD), which is becoming a leading cause of chronic liver disease worldwide. Its prevalence has rapidly increased in recent years, and is projected to increase even more. NAFLD is a leading cause of hepatocellular carcinoma (HCC), the sixth-most prevalent cancer worldwide and the fourth most common cause of cancer-related death. Although the molecular basis of HCC onset in NAFLD is not completely known, inflammation is a key player. The tumor microenvironment (TME) is heterogeneous in patients with HCC, and is characterized by complex interactions between immune system cells, tumor cells and other stromal and resident liver cells. The etiology of liver disease plays a role in controlling the TME and modulating the immune response. Markers of immune suppression in the TME are associated with a poor prognosis in several solid tumors. Immunotherapy with immune checkpoint inhibitors (ICIs) has become the main option for treating cancers, including HCC. However, meta-analyses have shown that patients with NAFLD-related HCC are less likely to benefit from therapy based on ICIs alone. Conversely, the addition of an angiogenesis inhibitor showed better results regarding the objective response rate and progression-free survival. Adjunctive diagnostic and therapeutic strategies, such as the application of novel biomarkers and the modulation of gut microbiota, should be considered in the future to guide personalized medicine and improve the response to ICIs in patients with NAFLD-related HCC.

Key Words: Hepatocellular carcinoma; Immunotherapy; Liver cancer; Nonalcoholic fatty

liver disease; Metabolic dysfunction-associated fatty liver disease; Obesity

Core tip: Complex interactions involving the immune system, angiogenesis and inflammation are associated with the pathogenesis of hepatocellular carcinoma (HCC). Recent reviews suggested lower efficacy of immunotherapy in patients with nonviral HCC. This calls into question the need to stratify patients to maximize the effectiveness of immunotherapeutic agents. In this study, we provided the latest report on the tumor microenvironment structure and its implications in response to immunotherapy in nonalcoholic fatty liver disease-related HCC and also discussed the efficacy of first-line systemic treatment in this patient population.

Citation: Costante F, Airola C, Santopaolo F, Gasbarrini A, Pompili M, Ponziani FR. Immunotherapy for nonalcoholic fatty liver disease-related hepatocellular carcinoma: Lights and shadows. *World J Gastrointest Oncol* 2022; 14(9): 1622-1636
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1622.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1622

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of hepatic abnormalities ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH)[1]. About 24% of adults globally suffer from NAFLD[2,3]. This percentage has considerably increased in recent years, almost doubling between 2005 and 2010[3]. NAFLD starts developing with the accumulation of lipids in hepatocytes, while progression to steatohepatitis occurs in 20%–30% of the cases[4,5]. Cirrhosis occurs in 10%–20% of the cases due to the deposition of fibrous tissue (fibrosis) and alterations in the regeneration of hepatocytes[4,5]. Although it is difficult to precisely determine the prevalence of NAFLD-related cirrhosis, NAFLD is one of the leading causes of cirrhosis worldwide[6,7], and it is currently the second most common indication for liver transplantation[2]. As NAFLD is associated with metabolic disorders in almost all patients, a change in the terminology from NAFLD to metabolic dysfunction-associated fatty liver disease (MAFLD) was proposed[8]. This new classification might lead to a further increase in the prevalence of this disease, but there is still a lack of complete agreement among experts about redefining NAFLD as MAFLD.

Besides increasing in prevalence, NAFLD is becoming one of the leading causes of hepatocellular carcinoma (HCC), being responsible for 1%–38% of HCCs globally[9-12]. The high variation in estimating the prevalence of NAFLD-related HCC is due to the heterogeneous definition of NAFLD used in different studies (histological *vs* radiological *vs* clinical)[9].

Chronic viral hepatitis, caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, accounts for 80% of HCC cases globally[13]. However, some studies suggest that NAFLD is the main cause of HCC in some areas of Europe, while in the USA, the number of NAFLD-related HCC cases is steadily increasing[10]. Considering the growing prevalence of NAFLD and the progressive reduction in viral-hepatitis-related HCC (due to vaccination and effective antiviral treatments)[14-16], NAFLD/MAFLD might become the main cause of HCC in the next 10 years[10,14]. Although most cases of NAFLD-related HCC occur in a cirrhotic liver, retrospective studies have shown that NAFLD-driven HCC can occur even in the absence of cirrhosis in 20%–50% of the cases, especially when NASH is present[10,13,17,18].

Many factors contribute to making NAFLD a leading cause of HCC, even in the absence of cirrhosis. Specifically, type 2 diabetes mellitus and obesity increase the risk of HCC in NAFLD patients with or without cirrhosis[19-23]. Moreover, NAFLD-related HCC has higher mortality than HCC associated with viral hepatitis[11]. This is probably because HCC in patients with NAFLD is generally diagnosed in more advanced stages and mostly outside surveillance programs[17].

Recently, immunotherapy with immune checkpoint inhibitors (ICIs) was found to be a good therapeutic option for advanced HCC, either as an alternative to tyrosine kinase inhibitors (TKIs) or along with them[24]. Patients need to be categorized according to their likelihood of response, considering that ICIs were found to be less effective in certain patient subpopulations, particularly those with NAFLD. In this review, we describe the mechanism behind the progression of NAFLD to HCC and discuss the efficacy of ICIs in patients with NAFLD to determine the factors that might elucidate the best therapeutic choice for this patient population.

HCCIMMUNOBIOLOGY AND LIVER MICROENVIRONMENT

Many factors contribute to the progression of NAFLD to HCC, including individual (*i.e.*, genetics, epigenetics and gut microbiota) and environmental (*i.e.*, diet) factors[25]. Although the molecular basis of this process is not completely known[26], most authors agree that chronic inflammation and immune system disorders play a crucial role[25,27-30]. These interactions occur in the tumor microenvironment (TME) (Figure 1).

The immune system: ally or enemy in the TME?

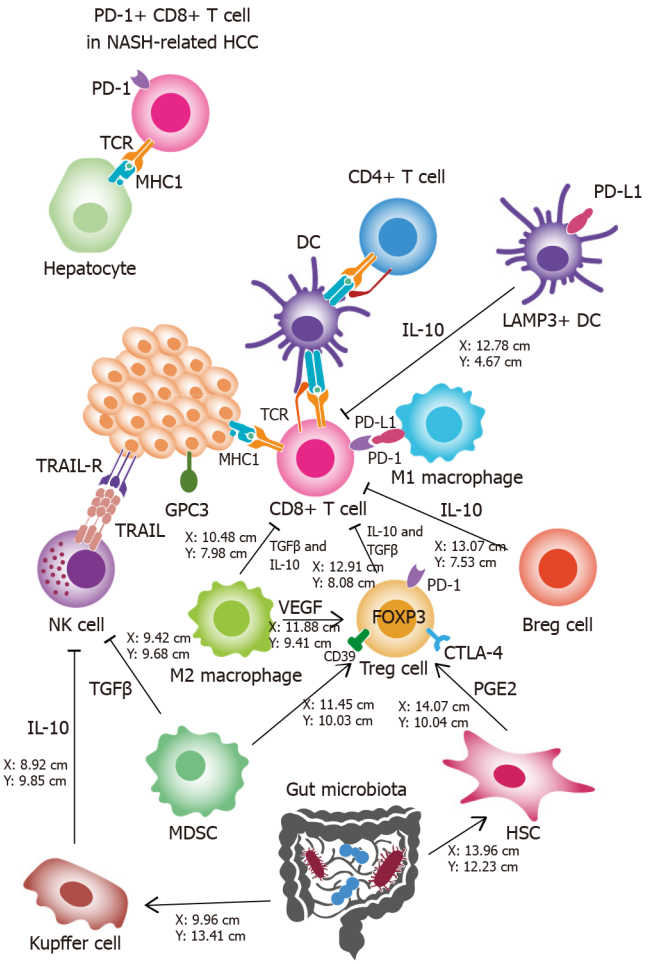
Based on the complex interactions with other cell types and cytokines, each cell in the TME has a different function. To provide an overview of the TME, a classification of HCCs based on immunological features was proposed for determining prognostic phenotypes and predicting therapeutic responses[29-31]. Two main types of HCC can be identified: the inflamed class (high immune infiltration, increased PD-1/PD-L1 signaling, and markers of $CD8^+$ T cell cytotoxic activity) and the noninflamed class (low abundance of tumor-infiltrating lymphocytes, low expression of immune checkpoints, and markers of $CD8^+$ T cell cytotoxic activity)[32]. The inflamed class can be divided into two subclasses[29,31]: (1) The active immune class, characterized by a high abundance of $CD8^+$ T cells, M1-phenotype macrophages, and overexpression of T cell effector genes, such as *CXCL9*, *CXCL10* and *IFNγ*, and granzymes, shows a favorable prognosis compared to the other classes; and (2) The exhausted immune class, characterized by an increase in M2 tumor-associated macrophages and T-cell exhaustion markers[29]. Transforming growth factor (TGF)-β signaling plays a key role in inducing T-cell exhaustion[33,34] and is associated with an increase in the expression of programmed cell death protein (PD)-1, TIM-3, LAG3 and TIGIT[35]. Another molecule involved in $CD8^+$ T-cell exhaustion is the thymocyte selection-associated high mobility group box (TOX) transcription factor, whose expression is induced by the vascular endothelial growth factor (VEGF) signaling pathway[35]. Noninflamed subclasses include: (1) The intermediate class, in which immune infiltration is lower than that in the inflamed class; and (2) The immune excluded class, characterized by immunosuppressive gene upregulation in tissues surrounding the tumor, which leads to immunological desertification[32,36]. The immune excluded class has the worst prognosis and is unlikely to respond to immunotherapy[36,37].

Another classification considers immune cell tumor infiltration, with three subtypes of HCC phenotype: (1) The immune-high subtype, which has a high rate of T-cell, B-cell and plasma cell infiltration; (2) The immune-mid subtype, which has a moderate rate of immune cell infiltration; and (3) The immune-low subtype, which has a low rate of immune cell infiltration[30]. As in the active immune class, the high-immune subtype is associated with an increase in T helper 1 cells and $CD8^+$ cell cytokines [30,32]. The high-immune subtype has a better prognosis than the other subtypes[30], particularly in poorly differentiated HCCs[30]. Furthermore, alterations in the T-cell count occur mostly during the shift from moderately to poorly differentiated HCC, resulting in immunological subtype differentiation in this phase[30].

Different roles of T cells in the TME

T cells play an important role in the progression of liver diseases to HCC. In an inflammatory setting, such as NASH, regulatory T (Treg) cells decrease while T-helper 17 cells increase[38]. IL-17 released by T-helper 17 exacerbates liver inflammation and promotes hepatocarcinogenesis[39]. After the development of HCC, the number of $Foxp3^+GARP^+$ $CTLA\text{-}4^+$ Treg cells increases in the TME, inhibiting the cytotoxic action of $CD8^+$ T cells against tumor cells[40,41]. The infiltration of Treg cells in the TME is associated with the immune excluded class[29].

$CD8^+$ T cells are directly cytotoxic to tumor cells. Two distinct phenotypes of $CD8^+$ T cells in HCC were described: one with low cytotoxic activity, associated with an upregulation of the *KLRB* gene and a poor prognosis[42]; the other, with a high cytotoxic capacity, associated with the overexpression of the *XCL1* gene and a better prognosis[43]. The participation of T cells in the TME is strongly determined by the etiology of liver disease. In NASH-related HCC, there is an excess of $CD8^+$ T cells expressing PD-1 [44,45]. These cells develop major-histocompatibility-complex-I-independent cytotoxicity against hepatocytes and lose tumor surveillance functions. This might be due to the metabolic dysregulation of immune system cells, which occurs in NASH[44,45]. In HBV-related HCC, the number of Treg cells increases with the overexpression of PD-1[46]. Furthermore, T cells are susceptible to Bcl-2-like protein 11-mediated apoptosis, which contributes to the tolerogenic milieu of HBV infection[47]. The number of $CD8^+$ T resident memory cells, which probably have cytotoxic activity[46,48], is also enhanced in HBV-related HCC, and they are associated with a favorable prognosis[46]. However, these cells overexpress PD-1, suggesting an immune-exhausted microenvironment[46]. Chronic HCV infection also induces an exhausted phenotype in $CD8^+$ T cells, causing a decrease in the production of interferon (IFN)γ, reduction in the expression of CD127, and overexpression of PD-1 and TIM[49]. Although the number of $PD\text{-}1^+CD8^+$ T cells increases in both virus-related and NASH-related HCC, the response to anti-PD-1 ICIs is different in these two scenarios, suggesting that $PD\text{-}1^+CD8^+$ T cells play a distinct function[44]. This is dependent on the type of cells with which $PD\text{-}1^+CD8^+$ T cells interact. In the high-immune HCC subtype, PD ligand (PD-L)1 is mainly expressed by macrophages, which suggests that the PD-1/PD-L1



DOI: 10.4251/wjgo.v14.i9.1622

Figure 1 Hepatocellular carcinoma immunological microenvironment. Different elements that contribute to the antitumor activity or limit antitumor immunity are illustrated schematically. The main effectors against tumor cells are CD8$^+$ T cells and natural killer (NK) cells. Dendritic cells (DCs), CD4$^+$ cells, and M1 macrophages enhance CD8$^+$ T cell cytotoxicity. Regulatory T cells, regulatory B cells, LAMP3$^+$ DCs, and M2 macrophages inhibit CD8$^+$ T cells and induce an immunosuppressive environment. Tumor cells attract M2 macrophages by expressing glypican-3 (GPC3). Myeloid-derived suppressor cells and Kupffer cells produce immunosuppressive cytokines in the tumor microenvironment and inhibit NK cells. The gut microbiota might play an indirect role in immunosuppression through persistent inflammation or other mechanisms leading to immune cell exhaustion. PD-1$^+$CD8$^+$ T cells in NASH-related hepatocellular carcinoma show cytotoxic activity against hepatocytes, instead of exhibiting antitumor function. Breg: Regulatory B cells; DC: Dendritic cell; HSC: Hepatic stellate cell; MDSC: Myeloid-derived suppressor cell; MHC I: Major histocompatibility complex class I; NASH: Nonalcoholic steatohepatitis; NK: Natural killer; TCR: T cell receptor; TME: Tumor microenvironment; Treg: Regulatory T cells; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; TRAIL-R: Tumor necrosis factor-related apoptosis-inducing ligand receptor; VEGF: Vascular endothelial growth factor.

interaction plays a role in T cell–macrophage crosstalk rather than in T-cell inhibition by the tumor[30]. Furthermore, macrophages from high-immune subtypes overexpress CD169[30], which is an M1 phenotype marker associated with macrophage-dependent T-cell activation and favorable prognosis in several cancers[50]. Other T cells, such as - T cells, might also be involved in antitumor surveillance, considering that their depletion in tumor tissues is associated with a higher incidence of postoperative recurrence[51].

B cells play an ambivalent role in the TME

B cells play a dual role in HCC, depending on their interaction with other components of the TME. B-cell infiltration occurs in high-immune subtypes and is associated with a better prognosis[30]. On the contrary, B-cell infiltration, when associated with elevated interleukin (IL)-17 production, is a poor prognostic marker[52]. Moreover, regulatory B cells inhibit the activation and cytotoxic activity of CD8$^+$ T cells in NASH-related HCC by expressing PD-L1 and producing IL-10[53].

Role of innate immunity in HCC TME

Along with CD8$^+$ T cells, natural killer (NK) cells act as the main cytotoxic effectors in HCC, contributing to innate immune system tumor surveillance[54]. However, due to the abundance of Treg cells in the TME, the number of intratumoral NK cells decreases and impairs their cytotoxic activity, as well as the production of IFNγ[55,56]. NK T cells play a role in the development of HCC in patients with NASH by

triggering the transformation of hepatic stellate cells (HSCs) into fibroblasts[28] and promoting liver inflammation and injury *via* nuclear factor-B signaling and the production of cytokines[57]. NK T cells show antitumor activity and cooperate with $CD4^+$ T cells to remove senescent hepatocytes from the liver following a chemically induced liver injury[58]. This antitumor property is associated with the chemokine CXC ligand (CXCL)16/CXCR6 hepatic chemokine pathway[58]. The interaction between bile acids and the gut microbiotaregulates CXCL16 expression in the liver sinusoidal endothelial cells[59], suggesting that microbiota the plays a role in the development of HCC.

Other innate immune cells, such as M2-phenotype tumor-associated macrophages and myeloid-derived suppressor cells, might suppress $CD8^+$ T cells by releasing TGF-β and IL-10[39,60,61]. Tumor-associated macrophage abundance in the HCC tissue is associated with a poor prognosis[62,63]. Dendritic cells play an important role in PD-1/PD-L1 crosstalk. In the TME and lymph nodes, $PD\text{-}L1^+$ $LAMP3^+$ dendritic cells inhibit circulating $CD8^+$ T cells activated by tumor antigens[64]. This immunoregulatory function primarily occurs through the secretion of IL-10 and the recruitment of Treg cells[65,66].

Tumor cells modulate the immune response

Tumor cells can effectively control the HCC TME. The overexpression of the MYC proto-oncogene in tumor cells is associated with upregulation of PD-L1 on the cell surface[67]. Furthermore, alterations in the WNT–β-catenin signaling pathway decrease the secretion of chemokine CC ligand 5, which affects the recruitment of dendritic cells[68]. The immune-excluded HCC class shows a higher rate of *WNT/CTNNB1* gene mutation[36]. Tumor cells in the HCC TME directly recruit immunosuppressive neutrophils by upregulating the chemokine CXCL5[69].

The gut–liver axis influences the TME in HCC

The inflammatory condition associated with the progression of HCC is not limited to the liver. Intestinal inflammation acts as a cofactor in the pathogenesis of HCC[70,71]. This was confirmed by finding a higher fecal calprotectin concentration in patients with cirrhosis and HCC compared to that in healthy subjects or in patients affected by cirrhosis without HCC[70]. The gut microbiota might play an important role in modulating intestinal inflammation with noticeable effects on hepatocarcinogenesis [70-72]. The dominant phyla associated with HCC include *Bacteroidetes*, *Firmicutes* and *Proteobacteria*[73]. Changes in the gut microbiota occur commonly in patients with NAFLD-related cirrhosis, where the microbial diversity decreases compared to that in healthy people[74]. Other proinflammatory bacteria, such as *Bacteroides* and *Ruminococcaceae*, are overabundant in individuals with NAFLD-related cirrhosis and HCC[70]. In contrast, depletion of bacteria such as *Akkermansia* and *Bifidobacterium*, which have an anti-inflammatory effect, might also occur[70]. The integrity of the gut epithelial and vascular barrier plays an important role in preventing bacteria from entering the portal circulation[75]. Alterations in the gut–liver axis that occur in cirrhosis (such as changes in the gut microbiota composition and impaired bile acid production) might result in the disruption of the gut barrier, thus, increasing intestinal permeability[76]. Hence, intestinal bacteria, along with their antigens and products, such as lipopolysaccharides, can easily reach the liver *via* the portal system[77]. By binding Toll-like receptors, bacterial antigens activate Kupffer cells and HSCs[78]. This interaction enhances liver inflammation and plays a crucial role in hepatocarcinogenesis[71]. For example, Toll-like receptor 4 activation in Kupffer cells can increase IL-10 production, which suppresses the cytotoxic functions of NK cells[79]. Gram-positive bacteria might also play an important role in NAFLD-related HCC. Their metabolic products, such as deoxycholic acid and lipoteichoic acid, cause senescence of HSCs[80]. Senescent HSCs have a distinct phenotype characterized by higher production of cytokines, chemokines, and matrix-remodeling proteins[81]. In a preclinical mouse model, prostaglandin E2 generated by senescent HSCs was shown to interfere with the TME through the prostaglandin E receptor 4 signaling pathway[82]. Prostaglandin E receptor 4 is a G-protein-coupled receptor that is mostly expressed by immune cells[83]. Its activation may have an immunosuppressive effect by enhancing the infiltration of Tregs and PD1+ CD8+ T cells in the TME[82].

IMMUNOTHERAPY IN NAFLD-RELATED HCC: EVIDENCE AND CONCERNS

Systemic therapies represent the standard of care for unresectable HCC, either in an advanced or intermediate stage, that is unsuitable for further treatment[84]. Over the last few years, ICIs have shown positive therapeutic results, leading researchers to shift their focus from tyrosine kinase inhibitors (TKIs) to ICIs. Several agents have been approved, either alone or in combination, as first-line or second-line treatments for HCC, with some variations in the treatment regimen found in different countries[85] (Table 1).

Effectiveness of immunotherapy in NAFLD-related HCC

Subgroup analyses of survival outcomes based on trials evaluating the efficacy of ICIs as first-line treatment revealed a discrepancy between HCC associated with HBV or HCV infection (viral HCC)

Table 1 The main immune checkpoint inhibitors approved for treatment of advanced hepatocellular carcinoma

Drug	Mechanism of action	Efficacy	Safety	Approval
First-line				
Atezolizumab (1200 mg, IV) plus bevacizumab (15 mg/kg, IV) every 3 wk	ICI, anti-PD-L1 antibody (atezolizumab) plus antiangiogenic, anti-VEGF-A antibody (bevacizumab)	Improved OS, PFS, ORR *vs* sorafenib (IMbrave-150 phase III trial[93])	irAEs[1], hypertension, fatigue, proteinuria, pruritus, gastrointestinal bleeding	Approved by FDA and EMA for patients with advanced HCC
Tremelimumab (300 mg, IV) plus durvalumab (1500 mg, IV) once, followed by durvalumab (1500 mg, IV) every 4 wk	ICI, anti-CTLA-4 antibody (tremelimumab) plus ICI, anti-PD-L1 antibody (durvalumab)	Improved OS *vs* sorafenib and favorable benefit-risk ratio (HIMALAYA phase III trial[89])	Pruritus, irAEs[1]	Under evaluation for approval. Granted orphan drug designation by FDA for HCC treatment (2020)
Sintilimab (200 mg, IV) plus IBI305 (bevacizumb biosimilar; 15 mg/kg, IV) every 3 wk	ICI, anti-PD-1 antibody (sintilimab) plus antiangiogenic, anti-VEGF-A antibody (IBI305)	Better OS and PFS in HBV-related advanced HCC *vs* sorafenib (ORIENT-32 phase II/III trial[113])	Proteinuria, irAEs[1], thrombocytopenia, leukopenia, hypertension, fatigue	Approved by NMPA in China for patients with advanced HCC (2021)
Second-line				
pembrolizumab (200 mg, IV) every 3 wk plus best supportive care	ICI, anti-PD-1 monoclonal antibody	Better OS, PFS and ORR in patients post-sorafenib *vs* placebo (KEYNOTE-394 phase III trial[114] and KEYNOTE-224 phase II trial[115])	irAEs[1], fatigue, pruritus, anorexia	Approved by FDA for advanced HCC post-sorafenib (2018)
Nivolumab (1 mg/kg, IV) plus ipilimumab (3 mg/kg, IV) every 3 wk for 4 cycles, followed by nivolumab (240 mg, IV) every 2 wk	ICI, anti-PD-1 monoclonal antibody (nivolumab) plus ICI, anti-CTLA-4 antibody (ipilimumab)	Promising OS and durable response post-sorafenib (cohort 4 of CheckMate-040 phase I/II trial[90]). CheckMate 9DW phase III trial ongoing[116]	Pruritus, irAEs[1]	Approval by FDA for advanced HCC post-sorafenib (2020)

[1]Immune-related adverse events include hepatitis, colitis, pneumonia, endocrinopathy, skin rash, neurological disorders.

IV: Intravenous administration; ICI: Immune checkpoint inhibitor; VEGF-A: Vascular endothelial growth factor-A; OS: Overall survival; PFS: Progression free survival; ORR: Objective response rate; irAEs: Immune-related adverse events; FDA: Food and Drug Administration; EMA: European Medicines Agency; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; NMPA: National Medical Products Administration.

compared to liver disease of other etiology (nonviral HCC), including NASH-related HCC (Table 2). To our knowledge, none of the clinical trials that evaluated the efficacy of immunotherapy for the treatment of HCC differentiated the nonviral HCC subgroup of patients, thus including cases of HCC associated with NASH, alcohol use disorder, autoimmune hepatitis, primary biliary cholangitis, or sclerosing cholangitis.

In the CheckMate-459 trial, nivolumab treatment was found to be associated with slightly lower median overall survival (OS) than sorafenib in the nonviral HCC group (16 mo *vs* 17.4 mo; HR: 0.91; 95%CI: 0.72–1.16), while the best results were obtained in the viral HCC group (HCV-HCC patients: 17.5 *vs* 12.7 mo; HBV-HCC patients: 16.1 *vs* 10.4 mo)[86]. In the KEYNOTE-240 trial, pembrolizumab showed higher OS in HCC of any etiology compared to placebo, but better results were reported in patients with HBV-HCC (HR: 0.57; 95%CI: 0.35–0.94) than in those with nonviral HCC (HR: 0.88; 95%CI: 0.64–1.20)[87]. In the study 22 phase 1/2 trial that evaluated the effectiveness of the combination of tremelimumab plus durvalumab, HBV-HCC and nonviral HCC patients showed comparable OS results (14.4 and 13.8 mo, respectively), which differed from the results of the HCV-HCC patients (22.3 mo)[88]. In the subsequent HIMALAYA phase 3 study[89], tremelimumab plus durvalumab showed longer OS than sorafenib in HBV-HCC patients (HR: 0.64; 95%CI: 0.48–0.86) and in nonviral HCC patients (HR: 0.74; 95%CI: 0.57–0.95), which was opposite to that found in the HCV-HCC patients (HR: 1.06; 95%CI: 0.76–1.49).

Regarding second-line regimens, in cohort 4 of the CheckMate-040 trial, which investigated the therapeutic efficacy of three different dosing regimens of nivolumab plus ipilimumab, the OS benefit was similar in the nonviral HCC (14.7 mo) and HBV-HCC (15.2 mo) groups, while the OS in the HCV-HCC group was significantly higher (21.9 mo)[90].

Thus, the results of several studies supported the hypothesis that the underlying etiology might influence tumor response to immunotherapy. As shown by Foerster *et al*[91], this is particularly relevant in the case of NASH-related HCC, which is frequently identified at an advanced stage when systemic therapy becomes necessary. Regarding this, the authors highlighted some clinical issues. First, there are no effective strategies to prevent the development of HCC in NASH. Second, many cases of NASH-related HCCs arise in the absence of cirrhosis, but it is not known which subgroup of NASH patients might have a higher oncogenic risk and benefit from a surveillance program. Third, the low efficacy of ICIs in NASH-related HCC was concluded from post hoc analyses of phase III studies, which prevented definitive inferences from being drawn.

Table 2 Overall survival of patients with hepatocellular carcinoma receiving first-line immunotherapy alone or in combination, based on the etiology of the liver disease

Treatment	HCC etiology	HR (95%CI)	Trial	Phase
Atezolizumab plus bevacizumab *vs* sorafenib in first-line	Nonviral HCC	1.05 (0.68-1.63)	IMbrave150[93]	III
	HBV-HCC	0.58 (0.40-0.83)		
	HCV-HCC	0.43 (0.25-0.73)		
Nivolumab *vs* sorafenib in first-line	Nonviral HCC	0.91 (0.72-1.16)	CheckMate-459[86]	III
	HBV-HCC	0.79 (0.59-1.07)		
	HCV-HCC	0.72 (CI 0.51-1.02)		
Atezolizumab plus cabozantinib *vs* sorafenib in first-line	Nonviral HCC	1.18 (0.78–1.79)	COSMIC-312[99]	III
	HBV-HCC	0.53 (0.33-0.87)		
	HCV-HCC	1.10 (0.72-1.68)		
Tremelimumab 300 mg × 1 dose + Durvalumab 1500 mg *vs* sorafenib in first-line	Nonviral HCC	0.74 (0.57-0.95)	HIMALAYA[89]	III
	HBV-HCC	0.64 (0.48-0.86)		
	HCV-HCC	1.06 (0.76-1.49)		

HCC: Hepatocellular carcinoma; OS: Overall survival; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

Tumor immune surveillance in NAFLD-related HCC and its association with efficacy of immunotherapy

Pfister *et al*[44] investigated the role of adaptive immunity in both NASH and NASH-related HCC and its effects on the efficacy of ICIs. In mice with steatohepatitis, an increase in hepatic resident-like $CD8^+$ $PD1^+$ T cells with characteristics such as exhaustion and effector functions were observed. Moreover, NASH severity was correlated with PD-L1 expression in hepatocytes and nonparenchymal cells. These results suggested that steatohepatitis-related HCC might significantly benefit from treatment with ICIs. However, when mice were administered anti-PD-1 immunotherapy, they did not show any tumor regression; instead, liver fibrosis increased. Conversely, mice affected by HCC of other origin had a positive response to anti-PD-1 immunotherapy. Furthermore, in NASH mice without HCC, the pre-emptive depletion of $CD8^+PD1^+$ T cells significantly decreased liver damage and, consequently, the incidence of HCC. Pre-emptive treatment with ICIs increased $PD1^+CD8^+$ T-cell infiltration in the liver and the incidence of HCC. When anti-tumor necrosis factor (TNF) or anti-CD8 antibodies were administered together with anti-PD-1 antibodies, liver damage and HCC incidence decreased compared to the reduction in liver damage after anti-PD-1 treatment alone. In humans, $PD1^+$ T cells were absent in healthy livers, but they were abundant in NASH livers and expressed the same gene profile as $PD1^+$ T cells found in mice. In summary, $CD8^+PD1^+$ T cells failed to provide adequate antitumor immune surveillance in NASH-related HCC after immunotherapy. Instead, they triggered the transition to liver cancer through a TNF-dependent mechanism that was further enhanced by anti-PD-1 treatment. Although the study included data from patients with NASH, further investigations need to be conducted to elucidate the true effect of ICIs on NASH-related HCC in the clinical setting.

Benefits of antiangiogenic drugs in NAFLD-related HCC

Retrospective studies analyzed the response of tumors to lenvatinib and sorafenib, two TKIs with anti-VEGF activity, associated with the etiology of HCC. While some studies[92-94] did not find significant differences, Shimose *et al*[95] reported that NAFLD/NASH etiology was associated with greater survival of the patients treated with sorafenib. This was also confirmed by the REACH-2 trial, which showed that second-line treatment with ramucirumab (an inhibitor of VEGF receptor 2) achieved higher OS in nonviral than in viral HCC patients compared to placebo (HR: 0.633; 95%CI: 0.379–1.057 *vs* HR: 0.762; 95%CI: 0.435–1.334 *vs* HR: 0.838; 95%CI: 0.522–1.347, respectively)[96]. However, another study did not find any difference in the OS of patients with NAFLD/NASH who received sequential therapy after sorafenib treatment compared to those with viral or alcohol-related etiology of liver disease[95]. The favorable effect of angiogenesis inhibition on nonviral HCC was confirmed by administering combination therapies. In phase 3 IMbrave150 trial, the combination of atezolizumab plus bevacizumab showed considerable improvement in the OS compared to the OS after treatment with sorafenib in the HCV-HCC group (24.6 *vs* 12.6 mo; HR: 0.43; 95%CI: 0.25–0.73) and in the HBV-HCC group (19.0 *vs* 12.4 mo; HR: 0.58; 95%CI: 0.40–0.83). However, no improvement in the OS was observed in the nonviral HCC group (17.0 *vs* 18.1 mo; HR: 1.05; 95%CI: 0.68–1.63)[97]. There was also a significant improvement

in the objective response rate (ORR) (26.5% *vs* 9.4%; HR: 3.47; 95%CI: 1.24–9.65) and the progression-free survival (PFS) (7.1 *vs* 5.6 mo; HR: 0.80; 95%CI: 0.55–1.17) compared to the Sorafenib in the nonviral HCC group. Moreover, upon comparing the effectiveness of atezolizumab plus bevacizumab treatment with that of atezolizumab treatment, it became clear that the improvement in the PFS was related to the addition of the anti-VEGF drug (6.3 *vs* 3.4 mo; HR: 0.49; 80%CI: 0.26–0.92)[98]. Cabozantinib is another TKI featuring an anti-VEGF effect. The phase 3 COSMIC-312 trial[99] investigated its efficacy in combination with the anti-PD-L1 atezolizumab *versus* sorafenib in the first-line treatment of advanced HCC. The PFS and preliminary interim OS results were assessed, while follow-up for the final OS analysis is ongoing. Overall, cabozantinib plus atezolizumab did not show any improvement in the OS compared to sorafenib at the interim analysis (15.4 *vs* 15.5 mo; HR: 0.90; 96%CI: 0.69–1.18), while the PFS was significantly higher in the subgroup treated with the combination therapy (6.8 *vs* 4.2 mo; HR: 0.63; 99%CI: 0.44–0.91). Specifically, compared to sorafenib, atezolizumab plus cabozantinib showed the best results in the HBV-HCC patients (PFS: 6.7 *vs* 2.7 mo; HR: 0.46, 95%CI: 0.29–0.73; OS: 18.2 *vs* 14.9 mo; HR: 0.53, 95%CI: 0.33–0.87), whereas, modest improvements were observed in the HCV-HCC patients (PFS: 7.9 *vs* 5.6 mo; HR: 0.64, 95%CI: 0.38–1.09; OS: 13.6 *vs* 14.0 mo; HR: 1.1, 95%CI: 0.72–1.68) and no benefit was found in the nonviral HCC subgroup (PFS: 5.8 *vs* 7.0 mo; HR: 0.92, 95%CI: 0.60–1.41; OS: 15.2 mo *vs* not reached; HR: 1.18, 95%CI: 0.78–1.79). Two meta-analyses of the CheckMate-459, KEYNOTE-240, and IMbrave150 phase 3 trials[44,100] confirmed that anti-PD-(L)1 therapy resulted in lower OS in nonviral HCC compared to that in viral HCC. Haber *et al*[100] also conducted a meta-analysis of five phase 3 trials to assess TKIs or anti-VEGF in the second-line setting (REACH, REACH-2, METIV-HCC, CELESTIAL, and JET-HCC) and showed that survival outcomes were not influenced by the HCC etiology (viral HCC-pooled HR: 0.81; 95%CI: 0.71–0.92; nonviral HCC-pooled HR: 0.82; 95%CI: 0.67–1.01). These results highlighted the synergistic effect of anti-VEGF and anti-PD-(L)1 agents for the treatment of HCC associated with liver disease of any etiology, especially in the nonviral setting.

Potential applications of biomarkers in immunotherapy for HCC

So far, PD-L1 expression assessed by immunohistochemistry on tumor tissue is the only approved biomarker to identify patients with higher probability to respond to ICIs[101]. However, as discussed above, PD-1/PD-L1 signaling is involved in complex and partially unclear molecular pathways that could limit the role of PD-L1 tissue expression as a reliable predictive factor. Some PD-L1-negative patients benefit from immunotherapy, whereas some PD-L1-positive patients do not. Hence, biomarkers identified through liquid biopsy, such as circulating tumor DNA[102], miRNAs[103], tumor cells[104], and extracellular vesicles[105], have been considered. However, no biological marker has demonstrated a strong predictive value in patients with HCC[106]. Scheiner *et al*[107] developed and proposed the C-reactive protein and -fetoprotein in immunotherapy (CRAFITY) score as an easily applicable clinical tool to predict response to ICIs in patients with HCC. The score ranges from 0 (C-reactive protein < 1 mg/dL and -fetoprotein < 100 ng/mL) to 2 (C-reactive protein ≥ 1 mg/dL and -fetoprotein ≥ 100 ng/mL). The authors found that higher scores indicated shorter OS and a worse radiological response. The gut microbiota might play a significant role in predicting the response to ICIs and modulating the immune response, thus, affecting the effectiveness of immunotherapy. Microbiological changes and intestinal permeability that occur in cirrhosis increase the interaction between hepatic cells and proinflammatory intestinal bacteria, enhancing inflammation in the liver[70]. Anti-inflammatory bacteria, such as *Akkermansia* and *Bifidobacterium*, are usually scarce in NASH-related HCC patients and might lead to a persisting inflammatory response with the suppression of immune system surveillance in the long term. An increase in the abundance of *Akkermansia* along with a reduction in Enterobacteriaceae occurs in patients who respond to ICI therapy[108,109]. Furthermore, the composition of the gut microbiota changes over time during immunotherapy, which might be associated with a modification in the expression of immunomodulating pathways or vice versa may be a result of the modulating effect of the immune system on the gut microenvironment. Whether these modifications can predict responses that are essential for making further treatment decisions[106] needs further confirmation. Based on these findings, the oral administration of *Akkermansia muciniphila* was suggested to enhance the effect of ICIs[110]. Similar findings were observed in patients affected by epithelial cancer[110], colorectal cancer[111], and lung cancer[112].

CONCLUSION

In patients with HCC related to NASH, antitumor immune surveillance is impaired. The weaker efficacy of ICIs in NASH-related HCC contradicts the obesity paradox, in which mild obesity predicts a better response in patients with melanoma and other cancers treated with immunotherapy. Tremelimumab plus durvalumab was the only combination of two ICIs tested against sorafenib in the first-line setting and the only one that showed a relatively higher OS in the nonviral and viral HCC subgroups. Some studies showed similar efficacy in the treatment of viral *versus* nonviral HCC for the combination of nivolumab plus ipilimumab. Thus, regimens based on the combination of two ICIs rather than ICI monotherapy are promising for HCC treatment, regardless of the etiology of the liver

disease. Similar results were reported for TKIs with anti-VEGF activity, including anti-VEGF and anti-VEGF receptor agents, and some studies found better results in NAFLD/NASH patients. Therefore, the addition of antiangiogenic agents might increase the efficacy of immunotherapy and improve responses in patients who have an impaired immune system, such as patients with nonviral HCC. This was confirmed by the effectiveness of atezolizumab plus bevacizumab in improving survival regardless of the liver disease etiology, along with a higher PFS in nonviral HCC. However, this preliminary evidence generated by *post hoc* analyses or meta-analyses needs validation. Indeed, no clinical trial could differentially address the outcome of NASH-driven HCC compared to HCC of other nonviral etiology, such as alcohol-related HCC. Future studies should distinguish patient populations based on the underlying liver disease for a specific analysis of clinical outcomes and a better understanding of the mechanisms that trigger tumor immune escape in these patients.

Overall, these findings suggest that changes are required in the current algorithm of advanced HCC treatment toward a strategy that involves the administration of highly specific and optimized therapies based on the etiology of liver disease. The stratification of the patients is hampered by intragroup molecular heterogeneity. Thus, a model based on histological or circulating biomarkers might be critical for predicting responses to immunotherapy and defining a personalized strategy. However, biomarkers that can predict the outcome of immunotherapy have not been identified yet; the CRAFITY score provided encouraging results but required prospective validation. Despite compelling evidence regarding the role of the gut–liver axis in NAFLD-associated HCC, putting the theoretical knowledge into practice, either to categorize patients or enhance the response to treatment, is still a work in progress.

ACKNOWLEDGMENTS

Thanks to Fondazione Roma for continuous support to our scientific research.

FOOTNOTES

Author contributions: Costante F and Airola C revised literature; Costante F, Airola C, Santopaolo F and Ponziani FR wrote the paper; Pompili M, Gasbarrini A, Santopaolo F and Ponziani FR supervised and revised the paper.

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

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S-Editor: Zhang H
L-Editor: Kerr C
P-Editor: Zhang H

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1637-1653

DOI: 10.4251/wjgo.v14.i9.1637

ISSN 1948-5204 (online)

REVIEW

Emerging role of caldesmon in cancer: A potential biomarker for colorectal cancer and other cancers

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A, A
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Bordonaro M, United States; Ji G, China; Liu J, China; Tanabe S, Japan; Wan XH, China; Yu T, China

Received: March 16, 2022
Peer-review started: March 16, 2022
First decision: April 17, 2022
Revised: May 5, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: September 15, 2022

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Abstract

Colorectal cancer (CRC) is a devastating disease, mainly because of metastasis. As a result, there is a need to better understand the molecular basis of invasion and metastasis and to identify new biomarkers and therapeutic targets to aid in managing these tumors. The actin cytoskeleton and actin-binding proteins are known to play an important role in the process of cancer metastasis because they control and execute essential steps in cell motility and contractility as well as cell

division. Caldesmon (CaD) is an actin-binding protein encoded by the CALD1 gene as multiple transcripts that mainly encode two protein isoforms: High-molecular-weight CaD, expressed in smooth muscle, and low-molecular weight CaD (l-CaD), expressed in nonsmooth muscle cells. According to our comprehensive review of the literature, CaD, particularly l-CaD, plays a key role in the development, metastasis, and resistance to chemoradiotherapy in colorectal, breast, and urinary bladder cancers and gliomas, among other malignancies. CaD is involved in many aspects of the carcinogenic hallmarks, including epithelial mesenchymal transition *via* transforming growth factor-beta signaling, angiogenesis, resistance to hormonal therapy, and immune evasion. Recent data show that CaD is expressed in tumor cells as well as in stromal cells, such as cancer-associated fibroblasts, where it modulates the tumor microenvironment to favor the tumor. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are generally not expressed in normal tissues, making these transcripts ideal targets for drug design. In this review, we will analyze these features of CaD with a focus on CRC and show how the currently available data qualify CaD as a potential candidate for targeted therapy in addition to its role in the diagnosis and prognosis of cancer.

Key Words: Bladder cancer; CALD1; Caldesmon; Chemoresistance; Colorectal cancer; Gastric cancer; Glioma; Epithelial to mesenchymal transition; Invasion; Metastasis

Core Tip: The actin-binding protein caldesmon (CaD) plays an important role in cancer development, metastasis, and resistance to chemotherapy. CaD has emerged as a significant player in carcinogenesis, as it features many cancer hallmarks, including epithelial mesenchymal transition, angiogenesis, and immune evasion. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are generally not expressed in normal tissues. These data qualify CaD as an attractive candidate for targeted therapy in addition to its role in the diagnosis and prognosis of cancer.

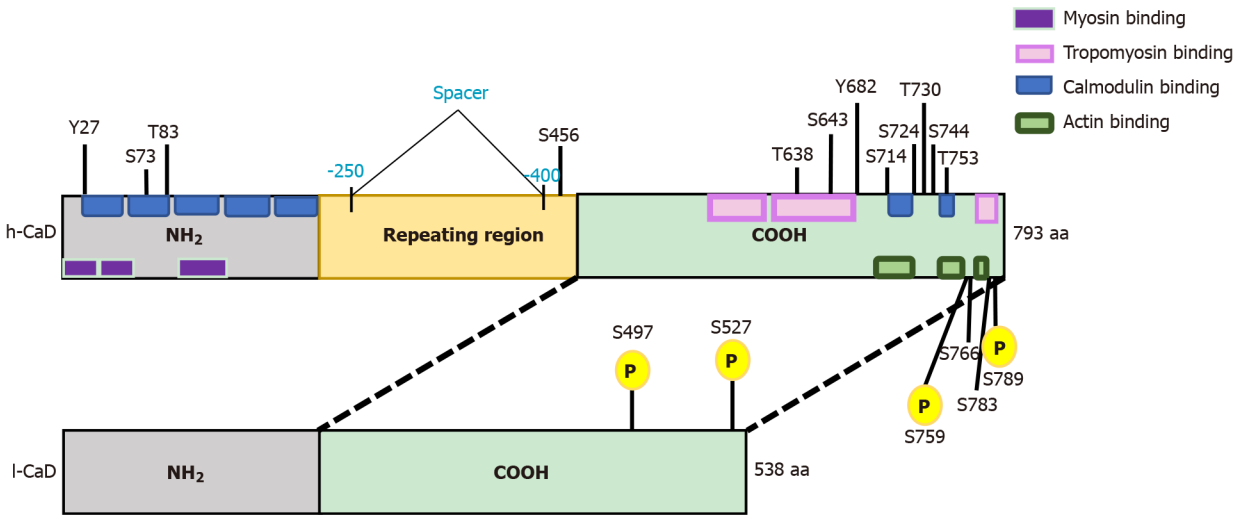
Citation: Alnuaimi AR, Nair VA, Malhab LJB, Abu-Gharbieh E, Ranade AV, Pintus G, Hamad M, Busch H, Kirfel J, Hamoudi R, Abdel-Rahman WM. Emerging role of caldesmon in cancer: A potential biomarker for colorectal cancer and other cancers. *World J Gastrointest Oncol* 2022; 14(9): 1637-1653
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1637.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1637

INTRODUCTION

The global cancer burden has increased to approximately 19.3 million cases and 10 million cancer deaths in 2020[1]. Colorectal cancer (CRC) is the second most common prevalent cancer, with 5253335 cases, and the third most common cancer worldwide, with 1931590 new cases, in 2020[1,2]. Almost half of the patients with CRC succumb to the disease[1,2]. Cancer morbidity and mortality are essentially due to the ability of cancer cells to invade, metastasize, and destroy normal tissues. Cancer cells, which undergo this complex process, have the ability to survive in the hostile microenvironment, a process mediated by the accumulation of multiple genetic and epigenetic mutations and the activation of a multitude of signaling pathways fueled, generally, by a state of genetic instability[3].

Cancers of epithelial origin (carcinomas), such as those of the colon, shed away their adhesion molecules and acquire mesenchymal markers that enable invasion and metastasis in a process known as epithelial to mesenchymal transition (EMT)[4-6]. The actin cytoskeleton is an important player in cell motility, division, and contractility among other cellular processes[7]. Multiple actin-binding proteins (ABPs) control these functions of the actin cytoskeleton[8]. ABPs form a growing family of more than 160 proteins that can bind actin monomers, polymers, or both[9]. ABPs can be divided into two broad categories, depending on their effect on actin filament dynamics[10]. The first category controls cytoskeletal responses to external stimuli by regulating G-actin/F-actin turnover. This category includes Arp2/3, ADF/cofilin, profilin, and gelsolin. The second category promotes the formation of higher-order structures, such as actin filament meshwork or bundles. This category includes tropomyosin, caldesmon (CaD), and filamin[10].

CaD is encoded by the CALD1 gene in multiple isoforms (Figure 1, and Supplementary Figure 1). High-molecular-weight CaD (h-CaD; 120-150 kDa) is restricted to smooth muscle cells of visceral and vascular origin, and it has been used in diagnostic histopathology as a specific marker for tumors of smooth muscle or myofibroblast origin. The low-molecular weight CaD (l-CaD; 70-80 kDa) isoforms are



DOI: 10.4251/wjgo.v14.i9.1637

Figure 1 The domain structures of high-molecular-weight caldesmon and low-molecular-weight caldesmon. Human caldesmon (CaD) has two major isoforms resulting from alternative splicing. The upper bar represents high-molecular-weight CaD (h-CaD) (793 aa), the full length protein, which contains an N-terminal domain (NH_2), a C-terminal domain, and a middle part (repeating domain). The lower bar represents low-molecular-weight CaD (l-CaD) (538 aa), which is missing the middle repeating region. All functional domains are shared between h-CaD and l-CaD, except the missing central spacer in l-CaD that separates the N-terminal myosin binding domain from the C-terminal actin binding domain. Common functional regions for myosin and calmodulin are located within the NH_2 terminal. The calmodulin binding site is also located in the C-terminal region. Tropomyosin and actin binding sites are found in the C-terminal region. Phosphorylation sites are shown and the shared phosphorylation sites for ERK and cdc2 are highlighted (yellow). h-CaD: High-molecular-weight caldesmon; l-CaD: Low-molecular weight caldesmon; NH_2: N-terminal domain; COOH: C-terminal domain.

expressed in nonsmooth muscle cells[11-13]. CaD, particularly l-CaD, has emerged as a significant player during the development and progression of many types of cancers. For some cancers, such as urinary bladder cancer, glioma, and glioblastoma, the literature consistently suggests an oncogenic role of CaD. However, the available data for some other cancers, such as stomach and breast cancer, show contrasting effects of l-CaD. Therefore, we set out to clarify the role of CaD during carcinogenesis, with a focus on CRCs. We will highlight the role of CaD in cancer development and progression, resistance to various therapeutic modalities, and immune evasion. We will also discuss the role of CaD in EMT, modulation of the tumor microenvironment, and tumor-specific splicing.

CAD AND THE ACTIN CYTOSKELETON

Cell motility, which is required for cancer cell invasion into surrounding tissue, intravasation, and metastasis, is driven by cycles of actin polymerization, cell adhesion, and acto-myosin contraction. The actomyosin system in smooth muscle cells is regulated by myosin-linked and actin-linked molecules. The myosin-linked mechanism is essentially based on myosin phosphorylation by Ca^{2+}/calmodulin-dependent myosin light chain kinase and dephosphorylation by a type 1 myosin phosphatase, which is targeted to myosin by a regulatory subunit[14]. The actin-linked mechanisms are mediated *via* complex interactions among a growing family of ABPs; a more detailed discussion of these proteins and mechanisms can be found in specialized reviews[15-19].

CaD and tropomyosin are crucial components of the actin-linked mechanism that regulates the acto-myosin contractile system in smooth muscle. CaD was initially identified as an inhibitory factor for the actin-myosin interaction, in which CaD-induced inhibition can be released by Ca^{2+}/calmodulin. Subsequently, CaD was found to play an important role in cell motility by regulating the contractile system in both smooth muscle and nonmuscle cells[12]. CaD is conserved in almost all vertebrate cells and stabilizes actin filaments directly by binding along the sides of F-actin; it also enhances the binding of tropomyosin to actin[20].

H-CaD has been used as a diagnostic biomarker of vascular smooth muscles[21], mesenchymal[22-24], and smooth muscle neoplasms[25,26] and related conditions[27-29], while nonmuscle l-CaD is broadly implicated in many aspects of cell motility, including cell migration[30], focal adhesion assembly[31], and podosome dynamics[32]. In cultured and transfected cells, overexpression of the actin-binding domain, or full length, of l-CaD promotes cell movement and facilitates the formation of cytoplasmic processes, while cell contractility is inhibited and the number of focal adhesions is decreased[31].

THE EMERGING ROLE OF CAD IN CARCINOGENESIS

CaD has emerged as an attractive molecule that potentially controls significant steps in tumor formation, cell division, invasion, metastasis, and response to therapy. Early work has shown that the expression and distribution of CaD are different in normal fibroblasts and their transformed counterparts[32]. In normal fibroblasts, myosin, CaD, and tropomyosin were distributed along the stress fibers as expected but were not seen at their termini known as 'focal adhesions/adhesion plaques'[33]. In contrast, these contractile proteins were concentrated within 'podosomes', which are cell-adhesive structures located within the protrusions of the ventral cell surface of transformed cells and are associated with high motility. Podosomes have previously been shown to have short F-actin bundles[34,35], together with actin-associated regulatory proteins, such as fimbrin[36] and gelsolin[37]. In transformed cells, CaD appears to play a major role in podosome structure and function due to its localization mainly in the podosome core domain with short F-actin bundles, in contrast to myosin and tropomyosin. Thus, CaD was associated with high motility of the podosomes of transformed cells, while the stable adherence of focal adhesions of normal cells was suggested to be due to the lack of this system [33]. The significance of these findings stems from observations of the podosomes of transformed cells being most dynamic adhesive structures with high motility (short half-life), leading to metastasis and invasion, while the focal adhesions of normal cells were not capable of performing these functions[38].

The role of CaD, particularly the light isoform (l-CaD), in solid tumors has been analyzed in various study types, including clinical, bioinformatics, and functional/experimental studies. A comprehensive summary of this literature is supplied in Tables 1 and 2. This summary does not include the classical use of CaD/h-CaD as a marker for smooth muscle and related tumors, which is not the focus of this review but can be found in other publications/reviews[25,26]. The majority of the publications suggest an oncogenic role of CaD, particularly l-CaD, in many cancer types, such as breast cancer[47], urinary bladder carcinoma[50,51], oral cavity squamous cell carcinoma[58], and CRC[39], including early onset [42], gastric cancer[45], and lung cancer[56], and it was associated with a poor prognosis in bladder cancer in an *in silico* analysis[52] (Table 1). Moreover, the serum level of l-CaD was found to be high in glioma patients; hence, it is suggested to be a potential serum marker for glioma[61]. Some of the aforementioned studies clearly indicated that the transcript studied or expressed was l-CaD, but others did not specify the transcript. Even in the last case, it is most likely that the transcript responsible for these actions is nonsmooth muscle l-CaD because h-CaD expression is most likely to be restricted to smooth muscles and their tumors.

In contrast, a smaller number of publications have reported contradictory results (Table 2). Following an earlier report that CaD is a cell motility suppressor[72], tumor suppressor functions were shown *in vitro* using breast[68,69], colon[68], thyroid, and prostatic cancer cells[70], and CaD was suggested to be a metastasis suppressor in gastric cancer[67]. Overall, the overwhelming majority of the recent literature supports the idea that l-CaD exerts multiple oncogenic potentials by upregulating tumor cell motility, angiogenesis, and cell division, as well as modulating the tumor microenvironment. Furthermore, l-CaD overexpression was associated with resistance to immunotherapy and chemotherapy and poor overall survival in multiple cancer types (Table 1).

CAD, TRANSFORMING GROWTH FACTOR-BETA SIGNALING, AND EMT

Cancer cells activate EMT to move and migrate from the primary tumor to other parts of the body. EMT is an essential process of cellular plasticity for normal tissue and organ development, yet it is also involved in an array of oncogenic processes, including proliferation and invasion, angiogenesis, stemness, and resistance to chemoradiotherapy[73,74]. The process involves major changes in the phenotype of cancer cells within the primary tumor marked by loss of an epithelial phenotype and gain of a mesenchymal phenotype. EMT is the first of many steps leading to metastasis. Different factors are involved in activating EMT, such as environmental factors, signaling molecules, and transcription factors. EMT is tightly controlled in normal tissues by maintaining a balance between EMT transcription factors, while in cancer, the process is much more complicated. Once the primary cancer is formed, different triggers stimulate the movement of tumor cells for nourishment, exchange of nutrients and/or immune escape. These factors, such as hypoxia, oxidative stress, nutrient deprivation, and inflammation, activate a set of transcription factors, including transforming growth factor-beta (TGF-β), Wnt, SNAIL, TWIST and MAPK/ERK-ZEB1, among others[73-77]. All of these signaling pathways participate in crosstalk with each other and share interconnected regulatory components, which together with their targets form a complex network[78]. Comprehensive transcriptomic analysis of a large cohort have shown that EMT is the most dominant program in CRC[79].

TGF-β signaling is a potent inducer and one of the best-characterized EMT pathways. Although TGF-β potently promotes tumor progression *via* mechanisms that include activation of the EMT program and the resulting invasion of carcinoma cells into surrounding nonneoplastic tissue, it may negatively control the initial stages of tumor formation through its antiproliferative effects. However, some tumor cells solve this problem by inactivating other components of the pathway, such as SMADs[80,81], rather

Table 1 Summary of the literature supporting an oncogenic role of caldesmon

	Cell/cancer type	Findings	Research method	Ref.
1	Colorectal cancer	L-CaD was expressed in colorectal cancer and liver metastasis, compared with normal tissue. L-CaD was associated with a poor response to chemotherapy. L-CaD was associated with resistance to 5-Fu treatment and caused an increase in p21 and cleaved-PARP and a decrease in the expression of NF-κB and p-mTOR *in vitro*	Clinical, functional	Kim *et al* [39], 2012
2	Colon, bladder, and prostate	CALD1 may indicate cancer-related splicing events. CALD1 was identified as a tumor-specific splicing variant in colon and urinary bladder cancer tissue samples	Bioinformatics, and experimental	Thorsen *et al* [40], 2008
3	Colorectal cancer	CALD1 was upregulated and associated with M2 macrophage infiltration, angiogenesis, and TGF-β in stage III/IV mismatch-proficient colorectal cancer. High expression of CALD1 was significantly correlated with transendothelial migration. Cancer cell proliferation, invasion, and migration abilities were suppressed after reducing CALD1 expression *in vitro*	Clinical, bioinformatics, functional	Zheng *et al* [41], 2021
4	Colorectal cancer-early-onset	CALD overexpressed in early-onset colorectal cancer	Bioinformatics, *in silico,* clinical	Zhao *et al* [42], 2019
5	Rectal cancer	CALD1 overexpressed in nonresponders to chemotherapy	Clinical	Chauvin *et al*[43], 2018
6	Colorectal cancer	Novel l-CaD isoforms produced by alternative splicing of CALD1 played a role in colorectal cancer metastasis	Bioinformatics, *in silico*	Lian *et al* [44], 2020
7	Gastric cancer	CALD1 is a novel target of TEA domain family member 4 that is involved in cell proliferation and migration	Bioinformatics, *in silico*	Lim *et al* [45], 2014
8	Gastric cancer	High expression of CALD1 is associated with poor overall survival and with immune infiltration in gastric cancer	Bioinformatics, *in silico*	Liu *et al*[46], 2021
9	Breast cancer, study of ER	Silencing of ER in MCF7 cells upregulated CALD1, concomitantly with the acquisition of a new phenotype that encompasses increased growth rates, loss of cell-to-cell adhesion and a redistribution of the cytoskeletal components, resulting in increased motility	Functional analysis, basic study	Al Saleh *et al* [47], 2011
10	Breast cancer-ER-positive	ANXA1 and CALD1 were associated with downregulation of ER *via* activation of NF-κB signaling, which blocks apoptosis and allows cancer cells to become independent of estrogen. ANXA1 and CALD1 proteins are independent markers for tamoxifen therapy outcome (resistance) and are associated with fast tumor progression	Clinical, association, pathway analysis	De Marchi *et al*[48], 2016
11	Normal mouse mammary cells	The expression level and phosphorylation state of CaD increase as a function of time after induction of EMT by TGF-β1, and these changes in CaD correlate with increased focal adhesion number and size and increased cell contractility	Functional analysis, basic study	Nalluri *et al* [49], 2018
12	Bladder cancer	L-CaD overexpression in primary nonmuscle invasive bladder cancer is significantly associated with tumor progression. L-CaD is implicated in increased cell motility and invasive characteristics through morphological changes in bladder cancer cells	Clinical, functional	Lee *et al* [50], 2015
13	Bladder cancer	CaD was identified as one of the proteins with significant differential expression between bladder cancer tissue and normal urothelial tissue, using antibody microarray profiling of tissue samples	Clinical	Lee *et al* [51], 2015
14	Bladder cancer	Low CALD1 in tumor is associated with a good prognosis	Bioinformatics, *in silico*	Liu *et al*[52], 2019
15	Bladder cancer	CALD1 was correlated with aggressive features and poor overall survival. CALD1 promotes tumor cell growth, migration, invasion, and the cell cycle; it inhibits tumor cell apoptosis *in vitro* and *in vivo*. CALD1 expression was positively correlated with JAK/STAT activation resulting in PD-L1 overexpression	Clinical, functional	Li *et al*[53], 2021
16	Bladder cancer	CALD1 was overexpressed in CAFs, as well as macrophages and T cells in the microenvironment of bladder tumors and was associated with oncogenic features	Bioinformatics, functional	Du *et al*[54], 2021
17	Bladder cancer	MIR100HG inhibits the expression of miR-142-5p, resulting in the upregulation of CALD1 and acquisition of aggressive features in bladder cancer	Clinical, bioinformatics, functional	Zhang *et al* [55], 2021
18	Lung cancer	CaD is overexpressed in brain metastases of lung cancer	Clinical, expression	Zhang *et al* [56], 2014
19	NSCLC	Activation of the anaphase-promoting complex by p53 induces a state of dormancy in NSCLC cells after 5-Fu. Subsequently, EMT and CaD upregulation were associated with dormant cancer stem cells	Experimental, functional	Dai *et al* [57], 2016
20	Squamous cell carcinoma of oral cavity	CaD expression is associated with a poor prognosis in patients with oral squamous cell carcinoma. CaD increased invasion and migration and was elevated in patients' serum	Clinical, functional	Chang *et al* [58], 2013
21	Nasopharyngeal carcinoma	Bone marrow-derived mesenchymal stem cells secreted nitric oxide in the nasopharyngeal carcinoma tumor environment, which resulted in translocation of	Functional	Zhang *et al* [59], 2014

		CaD to the podosome in a Ca^{2+}/calmodulin manner in tumor cells and promotion of their invasion and metastatic ability		
22	Glioma	CALD1 was upregulated in neoplastic cells. CALD1 was associated with a progressive vessel architecture. CALD1 may serve as marker of glioma progression	Clinical, functional	Cheng *et al* [60], 2021
23	Glioma, patients' serum	The serum level of l-CaD was significantly higher in the group of glioma patients as compared to any of the other brain tumor groups	Clinical	Zheng *et al* [61], 2005
24	Glioma-associated blood vessels	Splicing variants of CALD1 are differentially expressed in glioma neovascularization *versus* normal brain microvasculature. The mis-splicing of CALD1 correlated with the breakdown of tight junctions among vascular endothelial cells	Expression, functional	Zheng *et al* [62], 2004
25	Endothelial cells	L-CaD is involved in the migration of endothelial cells and/or endothelial progenitor cells into human neoplasms (gliomas, breast cancers, renal cell carcinomas) where they contribute to tumor angiogenesis	Expression, functional	Zheng *et al* [63], 2007
26	Kidney epithelial cells, mouse mammary cells	CaD is activated and upregulated upon TGF-β induction of EMT. CALD1 overexpression is a key component in TGF-β-driven EMT	Functional	Morita *et al* [64], 2007
27	Not specified	CaD maintains newly polymerized actin in a distinct state that has a higher affinity for the Arp2/3 complex	Functional	Jensen *et al* [65], 2012

h-CaD: High-molecular-weight caldesmon; l-CaD: Low-molecular weight caldesmon; EMT: Epithelial to mesenchymal transition; TGF: Transforming growth factor; CaD: Caldesmon; NSCLC: Non-small-cell lung cancer; 5-Fu: 5-fluorouracil; CAFs: Cancer-associated fibroblasts; JAK/STAT: Janus kinase/signal transducers and activators of transcription; PD-L1: Programmed death ligand 1; NF-κB: Nuclear factor kappa B; p-mTOR: Phosphorylated mammalian target of rapamycin; ER: Estrogen receptor.

Table 2 Summary of the literature supporting a tumor suppressor role of caldesmon

	Cell/cancer type	Findings	Research method	Ref.
1	Colorectal cancer	An alternatively spliced form of CALD1 was decreased in tissues from colorectal tumor as compared to adjacent normal tissues	Bioinformatics, *in silico*	Liu *et al*[66], 2018
2	Gastric cancer	CaD is decreased in metastasis-derived gastric cancer cell lines. Knockdown of CaD resulted in an increase in cell migration and invasion	Proteomics, clinical, functional	Hou *et al*[67], 2013
3	Breast, colorectal, and thyroid cancer cells	The ectopic expression of l-CaD reduced the number of podosomes/invadopodia and suppressed cell invasion	Basic, functional	Yoshio *et al*[68], 2007
4	Breast cancer, and rat aorta cell lines	PKGI-β enhances breast cancer cell motility and invasive capacity by phosphorylating CaD. Knockdown of endogenous CaD in MDA-MB-231 breast cancer cells had pro-migratory and pro-invasive effects	Basic, functional	Schwappacher *et al* [69], 2013
5	Prostate cancer	Leupaxin phosphorylates CaD leading to its downregulation, and this downregulation of CaD increased migration and invasion of prostate cancer cells	Basic experimental	Dierks *et al*[70], 2015
6	Vascular smooth muscle cells and NIH 3T3 fibroblast cells	CaD upregulation mediates p53 suppression of Src-induced podosome and rosette formation and cellular invasiveness. The study is based on normal cells and whether or not it applies to malignancy remains to be clarified	Basic, functional	Mukhopadhyay *et al*[71], 2009

PKGI-β: cGMP-dependent protein kinase I; CaD: Caldesmon; l-CaD: Low-molecular weight caldesmon.

than TGF-β itself. The expression levels of cytoskeletal-associated proteins, including the actin binding protein CaD, increase during TGF-β1-induced EMT[64]. CaD was shown to play a key role in TGF-β-driven EMT of normal murine mammary epithelial cells. Nalluri *et al*[49] found that induction of EMT by TGF-β1 is mediated by increased expression together with increased levels of phosphorylated CaD, which was associated with increased focal adhesion number and size and increased cell contractility. CALD1 appears to play a major role in CRC *via* EMT induction because its expression is significantly and specifically upregulated in the consensus molecular subtype 4, which is characterized by TGF-β signaling activation together with other EMT phenotype indicators, such as invasion of the stroma by malignant cells and marked angiogenesis[82]. Moreover, Calon *et al*[83] showed that the poor prognosis of CRC is linked to TGF-β signaling in stromal cells that results in CALD1 overexpression.

CAD CONTRIBUTES TO TUMOR ANGIOGENESIS

The HeLa l-CaD I and II splice variant and protein isoforms were initially cloned from HeLa S3 in 1992 [11]. L-CaD was found to be associated with actin filaments (stress fibers) and tropomyosin in quiescent

cells, but l-CaD, tropomyosin and myosin were not seen at the focal adhesions end of these fibers[33]. Endothelial cells (ECs) and endothelial progenitor cells (EPCs) are quiescent under normal conditions. However, these cells are activated in tumors under hypoxia and other environmental stimuli to start to proliferate and migrate in the process of angiogenesis. Upon activation of ECs/EPCs, changes in focal adhesions occur, and simultaneous remodeling of F-actin causes changes in cell shapes[84]. These events enable the navigation of EC tips during angiogenesis and the recruitment of circulating EPCs from bone marrow to the site of neoangiogenesis. The HeLa l-CaD-containing cell protrusions were found to be specific for tumor ECs/EPCs and have never been observed in normal ECs[63]. Consistent with this finding of podosomes in ECs[85], Zheng *et al*[63] found a variety of motility-related cell protrusions, such as filopodia, microspikes, lamellipodia, podosomes, membrane blebs and membrane ruffles, in the activated ECs/EPCs of various human tumors under a histologically preserved microenvironment. HeLa l-CaD appeared to be invariably expressed in the subregions of these cell protrusions. Furthermore, HeLa l-CaD-positive multinucleated ECs/EPCs were observed in the glioma samples, among other tumor samples. These cells appeared to be highly motile because they were ubiquitously distributed in the tumor tissue sections[63]. Multinucleation is considered to be a sign of aborted cytokinesis and is associated with the activation of aortic EC motility and podosome formation[85,86].

The expression of HeLa l-CaD was restricted to the tumor vasculature and was not found in normal blood vessels of cancers derived from various organs, including breast, lung, kidney, colon, stomach, ovary, uterus, prostate, thyroid, and liver[87]. HeLa l-CaD was preferentially expressed in the early stage of tumor neovascularization. The available data suggest that HeLa l-CaD can be considered a marker for angiogenic ECs during the early stages of tumor neovascularization[87]. Taken together, these findings suggest that HeLa l-CaD is implicated in the migration of ECs/EPCs in human neoplasms, where they contribute to tumor angiogenesis[63].

A recent study of the mechanisms underlying the effect of l-CaD on microvascular facilitation and architecture in glioma showed that l-CaD is associated with abnormal microvessels in anaplastic astrocytoma and glioblastoma (an aggressive grade IV astrocytoma)[60]. The mechanism of such action was suggested by biofunction prediction to occur by modulating tumor angiogenesis, as ECs and pericytes were more apparent in the tumor microenvironment of high CALD1 expression samples. Histological and immunofluorescence examination of tumor tissue showed that CaD was associated with vessel architecture in astrocytoma and glioblastoma[60-63]. In stage III/IV mismatch-proficient CRC, CALD1 was upregulated and associated with angiogenesis, as detected by bioinformatics 'Weighted gene coexpression network analysis' (WGCNA)[41].

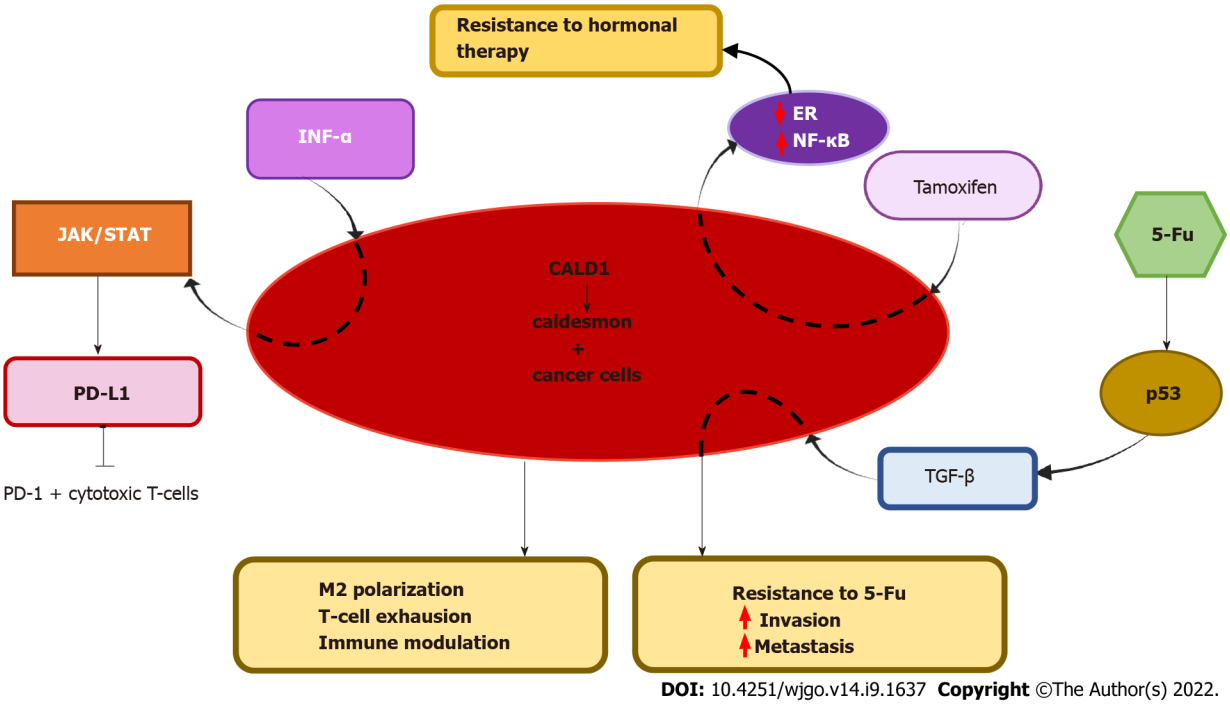
L-CAD IS A TUMOR-SPECIFIC SPLICE VARIANT

Alternative splicing is an attractive mechanism of mutation acquisition by cancer cells, as it has the potential to expand a limited number of genes into very complex proteomes and endow them with altered functions, localization, binding properties, and stability[66,88-90]. The CALD1 gene undergoes alternative splicing in cancer tissues, including colon, urinary bladder, and prostate tissues, and these variants are mostly tumor specific. Thorsen *et al*[40] found that the long CALD1 isoform, including an extended form of exons 5 and 6, was absent or reduced in bladder, colon, and metastatic prostate cancer. The dominant splice variant in these tumors is most likely to be transcript variant 2 encoded by WI-38 L-CADII[44]. Other cytoskeleton-associated proteins, such as Tropomyosin 1, ACTN1, and vinculin, were identified as significant candidates for alternative splicing in these tumors in the same study[40], supporting the role and importance of actin cytoskeleton modification in tumor progression[34,35,91]. It is known that splice variants can exert antagonistic functions in tumors, such as the well-known case of the B cell lymphoma (BCL)-X long isoform (BCL-X_L), which has an antiapoptotic function, and its short isoform BCL-X_S, which is proapoptotic[92]. Indeed, the identified cancer-specific splice variants of CALD1 are predicted to encode proteins with potentially altered functions[40]. Thus, the finding of CALD1 tumor-specific splice variants can explain the reported contrasting effects of the two isoforms, h-CaD and l-CaD, and could explain the oncogenic role of l-CaD in many types of cancers.

The splice variant identified by Thorsen *et al*[40] was confirmed to be tumor specific and associated with metastatic disease and poor overall survival in CRC[44]. Abnormal splicing was associated with upregulation of l-CaD in glioma tumor tissue samples and body fluids[61,62,93]. Cancer-specific splice variants may potentially be used as diagnostic, prognostic, and predictive biomarkers of various tumors. Moreover, the specificity of these isoforms to cancer cells compared with normal cells makes CaD an ideal selective therapeutic target in cancers[94].

CAD AND RESISTANCE TO THERAPY

CaD was implicated in resistance to multiple modalities of cancer therapy, including chemotherapy, radiotherapy, hormonal therapy and immunotherapy (Figure 2).



DOI: 10.4251/wjgo.v14.i9.1637

Figure 2 Caldesmon and resistance to various modalities of cancer therapy. INF: Interferon; JAK/STAT: Janus kinase/signal transducers and activators of transcription; PD-L1: Programmed death ligand 1; PD-1: Programmed death 1; 5-Fu: 5-fluorouracil; TGF: Transforming growth factor; ER: Estrogen receptor; NF-κB: Nuclear factor kappa B.

CaD and resistance to chemotherapy

It has long been shown that the F-actin associated with transformed cells is different from that of normal cells not only in morphology and function but also in its insensitivity to drugs[95]. The association between CaD and resistance to various forms of cancer therapy has been documented in many cancer types. Dai *et al*[57] showed that non-small-cell lung cancer (NSCLC) cells enter a state of dormancy upon exposure to 5-fluorouracil (5-Fu) and subsequently acquire resistance to this therapy. The mechanism of this resistance involves the accumulation of p53, activation of the ubiquitin ligase anaphase-promoting complex and TGF-β/SMAD signaling leading to EMT, followed by mesenchymal-epithelial transition. Chemotherapy-induced EMT-transformed NSCLC cells showed higher expression of CaD associated with increased invasion potential; however, these EMT-transformed NSCLC cells were arrested in the cell cycle in G0-G1 and lost their ability to divide during this phase[57]. The role of CaD in resistance to 5-Fu was documented in locally advanced rectal cancer patients[43].

CaD and antihormonal therapy

CaD was associated with resistance to the targeted antihormonal drug tamoxifen in estrogen receptor (ER)-positive recurrent breast cancer[48]. This study was based on a proteomic analysis to identify a predictive signature for tamoxifen therapy outcomes in recurrent breast cancer. CALD1 and annexin-A1 (ANXA) were the most differentially expressed proteins and were confirmed by immunohistochemical staining of an independent set of tumors. CALD1 expression showed a significant association with a shorter time to progression, independent of other clinicopathological predictive factors. The majority of proteins that were correlated with ANXA1 were also correlated with CALD1, but a direct link between the two genes (CALD1 and ANXA1) and the mechanism underlying the association have yet to be clarified. CALD1, in particular, was associated with ER downregulation and nuclear factor-kappa B (NF-κB) signaling[48].

CaD and immunotherapy

CALD1 was among the top genes associated with both overall survival and disease-free survival in bladder cancer according to bioinformatics analysis. Tumors with low levels of CALD1 expression had a better prognosis than tumors with high CLAD1 expression[52]. This finding was confirmed in a recent study, and the mechanism was linked to immunomodulation *via* upregulation of programmed death ligand 1 (PD-L1) in bladder cancer[53]. PD-L1 has the potential to suppress the immune response in both physiological and pathological pathways by interacting with its corresponding receptor, PD-1[96, 97]. PD-L1 expressed by tumor cells binds to PD-1 on the cytotoxic T-cell surface and thus attenuates immunosurveillance in the tumor microenvironment. Li *et al*[53] found that PD-L1 is associated with CALD1 in bladder cancer cells and that both are induced by interferon-gamma *in vitro*. CALD1 silencing

significantly reduced cell viability in T24 bladder cancer cells *in vitro* and *in vivo* in nude mouse xenografts. The authors suggested that CALD1 promoted the expression of PD-L1 *via* the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway[53]. It is likely that the CALD1 effect on PD-L1 is active in other cancers, such as colon cancer, and can exert immunomodulation through this axis because PD-L1 expression is also upregulated *via* JAK/STAT3 after fibroblast growth factor receptor 2 stimulation in CRC[98].

A recent bioinformatics-based report showed that CALD1 was highly expressed in gastric cancer compared with adjacent normal tissue and that this high expression was associated with poor overall survival in these patients. There was a strong correlation between CALD1 expression and gene markers of M2 macrophages (CD163, VSIG4, membrane-spanning 4A) and Treg and T-cell exhaustion markers (FOXP3, CCR8, STATA5B, TGF-β1, T cell immunoglobulin and mucin domain 3) in gastric cancer. These findings suggest that CALD1 plays an important role in M2 polarization, T-cell exhaustion, and immune modulation in gastric cancer[46].

CAD AND CRC

The available data show that CaD plays an important role in the development, progression and response to therapy of CRC, as detailed below (Figure 3).

CaD contributes to CRC development

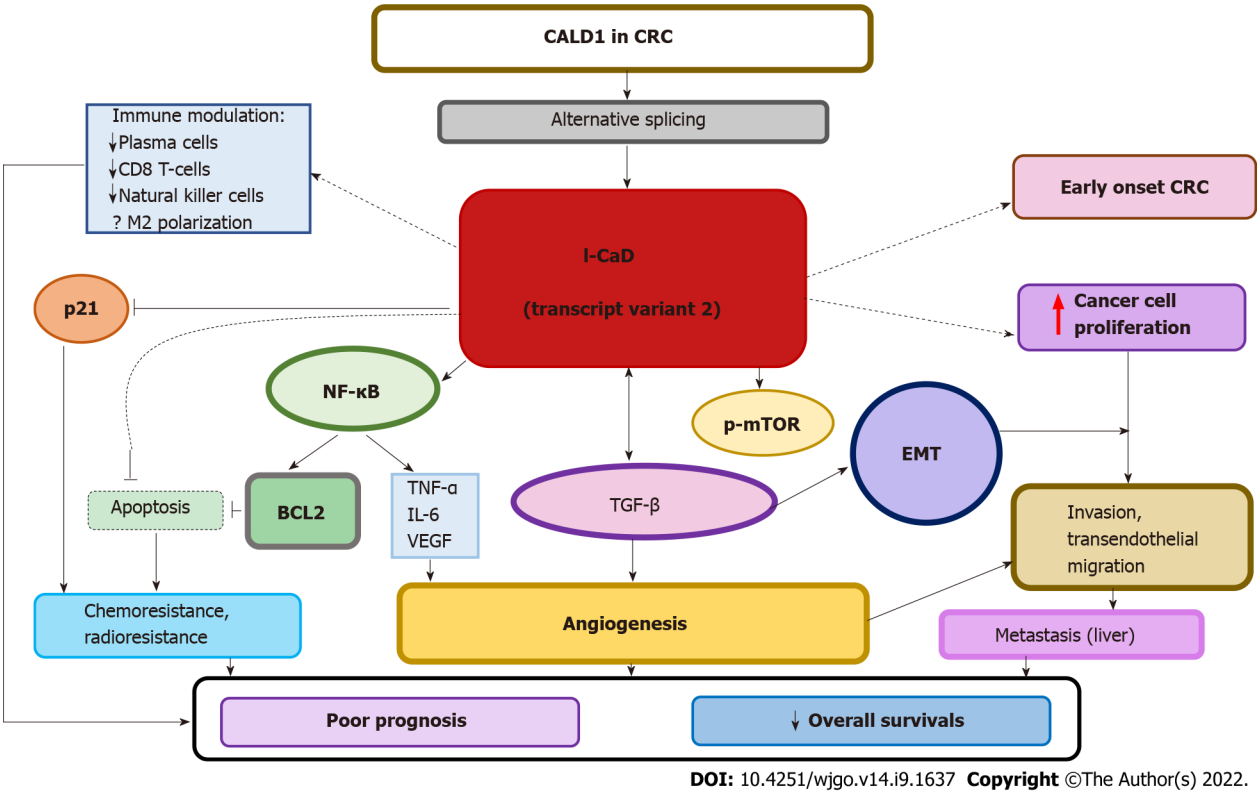
An early hint that CaD could play a role in CRC development came from the study in 2008 of alternative splicing in cancer by exon array analysis. Briefly, the identified tumor-specific CALD1 variant was missing an extended form of exons 5 and 6 and was predicted to encode proteins with potentially altered functions[40]. This finding implied an oncogenic role of l-CaD in colon cancer, as discussed in more detail above (see "l-Caldesmon is a tumor-specific splice variant").

Based on the proteomic finding of aberrant expression of CaD isoforms in colon cancer, Kim *et al*[39] set out to analyze the particular role of the short isoform l-CaD in CRC and liver metastasis. They observed a significantly higher expression level of l-CaD in primary colon cancer and liver metastasis than in the corresponding normal tissues. However, h-CaD did not differ among these groups. There was a tendency to have a poor response to chemoradiotherapy in patients with high expression of l-CaD in their tumors, which was confirmed *in vitro* by small interfering RNA (siRNA) silencing of l-CaD and monitoring the response to 5-Fu treatment in colon cancer cell lines[43]. L-CaD was suggested to exert these effects by relieving the cell cycle inhibition exerted by $p21^{Cip1}$ (cyclin-dependent kinase inhibitor 1, or CDK-interacting protein 1) and blocking apoptosis. Furthermore, silencing l-CaD downregulated NF-κB[39], an important signaling pathway that can stimulate tumor cell proliferation, survival, and angiogenesis by controlling a wide network of genes and molecules, such as tumor necrosis factor-α, interleukin-6, BCL2, and vascular endothelial growth factor[99]. Silencing l-CaD also downregulated phosphorylated mammalian target of rapamycin[39], a pathway that regulates not only tumor cell proliferation but also the tumor immune response and metabolism[100]. Collectively, Kim *et al*[39] showed that high expression of l-CaD in CRC is associated with increased metastatic properties and a decreased response to therapy.

A recent study confirmed that l-CaD transcript 2 is the dominant transcript and is associated with metastatic disease and poor overall survival in CRC[44]. Interestingly, CALD1 was among the top upregulated genes implicated in the development of early-onset CRC based on a comprehensive bioinformatics analysis[42]. This finding may shed light on the pathogenesis of early-onset CRC, which is a heterogeneous category of CRCs that is more common in Eastern than in Western countries[101, 102]. The association of CALD1 together with other genes involved in cellular mobility and vascular smooth muscle contraction with early-onset CRC can explain the aggressive nature of this subset of tumors[42].

A recent study by Zheng *et al*[41] utilized a new bioinformatics tool, WGCNA, to clarify the basis of the poor response to immunotherapy in mismatch-proficient, stage III/IV CRC and showed that CALD1 was upregulated and associated with protumorigenic M2 macrophage infiltration. M2 macrophages are believed to be an important contributor to the failure of immunotherapy due to their anti-inflammatory, immunosuppressive, and proangiogenic characteristics[103]. CALD1 was negatively correlated with fractions of plasma cells, CD8 T cells, CD4 memory-activated natural killer cells, and dendritic cells[41]. High expression of CALD1 was significantly correlated with angiogenesis, TGF-β, and trans-endothelial migration. Taken together, these data are consistent with the published literature on the importance of the crosstalk between angiogenesis and TGF-β in macrophage recruitment and M2 polarization[104, 105], but the role of CALD1 in this scenario remains to be clarified. Cancer cell proliferation, invasion, and migration abilities were suppressed after reducing CALD1 expression *via* siRNA silencing *in vitro* [41].

Only one article suggested that ectopic expression of CaD in a panel of cell lines of various lineages, including the HCA7 CRC cell line, reduced the number of podosomes/invadopodia and suppressed cell invasion, but no further functional analysis or clinical correlation was presented. The vector used,



DOI: 10.4251/wjgo.v14.i9.1637

Figure 3 Role of caldesmon in colorectal cancer. Dashed lines indicate reported associations, the mechanism of which has not been identified. CRC: Colorectal cancer; NF-κB: Nuclear factor kappa B; BCL-2: B cell lymphoma; TNF: Tumor necrosis factor; IL: Interleukin; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; p-mTOR: Phosphorylated mammalian target of rapamycin; EMT: Epithelial to mesenchymal transition; CaD: Caldesmon.

pcDNA3.1(+)-HA-CaD, was supposed to contain l-CaD[68]. However, the cell line used, HCA7, is an atypical CRC cell line with an unusual cytogenetic profile and other characteristics[106,107]. Overall, the available literature suggests that l-CaD, particularly splice variant 2, is a CRC splice variant that exerts protumorigenic characteristics and is associated with angiogenesis, invasion, metastasis, immune evasion, and poor prognosis in CRC.

CaD as a prognostic biomarker of CRC

As discussed above, Kim *et al*[39] showed that colon cancer patients with high expression of l-CaD in their tumors had a poor response to chemoradiotherapy. L-CaD could exert these effects by inhibiting $p21^{Cip1}$ and blocking apoptosis[39]. Lian *et al*[44] showed that l-CaD was associated with metastatic disease and poor overall survival in CRC. The WGCNA-based study of Zheng *et al*[41] showed that CALD1 was significantly associated with a worse prognosis in mismatch proficient, stage III/IV CRC. However, chemotherapy and tumor stage remained significantly correlated with overall survival. Both CALD1 and tumor stage were independent prognostic predictors in the GSE41258 validation dataset used in that study.

Calon *et al*[83] performed a comprehensive bioinformatics analysis to clarify the characteristics of the poor-prognosis subtypes of CRC in three common classification systems. Although these three classification systems were based on distinct global gene expression profiles in independent cohorts of CRC and differed regarding the number of the identified tumor subtypes[108-110], they all concluded that poor patient outcome in CRC is associated with the expression of stem cell and mesenchymal genes [111]. Calon *et al*[83] found that among the poor-prognosis gene sets common to at least two of the three molecular classifications, 31% (including CALD1) stained solely the tumor stroma, and 62% stained both stromal and tumor cells in the Human Protein Atlas Dataset[112]. Intriguingly, CALD1 mRNA and protein expression were upregulated in cancer-associated fibroblasts (CAFs) and other stromal cell populations in contrast to epithelial tumor cells[83]. However, CaD was identified in pure colon cancer parenchymal tissue from cell lines (containing no stroma), and both l-CaD and h-CaD were observed by western blot or transcriptomics analysis of colorectal carcinoma cells in other studies[39,41,113]. Moreover, the functional consequences of l-CaD silencing were shown to impact the mobility, response to therapy and signaling pathways in colorectal carcinoma cells in these studies[39,41]. Interestingly, Calon *et al*[83] showed that the poor prognosis of CRC is linked to TGF-β signaling in stromal cells that results in CALD1 overexpression, providing evidence linking CALD1 to TGF-β signaling in the tumor stroma.

Jensen *et al*[113] reported that the CALD1 gene was upregulated in the transcriptome of more than one CRC cell line (HT29, LoVo) that acquired resistance to SN38 (a potent irinotecan metabolite). Moreover, proteomic analysis of locally advanced, nonmetastatic CRCs treated with neoadjuvant chemoradiotherapy, including 5-Fu, showed that CALD1 was among the top genes overexpressed in nonresponders[43]. In this study, the authors verified the mRNA expression of CALD1, as well as the presence of gene sequence variants, in the CRC cell line set of the 'Colorectal Cancer Atlas' available from http://colonatlas.org/index.html.

ROLE OF CAD IN OTHER CANCER TYPES

Gastric cancer

Bioinformatics analysis suggested that CALD1 is a novel target of the TEA domain family member 4 gene that mediates gastric cancer development by stimulating cell proliferation and migration[45]. Another bioinformatics-based analysis showed that high expression of CALD1 is associated with poor overall survival and with immune infiltration in gastric cancer[46]. Conversely, Hou *et al*[67] showed that CaD expression was decreased in metastasis-derived gastric cancer cell lines as well as in resected biopsies of metastatic gastric cancer to lymph nodes compared with the primary tumors. Functional analysis showed that knockdown of CALD1 using siRNA in these cells resulted in an increase in cell migration and invasion. The first two studies[45,46], suggesting an oncogenic role of CaD in gastric cancer, were based upon bioinformatics analysis of a large series of gastric cancer, yet they did not supply a functional analysis of CALD1 action, while Hou *et al*'s study focused on metastatic gastric cancer[67]. Thus, controversy remains, and further work is needed to clarify the role of CaD in gastric cancer.

Breast cancer

Two independent studies have shown an inverse relationship between ER and CaD. In the first study, silencing of ER in an ER-positive breast cancer cell line upregulated CALD1, concomitantly with the acquisition of more aggressive oncogenic features, including increased growth rates, loss of cell-to-cell adhesion and increased motility[47]. The second study was based on clinical breast cancer samples and aimed to identify predictive markers of tamoxifen resistance in recurrent breast cancer. ANXA1 and CALD1 were the most differentially expressed proteins, and they were associated with the downregulation of ER *via* activation of NF-κB signaling, which blocks apoptosis and causes cancer cells to become estrogen-independent[48]. Another study suggested that CaD can exert its carcinogenic effects in mouse mammary cells *via* EMT induction. The expression level and phosphorylation state of CaD increased as a function of time after induction of EMT by TGF-β1, and these changes in CaD correlated with an increased focal adhesion number and increased cell contractility[49].

In contrast, two publications showed the tumor suppressive functions of CaD. In the first, ectopic expression of l-CaD reduced the number of podosomes/invadopodia and suppressed cell invasion in breast cancer cells[68]. The second showed that CGMP-dependent protein kinase I enhanced breast cancer cell motility and invasive capacity by phosphorylating CaD and that knockdown of endogenous CaD in MDA-MB-231 breast cancer cells exerted promigratory and proinvasive effects[69]. Thus, more work is needed to clarify the role of CaD in various molecular subtypes of breast cancer as well as in large cohorts of clinical samples.

Bladder cancer

The role of CaD in bladder cancer has been comprehensively studied, and the published literature consistently supports an oncogenic role of CaD in bladder cancer, as shown in Table 1. CaD is significantly overexpressed in bladder cancer tissue compared with normal urothelial tissue[51]. L-CaD is overexpressed in primary nonmuscle invasive bladder cancer and is significantly associated with tumor progression. Functional studies have shown that l-CaD mediates morphological changes associated with increased cell motility and invasive characteristics in bladder cancer cells and can inhibit apoptosis *in vitro* and *in vivo*[50,53]. CALD1 was significantly correlated with histological grade, stage, and lymphatic metastasis of bladder cancer in the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus databases[53]. High CALD1 expression was associated with a poor prognosis[52], including poor overall survival[53].

CALD1 has been linked to JAK/STAT activation and PD-L1 overexpression[53]. The role of CALD1 in promoting bladder cancer progression by remodeling the tumor microenvironment was supported by the recent finding of CALD1 expression in CAFs as well as macrophages and T cells in the bladder tumor microenvironment[54]. Finally, noncoding RNA regulation of CALD1 was studied in bladder cancer and was found to occur *via* MIR100HG, which can promote the proliferation, migration and invasion of bladder cancer cells. MIR100HG inhibits the expression of miR-142-5p, which targets CALD1, thus relieving CALD1 from this inhibitory effect. Consequently, upregulated CALD1 results in the induction of aggressive features in bladder cancer cells[55].

Glioma

CALD1 expression was associated with a high pathological grade and poor clinical outcome in a bioinformatics analysis of glioma samples from the TCGA and Chinese Glioma Genome Atlas databases. "Biofunction prediction" suggested that CALD1 modulated tumor angiogenesis in these tumors[60]. Single-cell RNA sequencing (scRNA-seq), a technique that can define cellular states within both normal and disease tissues, including the immune phenotypes in the tumor microenvironment [114], showed that CALD1 was upregulated in neoplastic cells and was involved in the tumorigenic processes of gliomas. Dysfunctional l-CaD also led to a decline in cell mobility in glioblastoma cells[60]. L-CaD is abnormally spliced in glioma vasculature, and the resultant altered expression of the protein isoforms in ECs/EPCs plays a role in the neoangiogenesis of various human tumor types[62]. Finally, the serum level of l-CaD was elevated in glioma patients, and this elevation was significantly higher than the l-CaD serum levels in other brain tumor patients[61].

CONCLUSION

Traditionally, scientific interest in CaD has been focused on its application in diagnostic histopathology to diagnose smooth muscle and related tumors using h-CaD or "total CaD" antibodies. However, the nonsmooth muscle isoform l-CaD has recently attracted much interest for its variable actions during carcinogenesis. In contrast to the initial expectation, based upon its role in inhibiting actin-myosin interaction and smooth muscle contraction, a growing list of studies are showing pro-oncogenic roles in various cancers. Some controversy remains, as a few studies suggested that CaD can exert a tumor suppressor role that needs to be clarified, together with the detailed mechanism of action of CaD in cancer cells of various lineages. The availability of new technologies for the study of ABP biology and functions could assist in these tasks[115-117]. Our comprehensive analysis of the available publications to date showed that CaD, particularly l-CaD, plays an important role in the development, metastasis, and resistance to chemotherapy in CRCs and other cancer types. Furthermore, CaD is implicated in angiogenesis and immune evasion in specific types of cancers, such as those of the urinary bladder. It is highly likely that the role of CALD1 in immune modulation in bladder cancer could be a general mechanism that is applicable to CRC and many other tumors. Few publications have focused on the analysis of the localization of CaD in the stroma and the role it plays in various components of the tumor microenvironment, which is an important research priority. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are not generally expressed in normal tissues. These data qualify CaD as a potential candidate for targeted therapy in addition to its role in diagnosis and prognosis.

FOOTNOTES

Author contributions: Alnuaimi AR performed the literature search; Abdel-Rahman WM proposed and designed the review, obtained funds, performed the literature search; Nair VA, Malhab LJB, Hamad M, Hamoudi R, Abu-Gharbieh E, Ranade A, Pintus G, Kirfel J, and Busch H edited the manuscript; Alnuaimi AR and Abdel-Rahman WM collected the data, and wrote parts of the manuscript; Alnuaimi AR, Abdel-Rahman WM, Nair VA, Malhab LJB, Hamad M, Hamoudi R, Abu-Gharbieh E, Ranade AV, Pintus G, Kirfel J, and Busch H contributed to the data analysis; and all authors revised/endorsed the final draft.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1654-1664

DOI: 10.4251/wjgo.v14.i9.1654

ISSN 1948-5204 (online)

MINIREVIEWS

Liquid biopsy to detect resistance mutations against anti-epidermal growth factor receptor therapy in metastatic colorectal cancer

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B, B, B, B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Herold Z, Hungary; Martin MJ, Argentina; Sun C, United States; Wan XH, China; Ye X, China; Ying HQ, China

Received: March 19, 2022
Peer-review started: March 19, 2022
First decision: May 12, 2022
Revised: May 19, 2022
Accepted: August 9, 2022
Article in press: August 9, 2022
Published online: September 15, 2022

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Abstract

Colorectal cancer (CRC) is a major cause of mortality worldwide, associated with a steadily growing prevalence. Notably, the identification of *KRAS*, *NRAS*, and *BRAF* mutations has markedly improved targeted CRC therapy by affording treatments directed against the epidermal growth factor receptor (EGFR) and other anti-angiogenic therapies. However, the survival benefit conferred by these therapies remains variable and difficult to predict, owing to the high level of molecular heterogeneity among patients with CRC. Although classification into consensus molecular subtypes could optimize response prediction to targeted therapies, the acquisition of resistance mutations to targeted therapy is, in part, responsible for the lack of response in some patients. However, the acquisition of such mutations can induce challenges in clinical practice. The utility of liquid biopsy to detect resistance mutations against anti-EGFR therapy has recently been described. This approach may constitute a new standard in the decision algorithm for targeted CRC therapy.

Key Words: Colorectal neoplasms; Precision medicine; Liquid biopsy; Cetuximab; Panitumumab

Core Tip: Contemporary management of metastatic colorectal cancer patients with wild type *KRAS* includes the use of anti-epidermal growth factor receptor (EGFR) agents, such as cetuximab or panitumumab, as first-line treatment. However, a significant number of patients receiving this treatment show disease progression. Some of the relapses could be explained by the presence of acquired resistance mutations in KRAS. Liquid biopsy of circulating tumor cells or circulating cell-free DNA is expected to improve the management of patients undergoing anti-EGFR therapy.

Citation: Valenzuela G, Burotto M, Marcelain K, González-Montero J. Liquid biopsy to detect resistance mutations against anti-epidermal growth factor receptor therapy in metastatic colorectal cancer. *World J Gastrointest Oncol* 2022; 14(9): 1654-1664
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1654.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1654

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality worldwide[1]. Disseminated disease (stage IV) with metastasis has been associated with poor prognosis, with a mean survival time of 15 mo[2]. The standard treatment for patients with metastatic CRC (mCRC) involves adjuvant chemotherapy with FOLFOX (leucovorin, 5-fluorouracil, and oxaliplatin) or FOLFIRI (leucovorin, 5-fluorouracil, and irinotecan). Furthermore, international guidelines recommend the analysis of *KRAS*/*NRAS* and *BRAF* mutations for targeted therapy[3,4]. Currently, the use of epidermal growth factor receptor (EGFR) inhibitor antibodies (anti-EGFR), such as cetuximab[5] or panitumumab[6], is recommended for patients with *KRAS* exon 2 wild-type (wt) mCRC. Both monoclonal antibodies exhibit a high affinity for the extracellular domain of EGFR; thus, they can prevent the ligand binding with EGFR[7]. Nevertheless, only 41% of patients with wt *KRAS* and left-sided colon disease reportedly attained partial or complete response to anti-EGFR treatments[8], as determined by RECIST criteria. The high level of variability in patient responses could be explained by the molecular and genomic variability of malignant colorectal neoplasms[9]. This heterogeneity could be explained by the consensus molecular subtype classification, which utilizes a transcriptomic approach to characterize the molecular heterogeneity of CRC[10]. This approach has opened new horizons by applying a novel classification to explain the distinct responses to conventional and targeted therapies in mCRC[11]. In addition to the heterogeneity of the primary tumor, the application of targeted therapies can lead to the selection of clonal tumor cells that acquire resistance mechanisms[12,13]. The emergence of activating *KRAS* mutations is a well-known (but not unique) mechanism of resistance to anti-EGFR therapy. For example, a retrospective analysis of the FIRE-3 clinical study (bevacizumab plus FOLFIRI or cetuximab plus FOLFIRI as first-line treatment for mCRC) has reported that a group of cetuximab-treated patients acquired activating mutations[14]. Furthermore, whole-exome sequencing studies have revealed that treatment with chemotherapy and cetuximab can be associated with a mutational signature (known as SBS17b) driving mutations in *KRAS*/*NRAS* and *EGFR* genes, resulting in resistance against this targeted therapy[15].

In real-world clinical settings, given that several patients are not considered suitable candidates for metastatic biopsies, it has been suggested that liquid biopsy could play a role in the early detection of mutations capable of inducing resistance to targeted therapies. Liquid biopsy is a recently described method that involves the analysis of genetic material from various sources, primarily blood (but also from urine, pleural fluid, and ascites). This method affords information on mutations and alterations in the copy number of genes related to the oncogenic process[16]. Several types of liquid biopsies are available, and the most widely used strategies involve the analysis of circulating tumor cells (CTC), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs) or exosomes, exhibiting both advantages and disadvantages[17]. In patients with mCRC, a high correlation has been noted between the primary metastatic tumor sample and ctDNA, approaching approximately 96.15% concordance for the analysis of *KRAS*, *NRAS*, and *BRAF*[18]. The objective of this review was to evaluate the role of liquid biopsy in the early identification of mutations that induce resistance to cetuximab or panitumumab therapy.

ADVANCES IN LIQUID BIOPSY DETECTION TECHNOLOGY

Liquid biopsy requires technology capable of extracting tumor genetic material (DNA or RNA) from the blood, along with a technique that can quantify and characterize the molecular sequence. Nucleic acids can be detected by polymerase chain reaction (PCR)-based techniques or next-generation sequencing (NGS)[19]. The advantages of PCR-based techniques include their lower cost, shorter processing time,

and easier bioinformatics analysis than NGS techniques[20]. Disadvantages of PCR techniques include the selection of a prior bound study target and the difficulty in examining rare genetic alterations[21].

Advances in PCR techniques have allowed the development of digital PCR and subsequent evolution toward more advanced technologies such as droplet digital PCR (ddPCR) and Beads, Emulsion, Amplification, Magnetics (BEAMing) digital PCR. Both technologies employ digital PCR principles, which involve sample division or partitioning, where each partition occurs *via* independent reactions. Subsequently, a digital system allows fluorescence quantification in each partition, and combining the value of each partition affords a final quantification of molecules of interest[22]. In ddPCR, sample reactions occur within water-in-oil droplets, which act as a system of encapsulated molecules, where millions of PCR reactions can be simultaneously quantified[22,23]. The BEAMing technique involves digital PCR in emulsions combined with flow cytometry to quantify DNA molecules. In emulsions, DNA molecules and primers are attached to magnetic beads. Subsequently, amplified fragments are recovered by magnets and recognized by flow cytometry to measure the DNA of interest[24].

NGS techniques are based on massively parallel sequencing of selected or unselected genes; thus, millions of DNA sequences can be read simultaneously[25]. One main advantage of NGS is its ability to detect new mutations or mutations that rarely appear[22]. In addition, NGS offers high sensitivity and specificity for mutation detection; however, it exhibits considerable variability, ranging from 0.1% to 1%, depending on the technique or platform used[26].

Using liquid biopsy, tumor DNA can be obtained from various sources, including ctDNA, CTC, and EV, found in the blood of patients with cancer. Cells normally release nucleotides into patient blood. This genetic material can be isolated and is known as cfDNA. ctDNA is a part of cfDNA derived from tumor cells and can harbor mutations, amplifications, and epigenetic modifications associated with cancer[27]. CTCs are rare tumor cells in the blood that originate from solid tumors or metastases. Enrichment processes allow the elimination of leukocytes from the blood and CTC selection to extract the genetic material to be investigated[28]. Finally, EVs or exosomes are vesicles in the blood and contain DNA, mRNA, or miRNA modulating receptor cells[29].

Advances in methods and technologies for attaining genetic material are expected to complement the limitations of tissue or metastasis biopsies to improve patient prognosis[30].

LIQUID BIOPSY FOR THE EXAMINING ANTI-EGFR RESISTANCE MUTATIONS

Frequency of appearance of resistance in the EGFR pathway

The EGFR receptor is a tyrosine kinase receptor, which, when ligand bound, activates the RAS, RAF, MEK, and ERK pathways[31]. The acquisition of activating mutations in any component of this pathway has been associated with oncogenesis[32]. Initial studies have focused on describing mutations in the *KRAS* oncogene in patients who relapsed following anti-EGFR therapy. Mutations in *KRAS*, a member of the small GTP-binding protein family, have been the focus of in-depth study, as the wt *KRAS* genotype is an indicator for anti-EGFR therapy. In a small number of patients with mCRC presenting disease progression, *de novo* mutations in *KRAS* measured by liquid biopsy[33] reached 38% (9/26). Reportedly, 40% of patients with mCRC exhibit *KRAS* mutations at diagnosis, most frequently in codons 12, 13, 61, and 146[34]. Mutations in codons 12 and 13 alter the position of the KRAS catalytic site at codon 61, reducing GTP hydrolysis and maintaining protein activity, even in the absence of a ligand[35,36]. These activating mutations can induce cellular proliferation and suppress apoptosis[34]. Numerous theories have been proposed to clarify how anti-EGFR antibodies allow the acquisition of resistance mutations. For example, cell culture studies have revealed that prolonged exposure to anti-EGFR treatment allows the survival and selection of clones harboring *KRAS* mutations[37,38]. In addition, it is postulated that *de novo* mutations in resistance genes can be generated by genomic instability in cancer [35]. Furthermore, it has been proposed that the same therapeutic drugs can induce mutagenesis[39]. For example, patient studies have revealed that anti-EGFR treatment can induce a distinctive mutational signature, SBS17b, with preferential mutations in *KRAS* Q61H[15], which is consistent with cell culture studies demonstrating anti-EGFR treatment-induced mutagenesis[40].

The acquisition of resistance mutations in *KRAS* is one of the most frequent mechanisms reported in liquid biopsy studies. In a small study, 4 of 11 patients with wt KRAS treated with anti-EGFR antibodies acquired *KRAS* mutations, as determined by ddPCR of ctDNA. In addition, mutations in other components of the EGFR pathway, such as *BRAF*, *MET*, and *ERBB2*, were detected in three patients[41]. These results were replicated in a study by Vitiello *et al*[18] (2019), in which 10 new *KRAS* mutations were identified by automated quantitative reverse-transcription PCR in the ctDNA of 30 mCRC patients with wt *KRAS* receiving anti-EGFR therapy. In a further study using the BEAMing method, analysis of ctDNA revealed that 7 of 34 patients with wt *KRAS*, who were treated with anti-EGFR, developed resistance mutations, mainly in KRAS codons 12, 13, and 61[42]. Similarly, a follow-up program using the same methodology showed that, among 31 patients with wt *KRAS* tumor tissue receiving anti-EGFR treatment, 5 presented mutations in *KRAS* and 3 in *NRAS*[43]. Furthermore, an analysis of 62 patients with mCRC treated with cetuximab or panitumumab revealed 27 resistance mutations in *KRAS* and 5 mutations in *EGFR* (detected in plasma); mutations in codons 12 and 61 of *KRAS* were the most

common. Interestingly, the authors reported that the longer EGFR inhibitors were discontinued, the more the allelic frequency of these mutations detected in plasma tended to decrease[44]. Finally, an NGS study of ctDNA demonstrated that 69% of 42 patients treated with anti-EGFR had mutations or amplifications in *KRAS*, with the *KRAS* Q61H mutation (exon 2) detected in 52% of patients. Extending the analysis to other elements of the EGFR pathway, 91% of patients showed alterations in several pathway components, such as *NRAS*, *BRAF*, *MAP2K1*, *ERBB2*, *MET*, and *KIT* mutations or extensions, with an average of five alterations *per* patient for these genes[45]. Mutations conferring resistance to anti-EGFR are frequent, specifically in *KRAS*/*NRAS*, estimated to account for approximately 30%–89% of patients with mCRC (Table 1).

Prognosis associated with the appearance of anti-EGFR resistance mutations

In addition, the prognostic utility of detecting resistance-acquired mutations during anti-EGFR therapy has been examined. Yamada *et al*[46] (2020) detected 20 acquired mutations in *RAS*, *BRAF*, or *EGFR* genes in ctDNA of 30 patients with mCRC treated with FOLFOX or FOLFIRI plus anti-EGFR. The authors reported that patients who developed measurable mutations in ctDNA had a worse prognosis for progression-free disease (PFS) than those with wt *RAS*. Follow-up analysis of patients with chemotherapy-refractory mCRC from the ASPECCT clinical trial[47] treated with panitumumab alone (conducted by liquid biopsy) revealed that 32% of 162 patients developed mutations in *RAS*. Mutations were found to primarily emerge in *KRAS* codons 2, 3, and 4 and less frequently in exon 2 of *NRAS*[48]. In contrast to previous studies, no significant differences were detected in patients with emerging *RAS* mutations in terms of PFS, overall survival (OS), or objective response rate. Subsequently, in the same cohort of patients, the authors found that the allelic frequency of resistance mutations in EGFR pathway genes, including *KRAS*, may be more closely associated with worse prognosis in panitumumab-treated patients[49]. These results are consistent with those of another study examining patients with wt *KRAS* CRC undergoing treatment with cetuximab or panitumumab; the emergence of mutations in *KRAS*, *NRAS*, or *BRAF* resulted in worse OS when compared with patients without mutations in these genes, as determined by analyzing CTC [hazard ratio (HR): 0.60, 95% confidence interval (CI): 0.40–0.91, P = 0.0028], but not when ctDNA liquid biopsy was used to analyze the same cohort (HR: 0.80, 95%CI: 0.59–1.33, P = 0.088)[50]. In summary, growing evidence indicates that the detection of mutations, as well as allelic frequency, can be linked to the prognosis of mCRC.

Importance of timing for anti-EGFR treatment and emergence of resistance mutations

It has been suggested that once disease progression is detected during anti-EGFR treatment, liquid biopsy can be used to evaluate the timing of reintroducing therapy[51]. This concept is known as rechallenge, whereby a period without treatment (such as anti-EGFR therapy) is followed by re-initiation of prior therapy, despite knowledge regarding the potential emergence of resistance mutations [8]. In a meta-analysis of patients who exhibited prior evidence of anti-EGFR benefits and rechallenge with anti-EGFR treatment (with a strategy of assessing *RAS* status by ctDNA liquid biopsy), up to 46% of patients converted from wt to mutant *RAS* following exposure to anti-EGFR treatment. Patients who maintained wt *RAS* before rechallenge had a better prognosis than those with a *de novo RAS* mutation [52]. Therefore, based on evidence suggesting a potential benefit in patients who maintain wt *RAS* prior to rechallenge, strategies have been proposed for patients who exhibit acquired resistance mutations in *RAS* following anti-EGFR treatment. Growing evidence indicates that resistance mutations decay over time after withdrawing anti-EGFR treatment; thus, withdrawing drug therapy eliminates the selective pressure on clones harboring resistance mutations[44]. An exploratory study of patients with wt *KRAS*/*BRAF* who acquired *RAS* or *EGFR* mutations during the course of anti-EGFR treatment showed that the frequency of mutant alleles decayed exponentially after discontinuing anti-EGFR treatment, with a mean of 4.4 mo[53]. In a retrospective cohort of 80 patients rechallenged after a longer interval, the authors reported a superior prognosis in terms of overall response[53]. Thus, considering the dynamics of the decay of clones with resistance mutations after treatment suspension, clinical studies have been proposed to corroborate the clinical utility of rechallenge therapies. For instance, it has been speculated that patients who previously progressed to chemotherapy and anti-EGFR antibodies could undergo second-line chemotherapy without anti-EGFR; if they progress, anti-EGFR rechallenge could then be performed based on *KRAS* allele frequency measurement[54]. This has been proposed in the REMARRY and PURSUIT phase II clinical trials; these studies suggested the reintroduction of FOLFIRI and panitumumab (which have an allelic frequency < 0.1% for mutated *KRAS*), allowing at least 4 mo without anti-EGFR administration[55]. Therefore, biopsies are not only useful for detecting resistance mutations, but could help determine the timing of treatment reintroduction once resistance-inducing mutations have declined.

Table 1 Frequency of acquired KRAS resistance mutations in patients with stage IV colorectal cancer treated with cetuximab or panitumumab

Ref.	*n* wt KRAS patients at baseline	Analysis technique	Mutations or amplifications in KRAS/NRAS	Most frequent mutations
Vitiello *et al*[18], 2019	30	ctDNA/RT-qPCR	10 (30%)	KRAS Q61x (4)
				KRAS G12x (3)
Diaz *et al*[33], 2012	24	ctDNA/BEAMing	9 (36%)	KRAS G12x (9)
Pietrantonio *et al*[41], 2017	11	ctDNA/ddPCR	4 (36%)	KRAS Q61H (2)
Vidal *et al*[42], 2017	18	ctDNA/BEAMing	7 (39%)	KRAS G12x (5)
				NRAS Q61x (3)
Morelli *et al*[44], 2015	62	ctDNA/BEAMing	27 (43%)	KRAS G12x (10)
				KRAS Q61x (9)
Strickler *et al*[45], 2018	42	ctDNA/NGS DNAseq	26 (62%)	KRAS Q61H (22)
				KRAS G12A (5)
Yamada *et al*[46], 2020	19	ctDNA/ddPCR	16 (84%)	KRAS Q61H (10)
				KRAS G12V (9)
Kim *et al*[48], 2018	164	ctDNA/NGS DNA seq	53 (32.3%)	KRAS exon 3 (A59x o Q61x) (20)
Takayama *et al*[75], 2018	25	ctDNA/ddPCR	9 (36%)	KRAS Q12S (5)
				KRAS Q12D (4)

ctDNA: Circulating tumor DNA; ddPCR: Droplet digital PCR; NGS: Next-generation sequencing; BEAMing: Beads, Emulsion, Amplification, Magnetics.

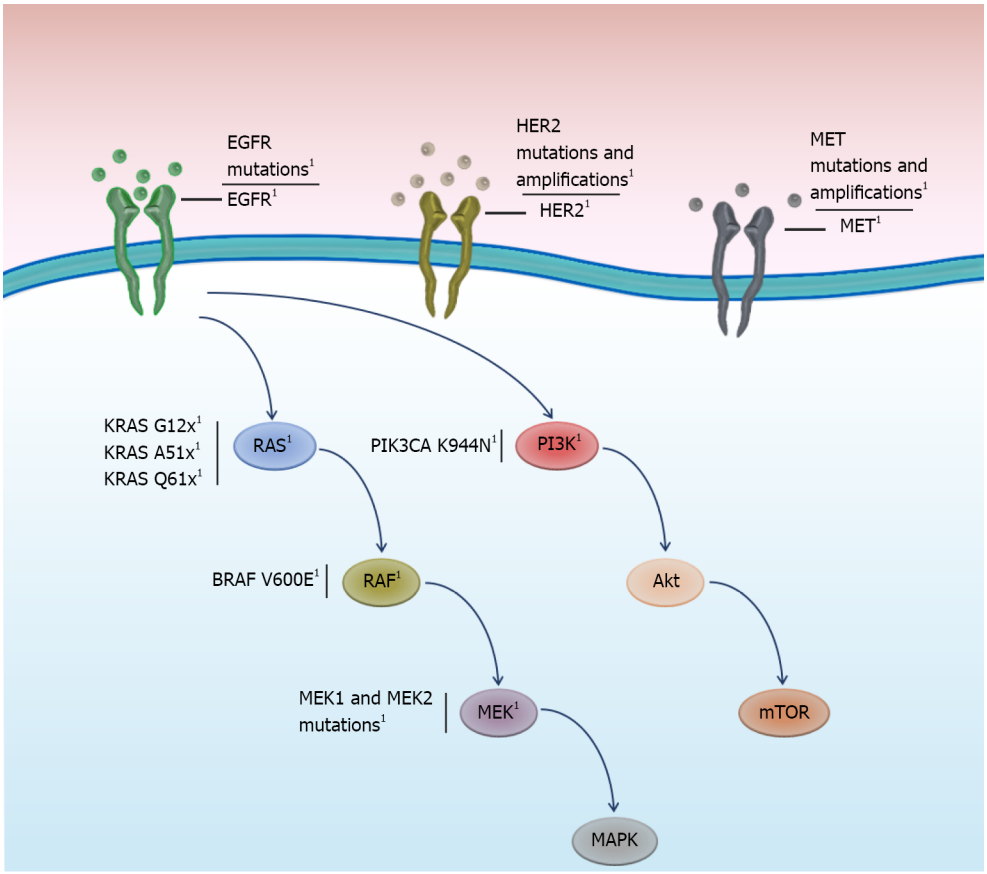
FUTURE PERSPECTIVES

Beyond KRAS/NRAS mutations

Resistance mutations to anti-EGFR treatment are frequent, particularly in *KRAS*, estimated to range between 30 and 89% (Table 1) in patients with mCRC. Although resistance mutations in *KRAS* are most frequent, mutations or amplification of other genes in the EGFR pathway, such as *ERBB2*, *MEK*, *BRAF*, and *MAP2K*, could also cause or contribute to anti-EGFR treatment resistance (Figure 1). Basic studies using patient-derived xenograft models, where the acquisition of natural resistance by chronic cetuximab exposure is reproduced, have reported the emergence of driver mutations in *EGFR*, *KRAS*, *MEK1*, and *MEK2*[56]. These results have been documented in real-world clinical settings, where patients were prospectively followed up by liquid biopsy. For instance, acquisition of *MET* amplification was frequent in wt *KRAS* mCRC (22.6%; 12/54 patients) that showed disease progression after anti-EGFR treatment, suggesting a possible mechanism of resistance[57]. Furthermore, a phase II clinical study proposed using a *MET* inhibitor to counteract the acquired resistance to anti-EGFR therapy. Tivantinib and cetuximab were administered to patients with histological evidence of *MET* overexpression. Although the combination did not afford superior benefit in patients, it was suggested that it might be more beneficial in patients with *MET* amplification[58]. Mutations acquired in PIK3CA (detected in ctDNA) could also induce resistance, based on analyzing a patient cohort with disease progression following cetuximab treatment[59]. A recent study suggested that the fusion of genes such as *FGFR2*, *FGFR3*, *RET*, *ALK*, *NTRK1*, and *ROS1* could emerge during anti-EGFR treatment; in particular, fusions involving *FGFR3* or *RET* could contribute to resistance to anti-EGFR therapy[60]. This finding allows the possibility of establishing liquid biopsy molecular panels to detect mutations causing resistance (beyond *KRAS*), which need to be validated in studies examining patients with mCRC undergoing anti-EGFR therapy.

ERBB2/HER2

HER2 is a tyrosine kinase receptor and member of the HER/ERBB receptor family that includes EGFR (HER1), HER3, and HER4[61]. HER2/ERBB2 activation induces cellular proliferation and activation of the RAS/RAF/ERK and PI3KCA/PTEN/AKT pathways[62]. Mutations or amplification of HER2/ERBB2 has been detected in various tumors. Although most HER2-based studies have primarily focused on breast cancer, the role of this receptor in mCRC has recently been described[63,64]. Previous



DOI: 10.4251/wjgo.v14.i9.1654

Figure 1 Main acquired resistance mutations detected by liquid biopsy. Key acquired resistance mutations are associated with the epidermal growth factor receptor (EGFR) pathway. Other mutations or amplifications in tyrosine kinase receptors, such as HER2/ERBB2 or MET, can potentially lead to resistance to anti-EGFR therapy. [1]Indicate acquired resistance mutations, as reported in previous studies. EGFR: Epidermal growth factor receptor.

in vitro and prospective patient studies have suggested that both the presence of mutations related to the active site of the receptor and *HER2* amplification are associated with a poor response to anti-EGFR therapy[62,63,65]. In addition, the acquisition of mutations in *HER2* may be an underlying mechanism of secondary resistance that can be detected early using liquid biopsy. In a liquid biopsy study, 1 of 11 patients who progressed on anti-EGFR treatment showed *HER2* amplification and simultaneous mutation of *KRAS*[41]. In a study evaluating ctDNA by NGS, one case of *HER2* amplification was identified in a series of 15 patients treated with cetuximab[66]. Nonetheless, a case-control study revealed that the presence of *HER2* amplification in patients with wt *KRAS* CRC (prospectively measured by ddPCR of ctDNA) was not associated with a worse prognosis when compared with those without *HER2* mutations. However, the number of cases of amplified *HER2* was markedly low (five cases) to establish meaningful conclusions[67]. A phase IB clinical study has proposed the use of neratinib (pan-ERBB kinase inhibitor) and cetuximab in patients who have progressed to anti-EGFR therapy[68]. This trial was based on the hypothesis that *HER2*-negative tumors acquire *HER2* amplification as a mechanism of resistance to anti-EGFR treatment; neratinib, an irreversible inhibitor of EFGR, HER2, and HER4, improved prognosis in this subgroup of patients[69]. Evidence of *HER2* amplification was reported in 6 of 16 patients (assayed by chromogenic immunohistochemistry of metastatic biopsies or by NGS in ctDNA). Importantly, combining cetuximab with 240 mg/day of neratinib was well-tolerated, with a low incidence of adverse side effects[68]. Overall, current evidence from clinical models regarding the detection of acquired mutations in *HER2/ERBB2* is at an early stage, although this gene represents an interesting potential therapeutic target in patients who develop *HER2* amplification during anti-EGFR treatment.

Toward liquid biopsy implementation in daily clinical practice

Liquid biopsies for monitoring anti-EGFR resistance mutations have not been performed in routine medical practice. Real-world studies on liquid biopsy programs indicate that the application of these techniques can effectively alter the management of patients with colon cancer[43]. However, implementing these programs can pose challenges, including the high cost associated with these methods (PCR-based or NGS) and the lack of reimbursement[70], lack of cut-off values for detecting mutations, and absence of monitoring protocols[71].

Therefore, it is necessary to establish protocols for the frequency of taking liquid biopsies, as well as their implications for clinical patient management. Clinical studies are currently being conducted to standardize the frequency of sampling and interpretation of results. Two prospective studies have attempted to establish the prognostic value of liquid biopsy protocols; both studies including periodic three-monthly ctDNA analyses and clinical follow-up in CRC wt *KRAS* patients exposed to 5-fluorouracil regimens plus anti-EGFR antibodies[72,73]. Finally, current international guidelines, such as ESMO, have concluded that although there is insufficient evidence to recommend follow-up with liquid biopsy, such analysis could be useful for detecting secondary resistance to anti-EFGR[4]. In contrast, the Japanese Society of Medical Oncology clinical guidelines recommend the use of liquid biopsy because of its usefulness in monitoring anti-EGFR therapy[74].

CONCLUSION

Based on current evidence, liquid biopsy could be developed as an innovative tool for managing patients with mCRC who receive anti-EGFR therapy. *De novo KRAS* mutations are one of the most commonly described mechanisms of acquired resistance and are associated with poor outcomes. However, establishing panels beyond *KRAS*, including genes related to the EGFR pathway, is crucial, given that such genes also potentially contribute to anti-EGFR resistance. Adequate strategies are needed to integrate liquid biopsy for the early detection of clinical progression of mCRC in patients undergoing anti-EGFR therapy. Future clinical studies will advance the routine use of liquid biopsy as a tool for reaching clinical decisions that benefit patients.

FOOTNOTES

Author contributions: Valenzuela G wrote the manuscript and created the figures and tables; Burotto M, Marcelain K, and González-Montero J performed data collection and literature review and critically revised the manuscript; All authors have approved the final version of the manuscript for publication.

Supported by Agencia Nacional de Investigación y Desarrollo de Chile, Fondo Nacional de Investigación en Salud (FONIS), No. SA20I0059.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: A
P-Editor: Fan JR

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1665-1674

DOI: 10.4251/wjgo.v14.i9.1665

ISSN 1948-5204 (online)

MINIREVIEWS

Implication of gut microbiome in immunotherapy for colorectal cancer

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Manojlovic N, Serbia; Popovic LS, Serbia

Received: March 20, 2022
Peer-review started: March 20, 2022
First decision: April 17, 2022
Revised: May 9, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

Colorectal cancer (CRC) constitutes the third most frequently reported malignancy in the male population and the second most common in women in the last two decades. Colon carcinogenesis is a complex, multifactorial event, resulting from genetic and epigenetic aberrations, the impact of environmental factors, as well as the disturbance of the gut microbial ecosystem. The relationship between the intestinal microbiome and carcinogenesis was relatively undervalued in the last decade. However, its remarkable effect on metabolic and immune functions on the host has been in the spotlight as of recent years. There is a strong relationship between gut microbiome dysbiosis, bowel pathogenicity and responsiveness to anti-cancer treatment; including immunotherapy. Modifications of bacteriome consistency are closely associated with the immunologic response to immunotherapeutic agents. This condition that implies the necessity of gut microbiome manipulation. Thus, creatingan optimal response for CRC patients to immunotherapeutic agents. In this paper, we will review the current literature

observing how gut microbiota influence the response of immunotherapy on CRC patients.

Key Words: Colorectal cancer; Gut microbiome; Immunotherapy; Checkpoint inhibitors; Tumor microenvironment

Core Tip: Colorectal cancer (CRC) constitutes the third most frequent malignancy. CRC is a complex, multistep process. The impact of environmental factors as well as the disturbance of the gut microbial ecosystem is associated with CRC development. There is a strong relationship between the gut microbiome and resistance to immunotherapy.

Citation: Koustas E, Trifylli EM, Sarantis P, Papadopoulos N, Aloizos G, Tsagarakis A, Damaskos C, Garmpis N, Garmpi A, Papavassiliou AG, Karamouzis MV. Implication of gut microbiome in immunotherapy for colorectal cancer. *World J Gastrointest Oncol* 2022; 14(9): 1665-1674
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1665.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1665

INTRODUCTION

Colorectal cancer (CRC) constitutes the third most frequently reported malignancy in the male population and the second most common in women in the last several decades, based off GLOBACAN epidemiological data[1]. Colon carcinogenesis is a complex, multifactorial event composed of genetic and epigenetic aberrations, which additionally causes the disturbance of gut homeostasis resulting from gut microbiota modifications[2]. The microbiome constitutes a multiplex ecosystem of microorganisms located in the gastrointestinal tract of many species, including humans[3].

The relationship between the intestinal microbiome and disease development, including carcinogenesis, was relatively undervalued in the last decade. However, the interrelation of gut microbiota with the main functions of the host has recently been in the spotlight[4]. The digestive tract contains the largest amount of microbiota colonization among other anatomical regions, accounting for approximately 70% of the human microbiota make-up[5], including viral and bacterial microorganisms, archaea and fungi[6,7]. The proximal parts of the GI tract, including the stomach and small intestine, present few microbiota species whereas the distal part, the colon, presents the largest number of species (microorganisms) in the colonic substance[7]. The six main phyla of the gut microbiome (90% of the population) include[8]: Bacteroidetes, Actinobacteria, Firmicutes, Proteobacteria, Verrucomicrobia, and Euryachaeota[9]. Of all the genera found in the human gut, Bacteroides makes up the majority of the population (30%)[10], implying its significant effecton the human functional system. Additionally, many genera from the Firmicutes phylum compose a high amount of the intestinal substance, such as lactobacillus, Clostridium, Faecalibacterium, Eubacterium and Ruminococcus[11]. The application of metagenomics on fecal specimens has given the opportunity for microbiome quantification and analysis, and potentially its use as a potent diagnostic tool[12].

LITERATURE SEARCH

PubMed was searched to identify studies on gut microbiome, immunotherapy and CRC. PubMed and *Reference Citation Analysis* (https://www.referencecitationanalysis.com/) were searched to identify studies on gut microbiome, immunotherapy and CRC. The literature review was completed on February 28, 2022. The following search terms were applied: "Colorectal cancer", "Immunotherapy", "Checkpoint inhibitors," "Tumor microenvironment," and "Gut microbiome". The reference lists of all related articles were screened for other potentially relevant studies. The search citation analysis is presented in the reference list. Finally, the authors similarly reviewed the reference lists of eligible articles to identify further eligible articles, books and other forms of publication. Publications that are written in any other language other than English were excluded. Publications of abstracts were also excluded.

THE FUNCTIONAL ROLE OF THE GUT MICROBIOME

Gut microbiota exhibits diverse functions in the human organism and are responsible for many metabolic processes and biosynthesis. Vitamin synthesis constitutes one of the key roles of gut microbiota, such as riboflavin, vitamin B1, biotin, vitamin K and cobalamin[13]. They also have a crucial role in non-digestible carbohydrate metabolism; to transform them into short-chain fatty acids (SCFAs), such as butyric acid, acetic acid and propionic[14], which are produced by the main phyla of bacteriome, this includes Bacteroidetes and Firmicutes[15]. Alteration of the above metabolic process leads to modification of the fatty acid production and overall metabolic imbalance[16]. Along with their involvement in vitamin and short fatty acids synthesis, they take part in bile acid production[17]. Neuromodulators are also produced by gut microbiota, with a significant implication for the gut-brain axis, which includes the peripheral and central nervous systems as well as the enteric nervous system [18]. Many neurological and psychiatric disorders are closely connected with the gut microbiome. This can occur because they are responsible for synthesizing many pro-inflammatory cytokines, amyloids and liposaccharides[18]. Based on metagenomics, genome disturbance and dysbiotic flora can cause a predisposition to develop a number of malignancies[19], including non-neoplastic disorders, such as atopy, functional intestinal disturbances, like irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and metabolic syndrome[20,21].

There is a strong relationship between gut microbiome dysbiosis and bowel pathogenicity. In the case of the bowel, functional disorders such as IBS have many studies illustrating an altered consistency of the bacteriome, with both an increase or decrease in the quantity of many bacteria. It is specifically observed as an aberrant increase of *Ruminococcus, Firmicutes*, and *Clostridium spp*. with an abnormal decrease of *Ruminococcusalbus* and *callidus, Bacteroidesfragilis* and *bulgatus*[18]. Additionally, the overproduction of SCFAs that deregulate the secretion of serotonin from the enteroendocrine cells leads to increased bowel movements and fermentation. This causes the symptomatology associated with meteorism[22]. Patients who suffer from organic bowel diseases, such as IBD, Ulcerative colitis and Crohn's disease (CD) have been observed to have an altered microbiome. The modification of the gut microbiome is closely associated to dietary habits[23]. Patients with CD specifically demonstrate increased amounts of *Neisseria caeacorrodens*, *E. coli* and proteobacteria[24], while enhanced amounts of fungal species such as *Candida albicans*, *Cyberlindnera jadinii* and *Saccharomyces cerevisiae*can also be observed[25]. In addition, a decreased number of some bacterial taxa, such as Firmicutes, *Faecalibacterium prausnitzii*, Bacteroidetes and Roseburia, is observed[26]. Dietary habits that include a high amount of fruit and vegetable consumption can lower the risk for developing CD[27].

Intestinal epithelial cell sare closely interrelated with the immune system *via* the existence of goblet and Paneth cells and their products. Goblet cells are located in intestinal mucosa and have a crucial role in producing mucus. Paneth cells are located in the crypts of Lieberkühn, secreting various immunomodulatory peptides with antimicrobial qualities[28]. Moreover, bacterial metabolites also take place in immune responses *via* the production of SCFAs and are closely associated with innate immunity and antibody production[29].

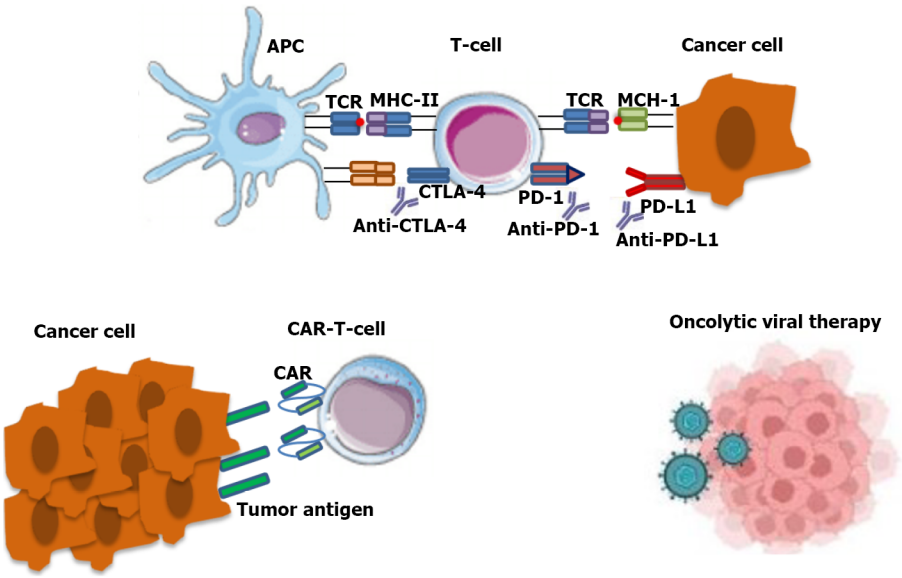
Immunotherapy constitutes a significant therapeutic option, including immune checkpoint inhibitors, cancer vaccines and chimeric antigen receptor-T cells[30]. This treatment modality makes use of the immune responses to create an anti-neoplastic effect. The main therapeutic agents include the following monoclonal antibodies: (1) Anti-cytotoxic TT-lymphocyte antigen-4 (anti-CTLA-4); (2) Anti-programmed cell death 1 ligand 1 (anti-PD-L1); and (3) Anti-programmed cell death protein 1 (anti-PD-1)[28,31]. The principal advantage of immunotherapeutic agents includes their aimed action on malignant cells appears in Figure 1.

This therapeutic modality is currently selected as an anti-cancer treatment specifically in cases of tumors that are characterized by high microsatellite instability (MSI-H)[32]. Tumors that present MSI-H arise from a defective DNA mismatch repair (MMR) mechanism that leads to the accumulation of genetic mutations. This can be seen in the case of mutant MSH2, PMS2, MSH6 and MLH1. Or by epigenetic aberration, such as genome hyper-methylation[33]. There are many reports that gut microbiota influences the response to anti-cancer treatment including immunotherapy[34]. It is observed that a significant number of CRC patients that lacked a specific taxa in their bacteriome, presented a limited response to immunotherapy agents such as anti-PD1.This condition implies the use for more personalized anti-cancer treatments that can prove to be potent. In this paper, we review the current literature on how gut microbiota influences the response of CRC patients to immunotherapy[35].

THE ROLE OF MICROBIOME IN COLON CARCINOGENESIS

There are many studies about the implication of gut microbiota in immunotherapeutic agents including immune checkpoint inhibitors for melanoma. Fewer studies exist about its role in CRC treatment management.

Modifications in the gut microbiome and microbial metabolites have been involved in many pathological processes and diseases, including colon carcinogenesis[36]. Many intestinal bacterial



DOI: 10.4251/wjgo.v14.i9.1665

Figure 1 Mechanism of action of both anti anti-cytotoxic TT-lymphocyte antigen-4 and anti-programmed cell death protein 1/programmed cell death 1 ligand 1 check point inhibitors. In the tumor microenvironment, antigen-presenting cells (APCs), such as dendritic cells processed specific tumor peptides (TAAs) and complexed them to major histocompatibility complex (MHC) molecules. Then, APC migrated to T cell-dependent areas of tumor presented TAA to naïve or quiescent T cells. Checkpoint inhibitor, such as anti-programmed cell death protein 1 (anti-PD-1)/anti-programmed cell death 1 ligand 1 (anti-PD-L1) and/oranti-cytotoxic TT-lymphocyte antigen-4 (anti-CTLA-4) on tumor cells, lead to re-activation of immune responses. The anti-PD-1 or anti-PD-L1 blocking by monoclonal antibodies (as nivolumab, pembrolizumab for PD-1 or atezolizumab for PD-L1) ipilimumab restore CD28 pro-activity signaling and restore effective anti-tumor T lymphocyte responses. The anti-CTLA-4 blocking by monoclonal antibodies as ipilimumab restore CD28 pro-activity signaling and result in effective anti-tumor T lymphocyte responses. The binding of PD-L1 to PD-1 and CTLA-4 to B7 keeps T cells from killing tumor cells in the body. Blocking the binding with an immune checkpoint inhibitor allows the T cells to kill tumor cells (upper panel).Chimeric antigen receptor (CAR) T cells are T cells that have been genetically engineered to produce an artificial T cell receptor for use in immunotherapy. CARs are receptor proteins that have been engineered to give T cells the new ability to target a specific protein (lower panel).

products have been implicated in malignant states in the intestinal tract[37]. Several studies demonstrate the presence of an altered microbiome either in CRC patients' fecal specimens or in malignant tissues compared to healthy patients[38]. These alterations in the microbiome which take place in the initial steps of CRC development can be utilized as predictive biomarkers as well as microbial diagnostic gene markers. This can be utilized in patients with an increased risk of developing colon adenomas that can potentially lead to CRC[39].

Environmental factors have a high influence on the gut microbiome along with idiosyncratic factors [40] which subsequently induce carcinogenesis and CRC development *via* the overgrowth of particular microbial species in the flora[41]. The formulation of colonic microbial substances is closely related to modifiable factors such as eating behavior and style of living[42]. While there is a key role in the metabolism of nutrients[43], there is also a diversity of environmental risk factors that are associated with colorectal carcinogenesis such as obesity, tobacco use, alcohol consumption and prepared meat products[44].

Many studies demonstrate the implication of specific bacterial taxa in carcinogenesis, such as *Enterococcus faecalis, Helicobacter hepaticus*, *Bacteroides fragilis* and *Fusobacterium nucleatum*. The products of the previously mentioned microbes lead to genomic alterations[45]. While in the case of the *Fusobacterium nucleatum*, the carcinogenesis indirectly occurs *via* the perpetual secretion of pro-inflammatory cytokines [46]. This phenomenon implies the close interrelation of the microbiome with immune response and metabolic processes[47].

There is a notable reduction of genera from the Firmicutes phylum, which produce a significant metabolite, the alleged butyrate. An enhanced reproduction of specific phyla, such as *Bacteroides fragilis, Peptostreptococcus stomatis* as well as *Tarvi monas micra, Fusobacterium nucleatum*[48] and *Solobacterium moorei*[49]. Additionally, there are reports that show an increased amount of *Enterococcus*, *Escherichia coli*, *Klebsiella* and *Streptococcus,* as well as a decrease in *Rothia*[2].

There is considerable evidence that CRC development is closely associated with the presence of Fusobacteriaceae family members, such as *Fusobacterium nucleatum*, *necrophorum* and *mortiferum*[37] *via* a mechanism that was reportedly observed in mice[50].

Generally, dysbiosis which includes the modification of microbial taxa in the gut ecosystem leads either to a limited variety of microbiota or the overgrowth of microbes. This can further lead to the development of opportunistic infections[51], destruction of the intestinal epithelial barrier, bacterial translocation to the mesenteric lymph nodes or the circulatory system, ultimately leading to a local and

systemic inflammatory response[52].

Recruitment of T lymphocytes is observed in CRC malignant tissues[53] *via* the secretion of chemotactic cytokines. This is further related to an abundance in proteobacteria Ruminococcaceae, *B. fragilis* and *E.coli*. Alternatively, a high number of Fusobacteriais is associated with a dismal prognosis. In *in vitro* it has been observed to express an increased number of recruited T cells and inflammatory modulators [interleukin (IL)-6, IL-8, IL-1][54], an inhibitory effect on Natural killer cells, as well as tumor-infiltrating lymphocytes[55]. Although *Fusobacterium nucleatum* is normally associated with a worse prognosis, it constitutes a promoter for differentiation in regulatory T cells leading to a decrease in expression of scurfin or forkheadbox P3 which is correlated to prolonged survival[56].

IMMUNOTHERAPY IN CRC

The therapeutic management of CRC is considered quite challenging due to the complex molecular basis including genetic and epigenetic alterations[57]. In recent years, immunotherapeutic agents are utilized for tumors that present high MSI-H which results from a defective DNA MMR or epigenetic modification[33]. An epigenetic aberration is genome hyper-methylation in addition tomutant genes such as PMS2, MLH1 as well as MSH2 and MSH6[58]. In the case of MSI-H colorectal tumors, there is evident methylation of CpG islands in the promoter of the BRAF proto-oncogene[59]. It is observed that patients with BRAF and RAS genetic mutations present resistance to immunotherapeutic treatments with a limited enhancement of survival[60]. It can occur in cases of epidermal growth factor receptor inhibitors, like cetuximab, as well as Panitumumab[61]. In comparison with MSI tumors, the microsatellite stable tumors present a more aggressive phenotype and poor prognosis[62]. Immunotherapeutic agents, such as pembrolizumab are commonly used in cases of chemo-resistant advanced colorectal malignant tumors despite the existence or lack of either MMR or MSI-H based off the KEYNOTE 028 clinical trial[63]. For tumors with MMR phenotype, the utilization of nivolumab alone or with ipilimumab is highly recommended[47]. The administration of cancer vaccines in CRC is still under study and it is limited solely to cases of end-stage CRC[64]. Talimogene laherparepvec vaccine uses Herpes virus type-1 as a vector which targets the *GM-CSF* gene. The combination of systemic use of atezolizumab (anti-PD-L1 immunotherapeutic agent) with the above vaccine is currently under assessment for tumors with microsatellite stability[63] or as a monotherapy in secondary liver cancer [65].

Tumor microenvironment and microbiome in CRC

Tumor microenvironment (TME) includes multiple types of cells, such as fibroblasts, immune cells, endothelial and stromal cells[66]. TME demonstrates a significant role in immune responses, particularly in CRC, and constitutes as a therapeutic target for many anti-cancer agents[67]. The stroma around the tumor has a key role in resistance to chemotherapy due to the fact that it includes a heterogeneous population of cells with various levels of differentiation. This contributes to invasive tumor behavior and dissemination. This is shown in the case of tumor-associated macrophages and cancer-associated fibroblasts. Both of these are related to a dismal prognosis and neoangiogenesis[68,69], as well as Myeloid-derived suppressor cells which are also implicated in tumor progression and invasion. Their effect is under the regulation of tumoral products like chemokine (C-C motif) ligand 2 and 5 (CCL2 and CCL5)[70].

It was previously stated that the gut microbiota exhibited various effects on the differentiation mechanism and tumor development. While they influence the tumor response to immunotherapeutics [71], the existence of intra-tumoral bacteria is reported in many solid tumors, especially in breast cancer. It was demonstrated that the microbiome is particular for each kind of malignant tumor presenting distinct metabolic functions[72]. Based on data that was collected by whole-transcriptome analysis, there is a distinct microbiome correlated with different malignant tumors, implying a specific microbial profile for each type of cancer[73]. Additionally, TME has a crucial role in the existence and multiplication of intra-tumoral bacteria[74]. Many studies illustrate the close relationship between immunotherapy and gut microbiota, and their implication in the anti-tumor mechanism such as immune-checkpoint inhibitors[72].

THE IMPLICATION OF GUT MICROBIOME IN IMMUNOTHERAPY

Resistance to immunotherapy is difficult to overcome in clinical practice[31]. Manipulation of gut microbiota constitutes a promising method for reducing the resistance to therapeutic agents. This is implied by the notable effect of intestinal microbial products on the malignant tumor where they could also be considered cancer-driving molecules[75].

Experimental studies on mice have shown that bacteria have a crucial role in the anti-cancer immune response. While the response was limited in the case of germ-free mice[28], it was primarily reported that intestinal microbiota have a significant role in the response especially to immune checkpoint

inhibitors. However, the previous observation was also demonstrated in humans when an immune checkpoint blockade was applied[28]. In mouse-model studies, fecal microbial transplantation (FMT) from mice that presented immune-responsive microbiota, to germ-free mice, provided a better anti-neoplastic response and tumor growth management. This result is associated with an increased amount of cytotoxic T lymphocytes (CD8+) in TME[76]. Whereas the transfer of fecal samples, including microbiota prone for carcinogenesis, provides the opposite results to physiological mice[77]. However, the correlation of the anti-tumor response with external factors must be taken into consideration.

Alterations in the consistency of bacteriome were reported in cases of patients with an active response to PD-1 inhibitors. More specifically, these patients presented a higher amount of *Enterococcus faecium*, *Bifidobacterium longum* and *Collinsella aerofaciens*. Fecal specimens that presented the above microbial taxa were characterized as "responder" stool samples and were transferred *via* FMT to germ-free mice. Subsequently, the germ-free mice started to express the stool phenotype of the responders[28].

Based on various human and animal-model cohort studies, intestinal microbiota could not only have been beneficial but also toxic effects on immune checkpoint inhibition[78]. Reduced toxicity was observed in specimens where Bacteroidetes genera were in abundance. Although they relate to unresponsiveness to immune checkpoint inhibitors (ICIs), in contrast to Firmicutes, and especially in the case of Ruminococcaceae, they were not only responsive to ICIs but also presented toxic effects. In cases of overgrown *Faecalibacterium prausnitzii*, patients had an increased risk of presenting colitis related with CTLA-4 inhibitors[79,80].

Manipulation of intestinal microbiota for immunotherapy-response improvement

Based on all the characteristics of the intestinal microbiota, they can either promote the anti-neoplastic response or induce inflammation and carcinogenesis[81]. A reduced anti-cancer response in the host was observed in germ-free mice or with antibiotic administration (broad-spectrum)[28,35]. In cases with urinary tract malignancies and lung cancer, antibiotics had a harmful effect on anti-PD1/PD-L1 treatment[35] in comparison to cyclophosphamide which presented a promoting effect on the overgrowth of *Barnesiella intestine hominis* in the intestinal tract and a stimulatory effect on anti-cancer immune response[82].

However, the manipulation of microbiota and utilization of antibiotics for the killing of bacteria is detrimental to the response to immunotherapeutic agents. This method includes the risk of killing favorable bacterial species. To avoid the non-elective effect of antibiotics, bacteriophage therapy is administered which permits a selective elimination of unfavorable bacteria[83].

Lastly, environmental and lifestyle habits could potentially alter the gut microbiome. These include physical exercise, proper dietary habits, sleep patterns, as well as *via* the utilization of FMT[84]. Bacterio-therapy or FMT includes the transferring of beneficial bacterial species such as Bacteroides, Bifidobacteria, *E. hirae* and *Akkermansia mucini philia*[85].

CONCLUSION

The relationship between the intestinal microbiome and disease development, such as carcinogenesis, was underestimated in the last decades. Nevertheless, the crucial role of intestinal microbiota has been in the spotlight as of recent years. Not only for their significant influence on the main metabolic functions of the host but also on the immune and anti-tumor responses. Immunotherapeutic agents are commonly used specifically for cases with chemo-resistant advanced colorectal malignant tumors. The implication of gut microbiota in the anti-cancer immune response is still under research. However, there are many reports supporting that the lack of specific bacterial taxa in CRC patients leads to a limited response to immunotherapy or complete unresponsiveness with the presence of specific phyla that could promote the anti-cancer response. Based on various human and animal-model cohort studies, intestinal microbiota could not only have beneficial effects on immune checkpoint inhibition but also have detrimental effects. The aforementioned phenomenon illustrates the necessity for the manipulation of intestinal microbiota. Specifically for the highest anti-neoplastic immune response, either *via* bacteriophage therapy or lifestyle habits modifications as well as FMT. Further research regarding the implication of gut microbiome on immunotherapy responses is needed for the identification of additional druggable targets, along with the manipulation of intestinal microbiota to achieve an optimal therapeutic response personalized for each patient.

FOOTNOTES

Author contributions: All authors participated in the writing and editing of the manuscript.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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S-Editor: Fan JR
L-Editor: Filipodia
P-Editor: Fan JR

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1675-1688

DOI: 10.4251/wjgo.v14.i9.1675

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Basic Study

Potential of six-transmembrane epithelial antigen of the prostate 4 as a prognostic marker for colorectal cancer

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kołat D, Poland; Mohamed SY, Egypt

Received: January 18, 2022
Peer-review started: January 18, 2022
First decision: March 8, 2022
Revised: March 23, 2022
Accepted: August 6, 2022
Article in press: August 6, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Immune cells play a role in the regulation of tumor cell behavior, and accumulating evidence supports their significance in predicting outcomes and therapeutic efficacy in colorectal cancers (CRC). Human six-transmembrane epithelial antigen of the prostate (STEAP) proteins have been recognized and utilized as promising targets for cell- and antibody-based immunotherapy. One STEAP family member, STEAP4, is expected to be an attractive biomarker for the immunotherapy of prostate and breast cancer. However, the immunotherapeutic role of STEAP4 for colorectal carcinomas has not been demonstrated.

AIM

To explore the expression pattern of STEAPs in CRC and their relationship with immune infiltration, and investigate the potential utilization of STEAPs as novel prognostic indicators in colorectal carcinomas.

METHODS

The expression level of STEAPs in CRC was evaluated using various open-resource databases and online tools to explore the expression characteristics and prognostic significance of STEAPs, as well as their correlation with immune-related biomarkers, such as immune infiltration. Immunohistochemical (IHC) experiments were subsequently performed to verify the database conclusions.

RESULTS

The levels of STEAPs in CRC were inconsistent. The expression of STEAPs 1-3 in CRC was not significantly different from that in normal tissues. However,

STEAP4 mRNA levels were significantly lower in CRC than in normal tissue and were positively correlated with immune-related biomarkers, such as immune cell infiltration, immune stimulation, major histocompatibility complex levels, and chemokines. Interestingly, the expression of STEAP4 in microsatellite instability-high CRC subtype was higher than that in microsatellite stability subtype. IHC staining was performed on colon cancer tissue samples and showed that high expression of STEAP4 in adjacent tissues positively correlated with immune-related biomarkers, including MLH1, MLH6, and PMS2, but negatively correlated with programmed death ligand 1, to varying degrees.

CONCLUSION

Our results provide an analysis of the expression of STEAP family members in CRC. Among different STEAP family members, STEAP4 plays a different role in CRC compared to STEAPs 1-3. In CRC, STEAP4 expression is not only lower than that in normal tissues, but it is also positively correlated with immune infiltration and immune-related biomarkers. These findings suggest that STEAP4 may be a potential biomarker for predicting CRC immune infiltration status.

Key Words: Six-transmembrane epithelial antigen of the prostate; Colorectal cancer; Immunotherapy; Target; Survival

Core Tip: This study analyzed the expression levels of six-transmembrane epithelial antigen of the prostate (STEAP) family members in colorectal cancer (CRC) and explored the potential biological function of STEAP4. It was found that the expression of STEAP4 in CRC tissues had a positive correlation with immune infiltration and immune-related biomarkers, such as MLH1, MLH6, and PMS2, and a negative correlation with programmed death ligand 1. STEAP4 is expected to be a novel and potential prognostic biomarker for CRC.

Citation: Fang ZX, Li CL, Chen WJ, Wu HT, Liu J. Potential of six-transmembrane epithelial antigen of the prostate 4 as a prognostic marker for colorectal cancer. *World J Gastrointest Oncol* 2022; 14(9): 1675-1688
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1675.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1675

INTRODUCTION

Colorectal cancer (CRC) ranks third in incidence and second in cancer mortality among malignant tumors worldwide[1]. Currently, large changes in lifestyle and dietary habits are thought to have contributed to the increased incidence and mortality of CRC. For example, in China, the annual average increase of new CRC cases was estimated to be 4.2%[2]. Although gender and regional differences are considered as the prognostic factors for patients with CRC[3], the etiology of CRC oncogenesis and development is still complex and unclear.

With the development of standardized treatment for patients with CRC, the prognosis of CRC has greatly improved. However, the control of progression of metastatic disease is still intractable. Immune cells play an important role in the regulation of tumor cell behavior, and accumulating evidence supports their significance in predicting outcomes and therapeutic efficacy in CRC patients[4]. In this regard, attention is currently being paid to the immune microenvironment and immunotherapy of CRC, mainly focusing on T cells and therapeutic response as related to promising treatment strategies[5].

Immunotherapy serves as an alternative treatment for cancer patients, especially for those whose tumors overexpress antigens recognized by immune cells. The human six-transmembrane epithelial antigen of the prostate (STEAP) family of proteins belongs to a class of cellular transmembrane proteins and has been used to derive epitope peptides that stimulate T lymphocytes in patients with renal cell or bladder cancer[6]. Importantly, STEAPs are present at the intercellular junctions of the prostate secretory epithelium, and are overexpressed in prostate cancer, serving as attractive targets for prostate cancer immunotherapy[7].

Although STEAPs have been reported to be overexpressed in CRC[8-11], research on STEAPs in CRC remains limited and the immunotherapeutic role of STEAPs in colorectal carcinomas has not been shown. This research investigated the biological function of STEAPs in CRCs, in addition to the relationship between STEAPs and immune infiltration, and demonstrated the potential of STEAPs to serve as novel and prognostic biomarkers for immunotherapy in colorectal carcinomas.

MATERIALS AND METHODS

Patient information and ethics statement

A tissue microarray with 87 matched primary CRC tissues and their corresponding adjacent normal colorectal tissue samples, and six extra samples of cancer cases without the corresponding paracancerous tissue were purchased from the Shanghai OUTDO Biotech Company, Shanghai, China (XT17-025, HColA180Su18). Pathological type was classified according to the prognostic degree of cancers. This study was approved by the Ethics Committee of Shantou University Medical College.

Comparison of STEAP expression in normal and cancerous tissues

TCGA datasets were used to evaluate the expression of STEAPs in normal and different cancerous tissues through the Tumor IMmune Estimation Resource (TIMER2.0) online source (http://timer.cistrome.org/)[12]. The UCSC Xena database (https://genome-cancer.ucsc.edu/)[13] was applied to analyze STEAP expression differences in colon adenocarcinomas (COAD) and rectal adenocarcinomas (READ) and related normal tissues. Regarding the different subtypes of CRC, namely, microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), and microsatellite stable (MSS)[14], MSI is a biomarker for response to immune checkpoint inhibitors (ICIs); high disease control rates and good progression-free survival were observed in patients with MSI-H CRC[15]. MSI-L tumors are phenotypically indistinguishable from MSS tumors, and the biological significance of MSI-L is unclear[16], so emphasis has been placed on MSI-H and MSS. The expression of STEAPs in MSI-H and MSS was also evaluated in the GEPIA2 database (http://gepia2.cancer-pku.cn/)[17].

Relationship between STEAPs and immune infiltration in CRC

Immune cells involved in CRC development were evaluated using the TIMER2.0 database to predict the association between the expression of STEAPs and the abundance of immune cells, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells, in the tumor microenvironment. In terms of immune characteristics of lymphocytes, immunostimulants, major histocompatibility complex (MHC), and chemokines, TISIDB, an integrated repository portal for tumor-immune system interactions (http://cis.hku.hk/TISIDB/)[18], was applied to explore the potential function of STEAPs in CRC immune infiltration. After being downloaded from the TISIDB, the dataset was analyzed and drawn with matrix2png (https://matrix2png.msl.ubc.ca/bin/matrix2png.cgi), an online mapping program[19].

Immunohistochemical staining

The immunohistochemical (IHC) staining for STEAP4 in tissue microarrays was conducted as described before[20]. The tissue microarray slide was dewaxed in xylene, hydrated in graded alcohols, and processed with 2% ethylenediamine tetraacetic acid antigen-repair solution (Fuzhou Maixin Biotechnology Development Co. LTD, Fuzhou, China) by microwave heating for epitope retrieval. After blocking endogenous peroxidase with 3% H_2O_2, the slide was incubated with anti-STEAP4 antibody (dilution: 1:1000, Proteintech 11944-AP) at 4 °C overnight. Stained tissues with DAB reagent were mounted and underwent nuclear counterstaining with hematoxylin for visualization.

Sections were visualized under a bright-field microscope (Axio Imager A2, Zeiss, Germany) and evaluated independently by two investigators with no prior knowledge of the CRC patient information. For tissue expression of STEAP4, staining intensity was scored as 0, 1, 2, and 3 for colorless, light yellow, brown yellow, and dark brown, respectively, while the percentage of positive cells, equaling 0%, 1%-25%, 26%-50%, 51%-75% and 76%-100%, was scored as 0, 1, 2, 3, and 4 points, respectively. The final staining score for STEAP4 expression was calculated as the sum of staining intensity and the percentage of positive cells, and divided into low expression (scores 0-4) and high expression (scores 5-7) groups. The expression levels of MLH1/2/6, PMS2, and programmed death ligand 1 (PDL1) were included in the patient information and the cutoffs were described before[21].

Statistical and survival analyses

SPSS 25.0 statistical software was used to analyze the results. Enumerated data are recorded as the number of cases (n = 93), and the relationship between STEAP4 and the clinicopathological parameters of CRC patients was analyzed by the χ^2 and Fisher's exact probability tests. The relationship between expression of STEAP4 in CRC and adjacent normal tissues (n = 87 cases) was examined by the χ^2 test. Likewise, the correlation between highly expressed STEAP4 in CRC and the immune-related factors MLH1, MLH2, MLH6, PMS2, and PDL1 was determined by the χ^2 test. To investigate the prognostic value of STEAP4 in CRC patients, the Kaplan-Meier survival curve and log-rank test were used to evaluate the association of STEAP4 expression with CRC patient prognosis by using SPSS 25.0 software. The difference was considered statistically significant at $P < 0.05$.

Table 1 Expression of six-transmembrane epithelial antigen of the prostate 4 in colorectal cancer and normal tissues

	Case (*n*)	STEAP4		χ^2	*P* value
		Low	High		
Tumor	87	42 (48.3%)	45 (51.7%)	8.866	0.003[a]
Normal	87	23 (26.4%)	64 (73.6%)		

[a]$P < 0.05$.

STEAP4: Six-transmembrane epithelial antigen of the prostate 4.

RESULTS

Expression of STEAPs in different types of malignant tumors

To determine the expression pattern of STEAPs in different types of malignant tumors, TIMER2.0 was used to analyze the difference between normal and cancerous tissues in TCGA. All STEAPs were found to be expressed at low levels in breast invasive carcinoma and kidney chromophobe compared to corresponding normal tissues, while in other types of malignancies, the expression patterns of STEAPs differed (Figure 1). Interestingly, in COAD, head and neck squamous cell carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, and READ, the expression of STEAP4 was lower than that in normal tissues. However, in such cancerous tissues, the levels of STEAPs 1-3 were higher or not significantly different from those in normal tissues, suggesting that STEAP4 may perform a different function from STEAPs 1-3 in patients with such cancers.

CRC tissues have lower STEAP4 levels compared with normal tissues

To verify the expression pattern of STEAPs in CRC, another database, UCSC Xena, was analyzed to confirm the findings. Although expression of STEAPs 1 and 2 in COAD and READ were not different compared to their corresponding normal tissues, STEAP4 expression was lower, while STEAP3 expression was higher compared to that in corresponding normal tissues (Figure 2).

Low STEAP4 levels are associated with the MSS subtype of CRC

CRC is highly heterogeneous at the genetic and molecular levels, which affects the efficacy of clinical therapy. A subset of CRCs exhibit MSI, indicating defective DNA mismatch repair and high mutational burden, different from the majority of MSS subtypes[22]. CRC patients, especially those with MSI-H tumors, are more sensitive to ICIs than those with MSS tumors, and MSI-H tumors have greater infiltration of immune cells, higher expression of immune-related genes, and higher immunogenicity than MSS tumors[23]. Since the expression of STEAP4 is consistently low in CRC, different from the other three genes, we focused on STEAP4 for the remainder of this study. To explore STEAP4 expression levels in different subtypes of CRCs, the GEPIA2 database was used. Interestingly, the mRNA level of STEAP4 was high in MSI-H CRCs compared to MSI-L and MSS tumors (Figure 3).

STEAP4 is decreased in cancer tissues

As the expression pattern of STEAP4 seems to be different from those of the other STEAP members, paired normal and CRC tissues were used to evaluate the protein level of STEAP4. Representative images of STEAP4 expression are shown in Figure 4. The percentage of CRC tissues with high levels of STEAP4 was 51.7%, which was still lower than that in normal tissue (73.6%, $P = 0.003$) (Table 1).

Relationship between STEAP4 levels and clinicopathological parameters of patients with CRC

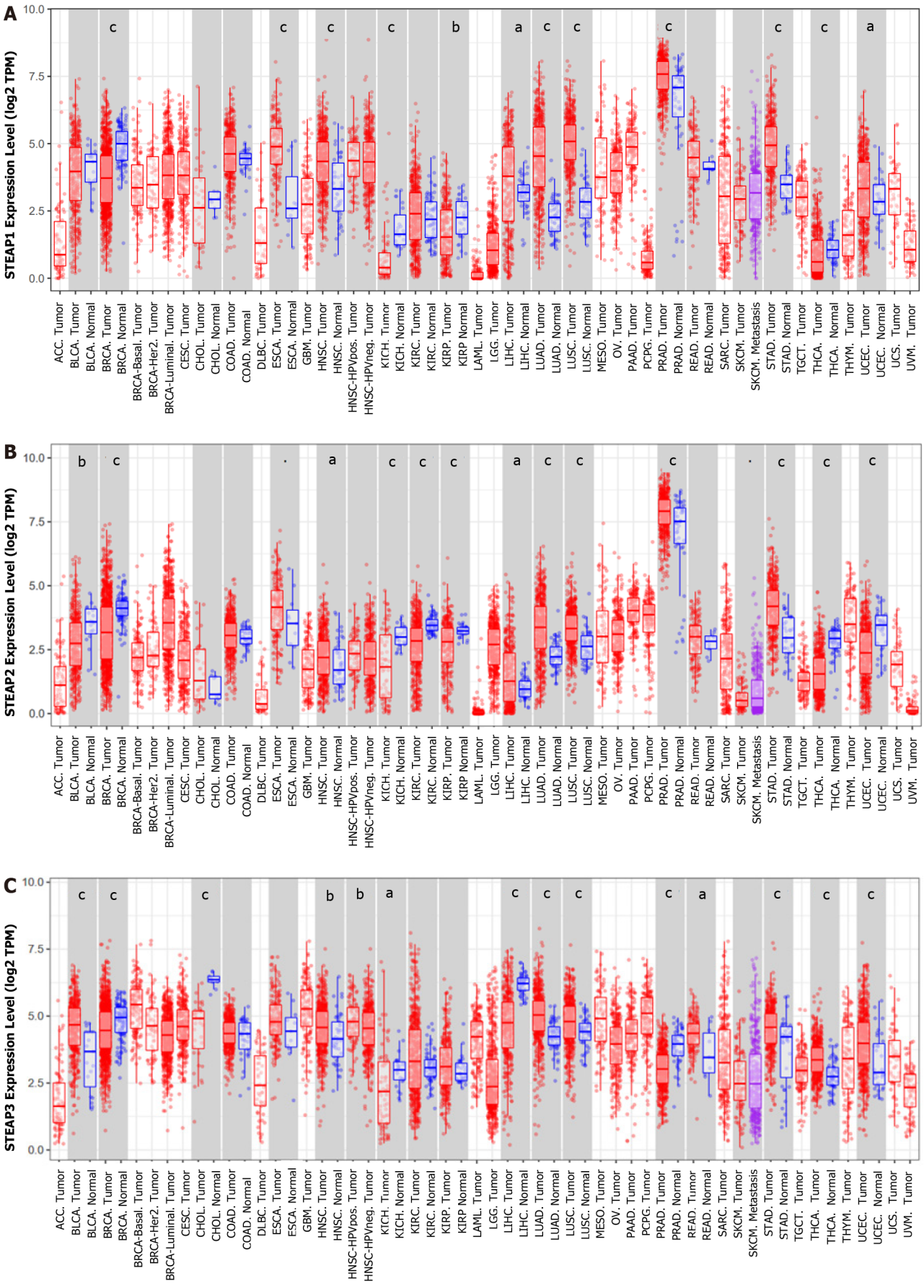
Clinicopathological analysis showed that STEAP4 expression was not associated with gender, primary tumor stage, lymph node status, American Joint Committee on Cancer (AJCC) stage, or pathological type (Table 2). The expression of STEAP4 decreased with the increase of primary tumor stage, lymph node status, and AJCC stage.

STEAP4 protein levels are positively associated with MLH1, MLH6, and PMS2, and negatively associated with PDL1

Based on the IHC results, the expression of STEAP4 and corresponding immune-related biomarkers in CRC was analyzed using χ^2 statistical analysis. As shown in Table 3, a low level of STEAP4 was positively related to low levels of MLH1, MLH6, and PMS2, but negatively associated with PDL1 level in CRC patients.

Expression level of STEAP4 is associated with the abundance of immune cell infiltration in CRC

Given the importance of antitumor immunity in the tumor microenvironment, the correlation between


A
STEAP1 Expression Level (log2 TPM)
B
STEAP2 Expression Level (log2 TPM)
C
STEAP3 Expression Level (log2 TPM)
ACC. Tumor
BLCA. Tumor
BLCA. Normal
BRCA. Tumor
BRCA. Normal
BRCA-Basal. Tumor
BRCA-Her2. Tumor
BRCA-Luminal. Tumor
CESC. Tumor
CHOL. Tumor
CHOL. Normal
COAD. Tumor
COAD. Normal
DLBC. Tumor
ESCA. Tumor
ESCA. Normal
GBM. Tumor
HNSC. Tumor
HNSC. Normal
HNSC-HPVpos. Tumor
HNSC-HPVneg. Tumor
KICH. Tumor
KICH. Normal
KIRC. Tumor
KIRC. Normal
KIRP. Tumor
KIRP Normal
LAML. Tumor
LGG. Tumor
LIHC. Tumor
LIHC. Normal
LUAD. Tumor
LUAD. Normal
LUSC. Tumor
LUSC. Normal
MESO. Tumor
OV. Tumor
PAAD. Tumor
PCPG. Tumor
PRAD. Tumor
PRAD. Normal
READ. Tumor
READ. Normal
SARC. Tumor
SKCM. Tumor
SKCM. Metastasis
STAD. Tumor
STAD. Normal
TGCT. Tumor
THCA. Tumor
THCA. Normal
THYM. Tumor
UCEC. Tumor
UCEC. Normal
UCS. Tumor
UVM. Tumor

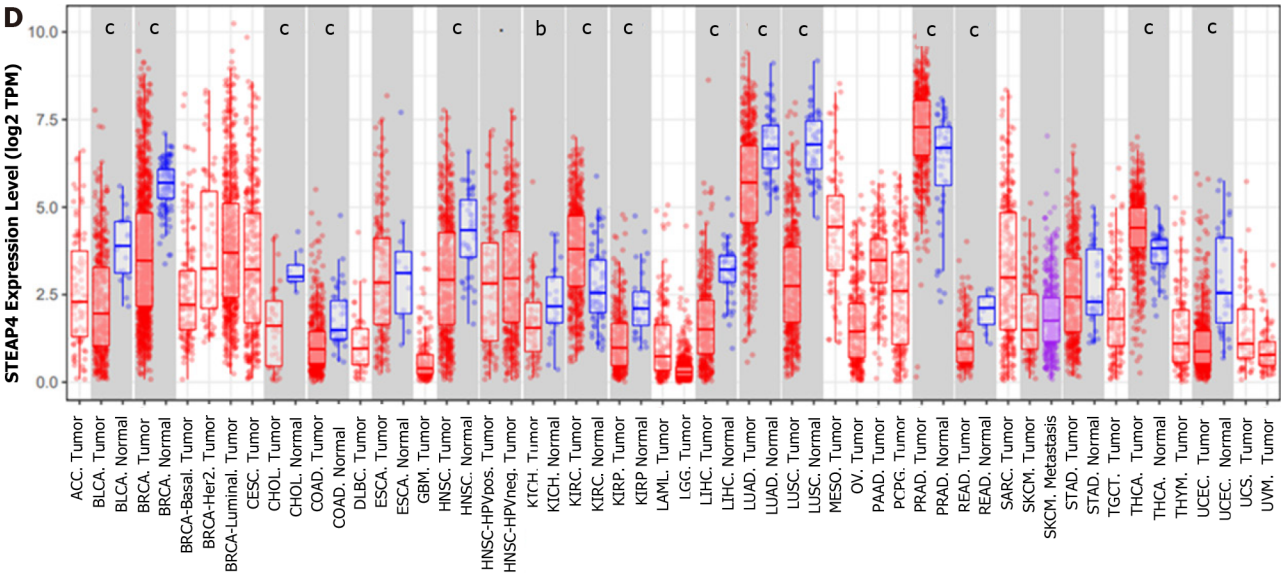


Figure 1 Expression of six-transmembrane epithelial antigen of the prostate in different normal and cancerous tissues from the TIMER2.0 database. A: Six-transmembrane epithelial antigen of the prostate (STEAP) 1; B: STEAP2; C: STEAP3; D: STEAP4. Student's *t*-test was used to estimate the significance of the differences in gene expression levels between groups. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. STEAP: Six-transmembrane epithelial antigen of the prostate; ACC: Adrenocortical carcinoma; BLCA: Bladder urothelial carcinoma; BRCA: Breast invasive carcinoma; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: Cholangiocarcinoma; COAD: Colon adenocarcinoma; DLBC: Lymphoid neoplasm diffuse large B-cell lymphoma; ESCA: Esophageal carcinoma; GBM: Glioblastoma multiforme; HNSC: Head and neck squamous cell carcinoma; KICH: Kidney chromophobe; KIRC: Kidney renal clear cell carcinoma; KIRP: Kidney renal papillary cell carcinoma; LAML: Acute myeloid leukemia; LGG: Brain lower grade glioma; LIHC: Liver hepatocellular carcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous cell carcinoma; MESO: Mesothelioma; OV: Ovarian serous cystadenocarcinoma; PAAD: Pancreatic adenocarcinoma; PCPG: Pheochromocytoma and paraganglioma; PRAD: Prostate adenocarcinoma; READ: Rectum adenocarcinoma; SARC: Sarcoma; SKCM: Skin cutaneous melanoma; STAD: Stomach adenocarcinoma; TGCT: Testicular germ cell tumors; THCA: Thyroid carcinoma; THYM: Thymoma; UCEC: Uterine corpus endometrial carcinoma; UCS: Uterine carcinosarcoma; UVM: Uveal melanoma.

expression of STEAP family members and immune cells in CRC was analyzed. Based on the TIMER database, it was found that the expression of STEAP1, STEAP2, and STEAP4 was negatively correlated with tumor purity and positively correlated with six types of immune cells, specifically, B cells, $CD8^+$ T cells, $CD4^+$ T cells, neutrophils, macrophages, and dendritic cells, in COAD and READ (Figures 5A, B, and D). However, the expression of STEAP3 was positively correlated with tumor purity, $CD4^+$ T cells, neutrophils, macrophages, and dendritic cells, and negatively correlated with B cells and $CD8^+$ T cells in COAD and READ (Figure 5C).

STEAP4 is positively correlated with immune characteristics in CRC

According to the TISIDB database, Spearman correlation analysis showed that the expression of STEAP1, STEAP2, and STEAP4 was positively correlated, but STEAP3 was negatively correlated with lymphocytes, immunostimulants, MHCs, and chemokines (Figures 6A-F), which is consistent with the results obtained based on the TIMER database (Figures 6C and G).

Low expression of STEAP4 in cancer tissues tends to predict poor overall survival in CRC patients

Based on the IHC results, the protein expression of STEAP4 was analyzed by the Kaplan-Meier method with log-rank test, which demonstrated that STEAP4 expression was not significantly associated with the overall survival (OS) ($P > 0.05$, Figure 7). Although the difference did not meet the statistical criteria, it was found that high expression of STEAP4 tended to predict a longer OS for CRC patients, suggesting that the protein level of STEAP4 could be a predictor of the survival of CRC patients.

DISCUSSION

Members of the STEAP family were originally identified as metalloreductases *in vivo*, playing an important role in maintaining iron homeostasis[24]. Abnormal accumulation of iron caused by unbalanced iron metabolism has been reported to lead to the occurrence, progression, and invasion of tumors[25]. Thus, the STEAP family bridges iron homeostasis and cancer[26]. As potential biomarkers and therapeutic targets of tumors, the STEAP family members play an important role in tumor therapy.

All four STEAP proteins are increased in prostate cancer and play important roles in the development and progression of prostate cancer[27]. Interestingly, although the structure of STEAP4 is similar to that

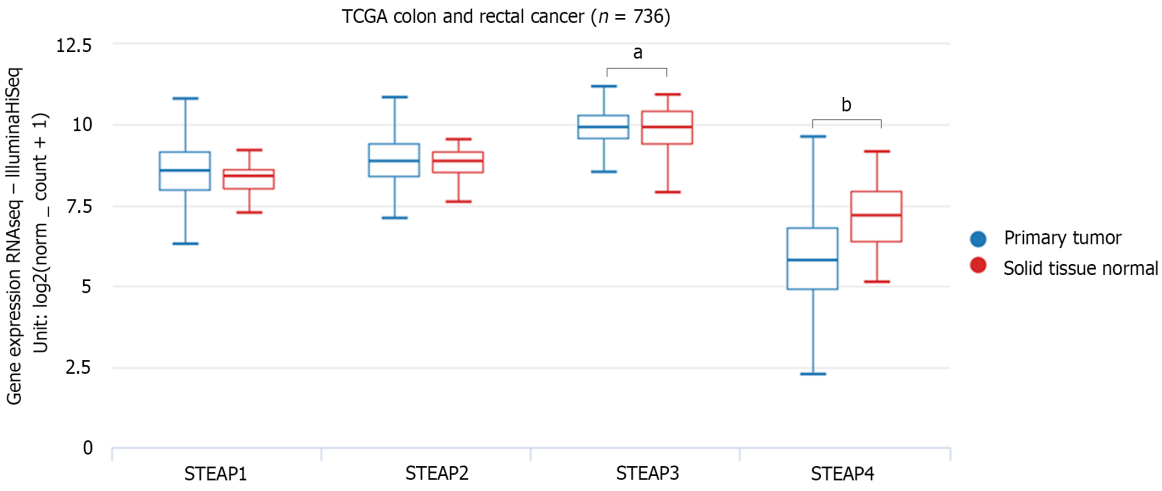


Figure 2 Six-transmembrane epithelial antigen of the prostate mRNA expression in colon adenocarcinoma and rectal adenocarcinoma tissues in the UCSC Xena database. Red represents normal tissue and blue is cancerous tissue. One-way ANOVA was utilized to estimate the significance of differences in six-transmembrane epithelial antigen of the prostate expression levels between groups. $^{a}P < 0.01$, $^{b}P < 0.001$. STEAP: Six-transmembrane epithelial antigen of the prostate.

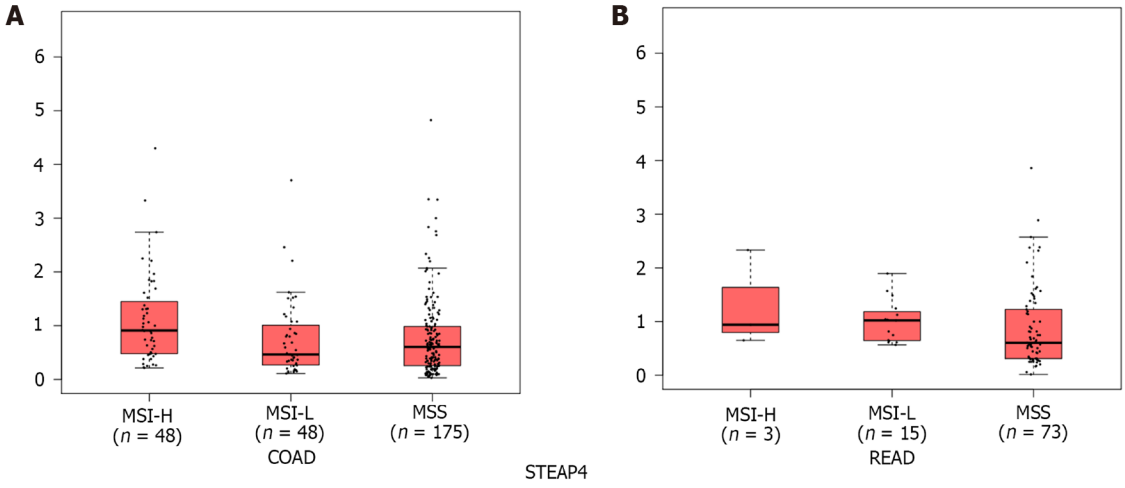


Figure 3 Expression patterns of six-transmembrane epithelial antigen of the prostate 4 in different subtypes of colorectal cancer. A: Colon adenocarcinoma; B: Rectal adenocarcinoma. MSI-H: Microsatellite instability-high; MSI-L: Microsatellite instability-low; MSS: Microsatellite stable; STEAP4: Six-transmembrane epithelial antigen of the prostate 4; COAD: Colon adenocarcinoma; READ: Rectal adenocarcinoma.

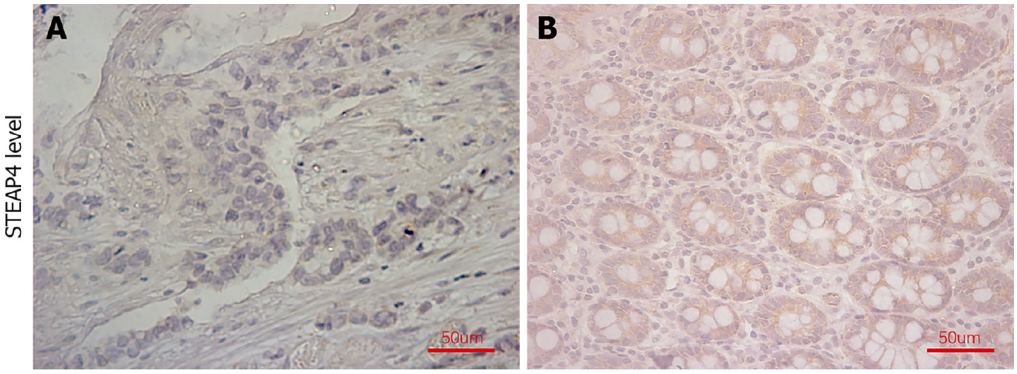


Figure 4 Representative images of six-transmembrane epithelial antigen of the prostate 4 expression in colorectal cancer and normal tissues. A: Six-transmembrane epithelial antigen of the prostate 4 (STEAP4) expression in colorectal cancer tissues; B: STEAP4 expression in adjacent normal tissues. Scale bar, 50 μm. STEAP4: Six-transmembrane epithelial antigen of the prostate 4.

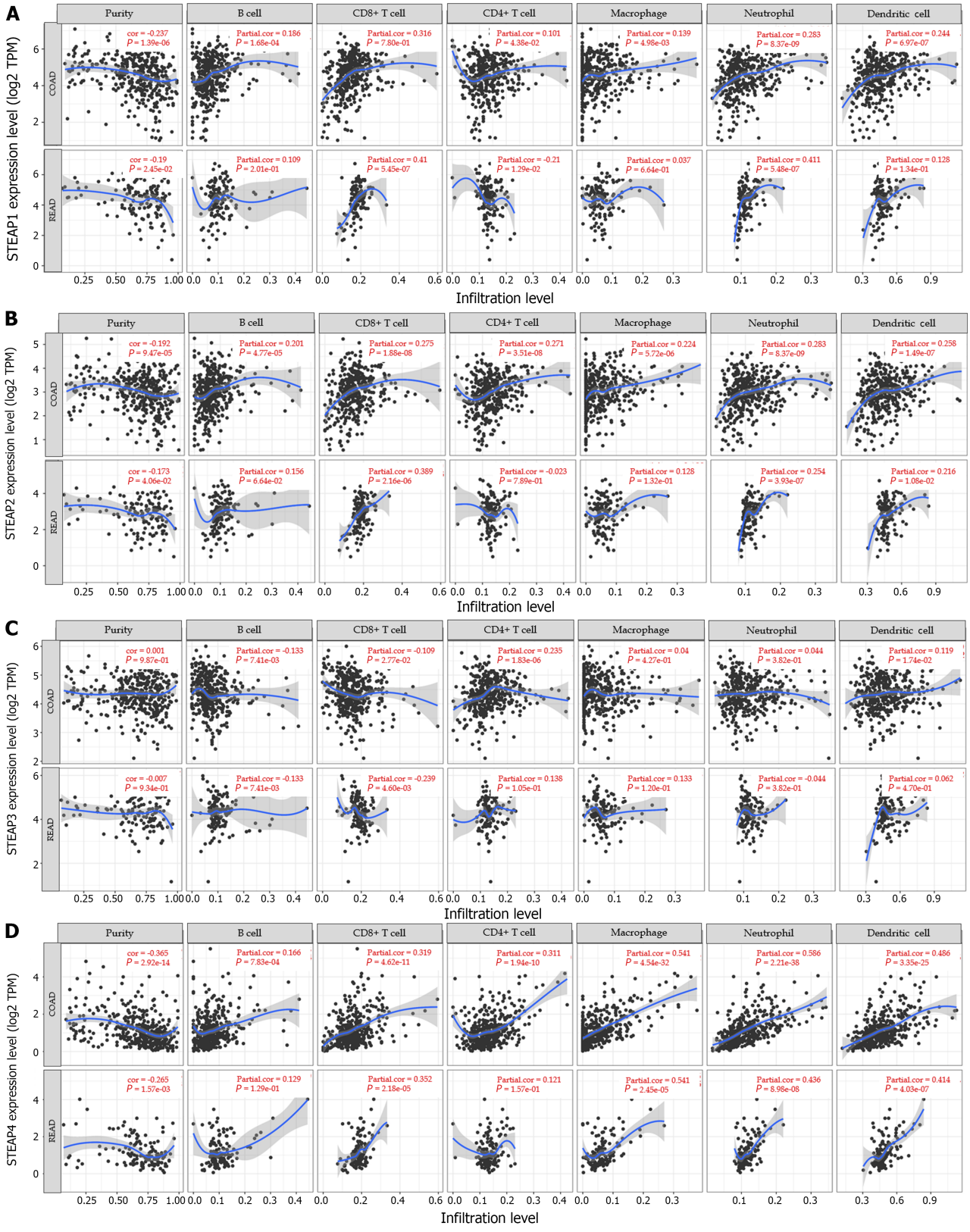


Figure 5 Relationship between six-transmembrane epithelial antigen of the prostate family member expression levels and immune infiltrates validated by the TIMER database. A: Six-transmembrane epithelial antigen of the prostate (STEAP1); B: STEAP2; C: STEAP3; D: STEAP4. STEAP: Six-transmembrane epithelial antigen of the prostate.

of the three other STEAP family members, the function of STEAP4 may diverge in different types of cancers[24]. STEAP1 antibody can effectively activate CD8$^+$ T cells, natural killer cells, and other immune-related factors against a broad spectrum of tumors[28]. In this study, there was a great difference between STEAP4 and STEAP1-3; STEAP4 promoted androgen receptor (AR)-positive prostate cancer (PC) and inhibited AR-negative PC, while it was very different from STEAP1-3 in CRC, so there

Table 2 Correlation between six-transmembrane epithelial antigen of the prostate 4 expression and clinicopathological parameters in colorectal cancer patients

Clinical parameter	STEAP4		*P* value
	Low (%)	High (%)	
Gender			
Female	29 (59.2)	20 (40.8)	0.123
Male	19 (43.2)	25 (56.8)	
Primary tumor stage[1]			
T1-T3	36 (48.0)	39 (52.0)	0.213
T4a-T4b	11 (64.7)	6 (35.3)	
Lymph node status			
N0-N1	41 (48.8)	43 (51.2)	0.160
N2	7 (77.8)	2 (22.2)	
AJCC stage			
Phase 1-2	28 (43.1)	30 (56.9)	0.407
Phase 3	20 (57.1)	15 (42.9)	

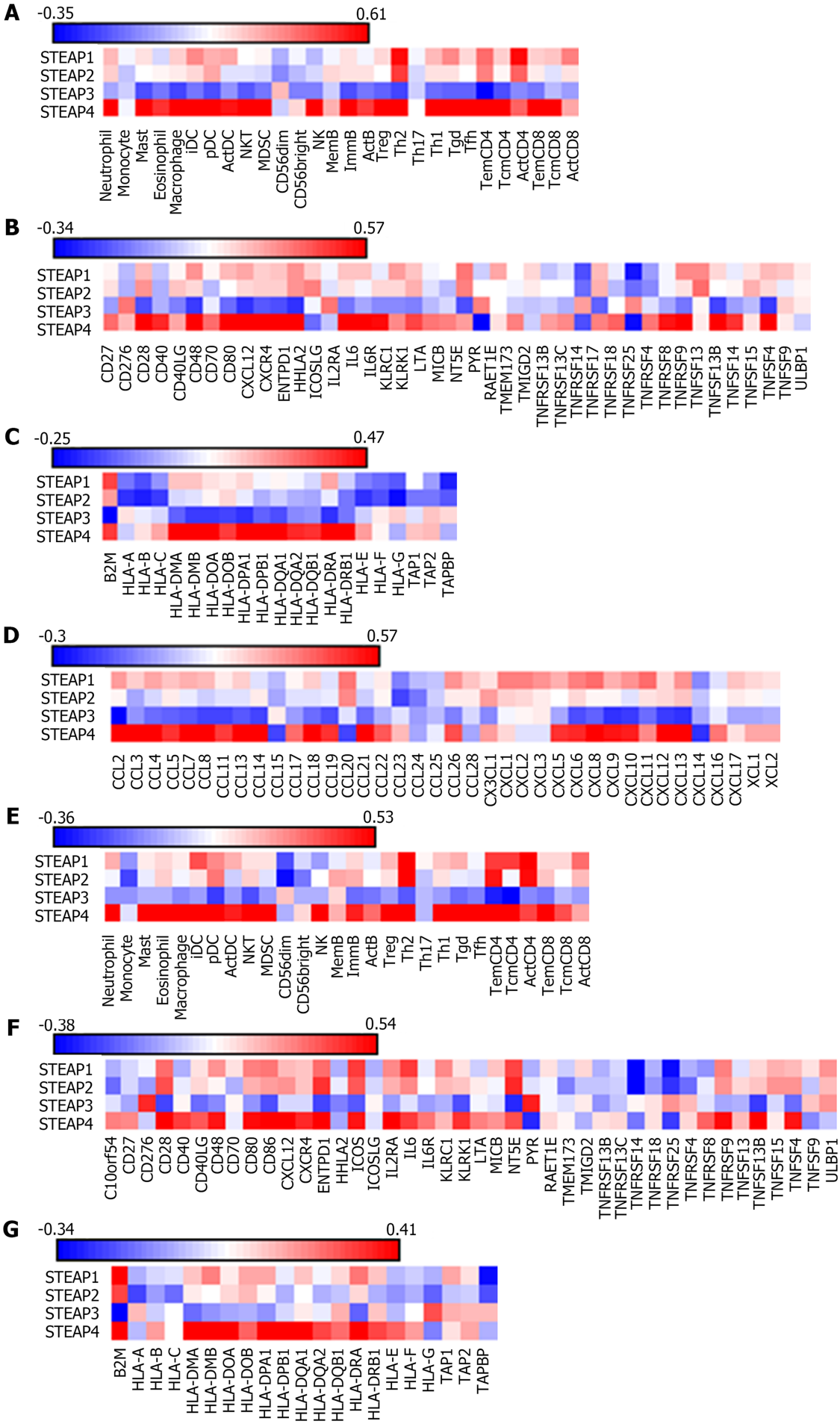
[1]One colorectal cancer patient with undetected primary tumor (Tx) was excluded from the primary tumor stage.
STEAP4: Six-transmembrane epithelial antigen of the prostate 4; AJCC: American Joint Committee on Cancer.

Table 3 Correlation of six-transmembrane epithelial antigen of the prostate 4 expression with MLH1/2/6, PMS2, and programmed death ligand 1 expression in colorectal cancer

		STEAP4		χ^2	*P* value
		Low	High		
MLH1	Low	6	5	18.42	0.000004[a]
	High	33	36		
MLH2	Low	18	10	3.90	0.071
	High	21	30		
MLH6	Low	8	7	13.44	0.0003[a]
	High	29	33		
PMS2	Low	12	8	10.31	0.017[b]
	High	27	30		
PDL1	Low	43	41	35.37	0.0019[c]
	High	2	4		

[a]$P < 0.001$.
[b]$P < 0.05$.
[c]$P < 0.01$.
PDL1: Programmed death ligand 1; STEAP4: Six-transmembrane epithelial antigen of the prostate 4.

might be different mechanisms. Interestingly, we found dual anti-STEAP1 antibody targeting T cells for cancer immunotherapy[29]. Combined with our study, it is suggested that STEAP4 can be developed as a new therapeutic strategy. Therefore, this study explored the role of STEAP4 at the mRNA and protein levels. STEAP4 was rarely studied in CRC. The current study used clinical tissues from CRC patients and characterized the mRNA and protein levels of STEAP4 to determine the expression of STEAP4 in CRC. Not surprisingly, low expression of STEAP4 was found in CRC tissues compared with adjacent tissues, which is consistent with the low mRNA expression of STEAP4 in CRC and a potential tumor suppressor role for STEAP4 in CRC patients.


A
-0.35
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STEAP1
STEAP2
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Neutrophil
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CD56bright
NK
MemB
ImmB
ActB
Treg
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ActCD4
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CD40
CD40LG
CD48
CD70
CD80
CXCL12
CXCR4
ENTPD1
HHLA2
ICOSLG
IL2RA
IL6
IL6R
KLRC1
KLRK1
LTA
MICB
NT5E
PYR
RAET1E
TMEM173
TMIGD2
TNFRSF13B
TNFRSF13C
TNFRSF14
TNFRSF17
TNFRSF18
TNFRSF25
TNFRSF4
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ULBP1
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-0.25
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HLA-DOB
HLA-DPA1
HLA-DPB1
HLA-DQA1
HLA-DQA2
HLA-DQB1
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Monocyte
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ActDC
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MDSC
CD56dim
CD56bright
NK
MemB
ImmB
ActB
Treg
Th2
Th17
Th1
Tgd
Tfh
TemCD4
TcmCD4
ActCD4
TemCD8
TcmCD8
ActCD8
F
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0.54
STEAP1
STEAP2
STEAP3
STEAP4
C10orf54
CD27
CD276
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CD40
CD40LG
CD48
CD70
CD80
CD86
CXCL12
CXCR4
ENTPD1
HHLA2
ICOS
ICOSLG
IL2RA
IL6
IL6R
KLRC1
KLRK1
LTA
MICB
NT5E
PYR
RAET1E
TMEM173
TMIGD2
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TNFRSF13C
TNFRSF14
TNFRSF18
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TNFRSF4
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TAP1
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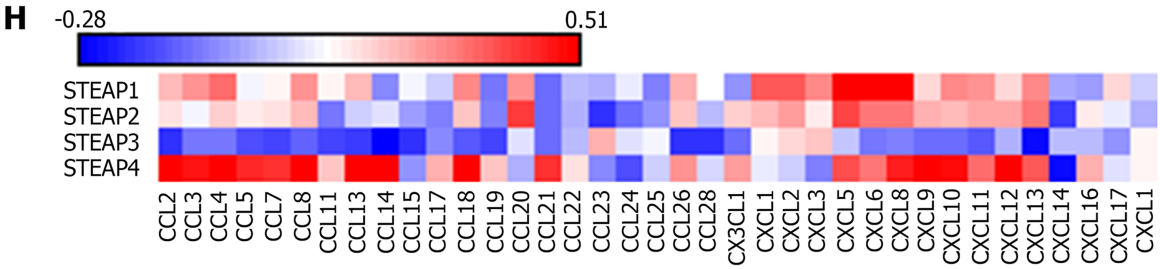

Figure 6 Spearman correlation between six-transmembrane epithelial antigen of the prostate family member expression and immune factors in colon adenocarcinoma and rectal adenocarcinoma. A-D: Colon adenocarcinoma; E-H: Rectal adenocarcinoma. STEAP: Six-transmembrane epithelial antigen of the prostate; iDC: Immature dendritic cell; pDC: Plasmacytoid dendritic cell; ActDC: Active dendritic cells; NKT: Natural killer T cell; MDSC: Myeloid derived suppressor cell; NK: Natural killer cell; Th: T helper cell; Tgd: Gamma delta T cell; Tfh: T follicular helper cell; B2M: Beta-2-microglobulin; HLA: Major histocompatibility complex; HLA-DOA: Major histocompatibility complex, class II, DO alpha; HLA-DOB: Major histocompatibility complex, class II, DO beta; HLA-DPA1: Major histocompatibility complex, class II, DP alpha 1; HLA-DPB1: Major histocompatibility complex, class II, DP beta 1; HLA-DQA1: Major histocompatibility complex, class II, DQ alpha 1; HLA-DRA: Major histocompatibility complex, class II, DR alpha; TAP: Transporter; TAPBP: Transporter Binding Protein; CD: Cluster of differentiation; CXCL: C-X-C motif chemokine ligand; ENTPD: Ectonucleoside triphosphate diphosphohydrolase; HHLA: HERV-H LTR-associating; ICOS: Inducible T cell costimulator; ICOSLG: Inducible T cell costimulator ligand; IL2RA: Interleukin 2 receptor subunit alpha; IL: Interleukin; IL6R: Interleukin 6 receptor; KIRC1: KIR3DL3, Homo sapiens killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 3; KIRK1: Killer cell lectin like receptor subfamily K, member 1; LTA: Lymphotoxin alpha; MICB: MHC class I polypeptide-related sequence B; NT5E: 5'-nucleotidase ecto; PVR: Poliovirus receptor; RAET1E: Retinoic acid early transcript 1E; TMEM173: STING, stimulator of interferon response CGAMP interactor; TMIGD: Transmembrane and immunoglobulin domain containing; TNFRSF: TNF receptor superfamily member; ULBP: UL16 binding protein; CCL: C-C motif chemokine ligand; CX3CL: C-X3-C motif chemokine ligand; XCL: X-C motif chemokine ligand.

To uncover the potential function of STEAP4 in CRC, clinicopathological parameters were analyzed. However, no statistical significance was found between the protein level of STEAP4 and primary tumor stage, lymph node metastasis, or AJCC stage. Reduced STEAP4 expression tended to be associated with advanced CRC stage and increased lymph node metastases, suggesting that the suppression of STEAP4 could play a role in promoting the progression and metastasis of CRC.

Recently, Xue *et al*[11] investigated the molecular mechanism of STEAP4 involvement in the hypoxic metabolism of inflammatory bowel disease and the linkage with mitochondrial dysfunction in colon cancer. Increasingly, high levels of STEAP4 result from increased levels of hypoxia and are associated with colitis in mouse models and inflammatory bowel disease patients. Inflammatory factors were not examined in the current study, which may influence STEAP4 levels in different types of CRC. Based on the inflammatory environment, hypoxic conditions can be associated with mitochondrial iron dysfunction caused by increased STEAP4[30]. However, in the absence of inflammatory infiltration, the inhibition of STEAP4 was reversed to be a tumor suppressor through interactions with protein kinases [31].

Further analyses were performed to uncover the potential relationship of STEAP4 with immune infiltration. As expected, both the protein and mRNA levels of STEAP4 are associated with immune-related factors, predicting a potential role of STEAP4 in stimulating immune infiltration. MLH1 deficiency has been shown to be associated with cetuximab resistance in CRC[21]. The positive relationship between STEAP4 and MLH1 in CRC suggests an involvement of STEAP4 in the immune response in the tumor microenvironment. Recently, Ijsselsteijn *et al*[32] reported that, for a cell-based model for Lynch syndrome, DNA mismatch repair deficiency was related to the core DNA mismatch repair genes MSH2, MSH6, MLH1, and PMS2[32]. The protein level of STEAP4 was found to be positively correlated with such DNA mismatch repair genes in the current study. The above results support a role for STEAP4 in the immune response to CRC to prevent further development and metastasis.

CONCLUSION

In the current study, STEAP4 was found to be a protective factor in the intestinal tract and could be used as a prognostic indicator for patients with CRC. CRC patients with high STEAP4 expression tend to have a longer survival.

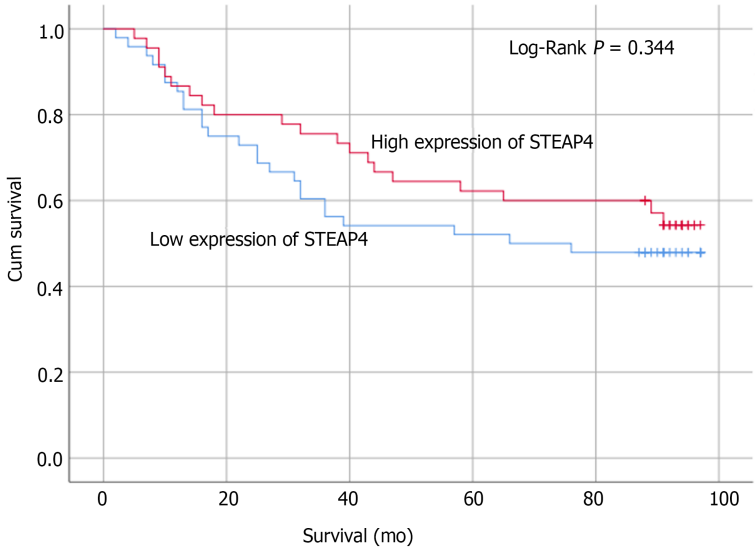

Figure 7 Low six-transmembrane epithelial antigen of the prostate 4 protein level tends to predict a poor overall survival in colorectal cancer patients. STEAP4: Six-transmembrane epithelial antigen of the prostate 4.

ARTICLE HIGHLIGHTS

Research background

Six-transmembrane epithelial antigen of the prostate 4 (STEAP4) is an attractive biomarker for the immunotherapy of prostate and breast cancers. However, the immunotherapeutic role of STEAP4 for colorectal carcinomas has not been demonstrated.

Research motivation

Immunotherapy emerges with predicting outcomes and therapeutic efficacy in colorectal cancers (CRC).

Research objectives

To explore the expression pattern of STEAPs in CRCs and their relationship with immune infiltration, and investigate the potential utilization of STEAPs as novel prognostic indicators in colorectal carcinomas.

Research methods

CRC patients' tissues and online datasets were used to analyze the expression level of STEAP4 in different types of CRC and their relationship with immune characteristics.

Research results

The expression of STEAP4 was significantly decreased in CRC tissues compared with adjacent normal ones, and was related to immune-related biomarkers. Low STEAP4 level predicted a poor overall survival of CRC patients.

Research conclusions

STEAP4 was found to be a protective factor in the intestinal tract and could be used as a prognostic indicator for patients with CRC.

Research perspectives

STEAP4 may be a potential biomarker for predicting CRC immune infiltration status.

FOOTNOTES

Author contributions: Liu J and Wu HT designed the research study; Fang ZX performed the research; Fang ZX, Li CL, Chen WJ, and Wu HT analyzed the research and wrote the manuscript; Liu J revised the manuscript critically; and all authors have read and approved the final manuscript.

Supported by the National Natural Science Foundation of China, No. 81501539; the Natural Science Foundation of Guangdong Province, No. 2021A1515012180 and 2016A030312008; the Special Grant for Key Area Programs of

Guangdong Department of Education, No. 2021ZDZX2004; the Science and Technology Special Project of Guangdong Province, No. 210715216902829; and "Dengfeng Project" for the Construction of High-level Hospitals in Guangdong Province-First Affiliated Hospital of Shantou University College Supporting Funding, No. 202003-10.

Institutional review board statement: The current study was reviewed and approved by the Ethics Committee of Shantou University Medical College.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Wang JJ
L-Editor: Wang TQ
P-Editor: Cai YX

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1689-1698

DOI: 10.4251/wjgo.v14.i9.1689

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Case Control Study

Inverse relations between *Helicobacter pylori* infection and risk of esophageal precancerous lesions in drinkers and peanut consumption

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Chen LW, Taiwan; Reshetnyak VI, Russia

Received: April 7, 2022
Peer-review started: April 7, 2022
First decision: June 2, 2022
Revised: June 16, 2022
Accepted: August 5, 2022
Article in press: August 5, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium found in the upper digestive tract. Although *H. pylori* infection is an identified risk factor for gastric cancer, its role in esophageal squamous cell carcinoma (ESCC) remains a topic of much debate.

AIM

To evaluate the association between *H. pylori* infection and the risk of precancerous lesions of ESCC, and further explore the association between dietary factors and the risk of *H. pylori* infection.

METHODS

Two hundred patients with esophageal precancerous lesions (EPL) aged 63.01 ± 6.08 years and 200 healthy controls aged 62.85 ± 6.03 years were included in this case-control study. Epidemiological data and qualitative food frequency data were investigated. Enzyme-linked immunosorbent assay measuring serum immunoglobulin G antibodies was used to determine *H. pylori* seropositivity. An unconditional logistic regression model was used to assess the association between *H. pylori* infection and EPL risk dichotomized by gender, age, and the use of tobacco and alcohol, as well as the association between dietary factors and the

risk of *H. pylori* infection.

RESULTS

A total of 47 (23.5%) EPL cases and 58 (29.0%) healthy controls had positive *H. pylori* infection. An inverse relation between *H. pylori* infection and the risk of EPL was found in the group of drinkers after adjustment for covariates [odds ratio (OR) = 0.32, 95% confidence interval (95%CI): 0.11-0.95]. Additionally, peanut intake was significantly associated with a decreased risk of *H. pylori* infection (OR = 0.39, 95%CI: 0.20-0.74).

CONCLUSION

Our study suggested that *H. pylori* infection may decrease the risk of EPL for drinkers in a rural adult Chinese population, and the consumption of peanut may reduce the risk of *H. pylori* infection. These findings should be framed as preliminary evidence, and further studies are required to address whether the mechanisms are related to the localization of lesions and alcohol consumption.

Key Words: *Helicobacter pylori*; Esophageal precancerous lesions; Peanut consumption; Esophageal squamous cell carcinoma

Core Tip: The association between *Helicobacter pylori* (*H. pylori*) infection and esophageal squamous cell carcinoma (ESCC) remains a topic of much debate. This study aimed to evaluate the association between *H. pylori* infection and the risk of precancerous lesions of ESCC, and further explore the association between dietary intake and the risk of *H. pylori* infection. Our findings suggested an inverse association between *H. pylori* infection and the risk of esophageal precancerous lesions in the group of drinkers [odds ratio (OR) = 0.32, 95% confidence interval (95%CI): 0.11-0.95]. Additionally, peanut consumption was significantly associated with a reduced risk of *H. pylori* infection (OR = 0.39, 95%CI: 0.20-0.74).

Citation: Pan D, Sun GJ, Su M, Wang X, Yan QY, Song G, Wang YY, Xu DF, Wang NN, Wang SK. Inverse relations between *Helicobacter pylori* infection and risk of esophageal precancerous lesions in drinkers and peanut consumption. *World J Gastrointest Oncol* 2022; 14(9): 1689-1698
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1689.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1689

INTRODUCTION

Esophageal cancer and gastric cancer are upper gastrointestinal cancers that share many risk factors[1-3]. However, their associations with *Helicobacter pylori* (*H. pylori*) infection can be completely different. It has been determined that *H. pylori* infection is an identified risk factor for gastric cancer[4], whereas the role of *H. pylori* in the risk of esophageal cancer remains controversial. Previous meta-analyses summarized that *H. pylori* infection is likely to be related to a reduced risk of esophageal adenocarcinoma (EAC)[5-9]. One of the reliable assumptions related to this phenomenon is that *H. pylori* infection causes gastric atrophy and parietal cell loss, thus leading to alleviated reflux and consequently, a decreased incidence of reflux esophagitis and Barrett's esophagus (precursor for EAC)[10-12]. However, the impact of *H. pylori* infection on esophageal squamous cell carcinoma (ESCC) is not well understood, and research is inconclusive as to what population may be significantly influenced[9,13-15]. Previous meta-analyses also reported that in the general population, no significant association was found between *H. pylori* infection and ESCC risk[6-8], whereas an inverse association was observed in the Middle East[9]. In the other populations, the inverse relationship was found to be highly associated with age, smoking status, and drinking status[15].

H. pylori is a Gram-negative bacterium found in the upper digestive tract. In spite of the fact that *H. pylori* infection may reduce the risk of EAC, it may also cause an adverse effect on human health. Apart from the elevated risk of gastric cancer, *H. pylori* infection is also etiologically related to peptic ulcers, atrophic and non-atrophic gastritis, and lymphoma associated with gastric mucosa, and is able to induce reduced bioavailability and malabsorption of nutrients including iron and vitamin B_{12}[16-18]. This case-control study aimed to investigate the association between *H. pylori* infection and the risk of precancerous lesions of ESCC, which is an identified early stage of carcinogenesis, and further examine the association between dietary factors and the risk of *H. pylori* infection.

MATERIALS AND METHODS

This study was carried out in a high-incidence area for ESCC located in Huai'an District, Huai'an City, Jiangsu Province, China, where the crude incidence rate from 1998 to 2016 was 91.85/100000[19]. As described in our previous studies[20-22], the Early Diagnosis and Early Treatment Project of Esophageal Cancer (EDETPEC) supported by the government and Cancer Foundation of China has been carried out in the endemic regions including Huai'an District since 2010. Local residents were required to undergo routine endoscopies. A detailed introduction to esophageal precancerous lesions (EPL) based on histological criteria for dysplasia and methods for EPL diagnosis has already been given in a previous study[21]. The localization of EPL was based on the definition of upper thoracic esophagus (from thoracic inlet to level of tracheal bifurcation; 18-23 cm from incisors), mid thoracic esophagus (from tracheal bifurcation midway to gastroesophageal junction; 24-32 cm from incisors), and lower thoracic esophagus (from midway between tracheal bifurcation and gastroesophageal junction to gastroesophageal junction, including abdominal esophagus; 32-40 cm from incisors)[23]. Figure 1 shows the flowchart of the study population and data collection process. This study included 200 EPL cases aged 62.85 ± 6.03 years and 200 healthy controls aged 63.01 ± 6.08 years matched by gender, age (± 2 years), and villages. The collection of epidemiological data and dietary intake data based on questionnaire method has been introduced in detail previously[21]. Subjects were required to provide the amount of beer/wine/liquor/any other alcoholic drinks consumed per day, which meant that the average alcohol units consumed per day could be estimated. Separated serum samples were obtained by centrifuging collected fasting blood samples at 3000 rpm for 5 min. Enzyme-linked immunosorbent assay (ELISA, KingMed Diagnostics Group Co., Ltd. Guangzhou, China) measuring serum immunoglobulin G (IgG) antibodies was used to determine *H. pylori* seropositivity. Sensitivity of the ELISA test was 97.9% [95% confidence interval (95%CI): 88.9%-99.9%] and specificity was 100% (95%CI: 86.8%-100%).

Epidata version 3.1 (EpiData Association, Odense, Denmark) was used for inputting and validating the epidemiological data and dietary intake data. Then, SPSS version 22.0 (SPSS, Chicago, IL, United States) was used to establish a database and perform statistical analyses. Two independent samples *t*-test and conditional logistic regression model were used to evaluate the differences in general characteristics and potential factors between healthy controls and EPL cases, wherever appropriate. The Fisher's exact test was used to analyze the difference in localization of EPL and *H. pylori* infection. An unconditional logistic regression model was used to assess the association between *H. pylori* infection and EPL risk dichotomized by gender, age, and tobacco and alcohol use, as well as the association between dietary factors and *H. pylori* infection. Covariates including gender, age, body mass index (BMI), education level, annual income, number of cigarettes per day, and alcohol units consumed per day were adjusted in the logistic regression model. Meanwhile, odds ratio (OR) and 95%CI were calculated accordingly. Statistical significance was defined as $P < 0.05$ (two-tailed).

The study protocol was approved by the Institutional Review Board of Southeast University Zhongda Hospital (Approval No. 2016ZDKYSB017), and the written informed consent was obtained.

RESULTS

Two hundred EPL cases aged 63.01 ± 6.08 years and 200 healthy controls aged 62.85 ± 6.03 years were enrolled. Among the pairs, 100 were males and 100 were females. Table 1 shows that 47 (23.5%) and 58 (29.0%) out of 200 cases and 200 controls, respectively, had *H. pylori* infection. Two independent samples *t*-test and conditional logistic regression analysis indicated that there were no statistically significant differences in age, BMI, education level, annual income per person, current drinking status, or *H. pylori* infection between the two groups after adjustment for covariates ($P > 0.05$). Compared with non-smokers, a smoking habit of more than 20 cigarettes a day was significantly associated with an elevated risk of EPL ($P < 0.05$).

Based on routine endoscopy examination, the study found that the number of cases whose EPL developed in upper, mid, and lower thoracic esophagus was 3, 130, and 67, respectively. Table 2 shows that the control group had the highest positive rate of *H. pylori* infection (29.0%), followed by EPL cases of upper and mid thoracic esophagus (24.8%) and EPL cases of lower thoracic esophagus (20.9%), but there was no statistically significant differences.

As shown in Table 3, when subjects were dichotomized according to gender, age, and the use of tobacco and alcohol, the protective effect of *H. pylori* infection against the risk of EPL was found in the group of drinkers after adjustment for covariates (OR = 0.32, 95%CI: 0.11-0.95). Supplementary Tables 1 and 2 shows that there may be a nonsignificant decreasing trend of *H. pylori* infection rate when alcohol consumption is increasing.

Figure 2 illustrates the association between dietary factors and the risk of *H. pylori* infection after the adjustment for covariates *via* the unconditional logistic regression model. The result indicated that peanut intake was significantly associated with a reduced risk of *H. pylori* infection (OR = 0.39, 95%CI 0.20-0.74). Supplementary Table 3 shows that there may be a significant positive association between peanut consumption and alcohol drinking (P for trend < 0.05).

Table 1 Characteristics and potential factors in cases with esophageal precancerous lesions and controls

Category	Cases, *n* = 200	Controls, *n* = 200	Adjusted OR (95%CI)[1]	*P* value
Age (yr), mean ± SD	63.01 ± 6.08	62.85 ± 6.03		0.792[a]
BMI (kg/m^2), mean ± SD	24.52 ± 3.33	24.36 ± 3.37		0.631[a]
Normal (18.5-23.9)	82 (41.0%)	84 (42.0%)	1.00 (reference)	
Underweight (< 18.5)	4 (2.0%)	5 (2.5%)	0.61 (0.12-3.02)	0.545
Overweight (24.0-28.0)	84 (42.0%)	89 (44.5%)	0.95 (0.61-1.49)	0.836
Obese (> 28.0)	30 (15.0%)	22 (11.0%)	1.61 (0.83-3.12)	0.164
Education level				
Illiterate	100 (50.0%)	96 (48.0%)	1.00 (reference)	
Primary school education	74 (37.0%)	77 (38.5%)	0.79 (0.45-1.39)	0.413
Middle school education and higher	26 (13.0%)	27 (13.5%)	0.81 (0.36-1.79)	0.599
Annual income/person (RMB)				
1-5000	53 (26.5%)	42 (21.0%)	1.00 (reference)	
5001-10000	88 (44.0%)	86 (43.0%)	0.75 (0.45-1.25)	0.267
> 10000	59 (29.5%)	72 (36.0%)	0.67 (0.37-1.21)	0.183
Current smoking status (number of cigarettes/d)				
Non-smoker	126 (63.0%)	134 (67.0%)	1.00 (reference)	
1-10	20 (10.0%)	17 (8.5%)	1.39 (0.67-2.87)	0.381
11-20	38 (19.0%)	41 (20.5%)	1.10 (0.61-1.97)	0.755
> 20	16 (8.0%)	8 (4.0%)	3.11 (1.00-9.63)	0.049
Current drinking status (alcohol units consumed/d, 1 unit is 8 g or 10 mL of pure alcohol)				
Non-drinker	147 (73.5%)	151 (75.5%)	1.00 (reference)	
< 4	10 (5.0%)	10 (5.0%)	1.03 (0.41-2.59)	0.954
4-	26 (13.0%)	23 (11.5%)	1.02 (0.52-2.02)	0.946
8-	17 (8.5%)	16 (8.0%)	1.06 (0.47-2.42)	0.885
H. pylori infection				
Negative	153 (76.5%)	142 (71.0%)	1.00 (reference)	
Positive	47 (23.5%)	58 (29.0%)	0.75 (0.46-1.24)	0.265

[a]*P* value of two independent samples *t*-test.
[1]Conditional logistic regression model with adjustment for gender, age, BMI, education level, annual income, number of cigarettes per day, and alcohol units consumed per day, except the specific variable itself.
H. pylori: *Helicobacter pylori*; BMI: Body mass index; OR: Odds ratio.

DISCUSSION

This study revealed that in drinkers, there was an association between *H. pylori* infection and a reduced risk of EPL, which is an identified early stage of esophageal carcinogenesis. However, the relationship between *H. pylori* infection and ESCC is still subject to much discussion. Some researchers believed that infection with *H. pylori* can increase the risk of ESCC by causing gastric atrophy that promotes excessive bacterial growth and causes endogenous nitrosamine production[24-26]. However, other studies which held that *H. pylori* infection probably plays a protective role in ESCC postulated that the protection is mediated *via* gastric atrophy, whereas the mechanism is related to a reduced load of esophageal acid[27, 28]. Therefore, it is likely that ESCC might be affected in a double-edged manner by *H. pylori* infection, which is dependent on population and other possible external factors. For example, previous studies have indicated that acid regurgitation may be facilitated by the reduction in lower esophageal sphincter's pressure and the retard of both esophageal motility and gastric emptying due to large consumption of alcoholic beverages[29-33]. Therefore, the current hypothesis is that *H. pylori* infection just alleviates esophageal reflux caused by alcohol to some extent, thus reducing the risk of esophageal

Table 2 Difference in *Helicobacter pylori* infection among controls and cases with esophageal precancerous lesions

Group	*H. pylori* infection			*P* value[a]
	Negative	Positive	Positive rate	
Controls	142	58	29.0%	
EPL cases (upper and mid thoracic esophagus)	100	33	24.8%	0.384
EPL cases (lower thoracic esophagus)	53	14	20.9%	

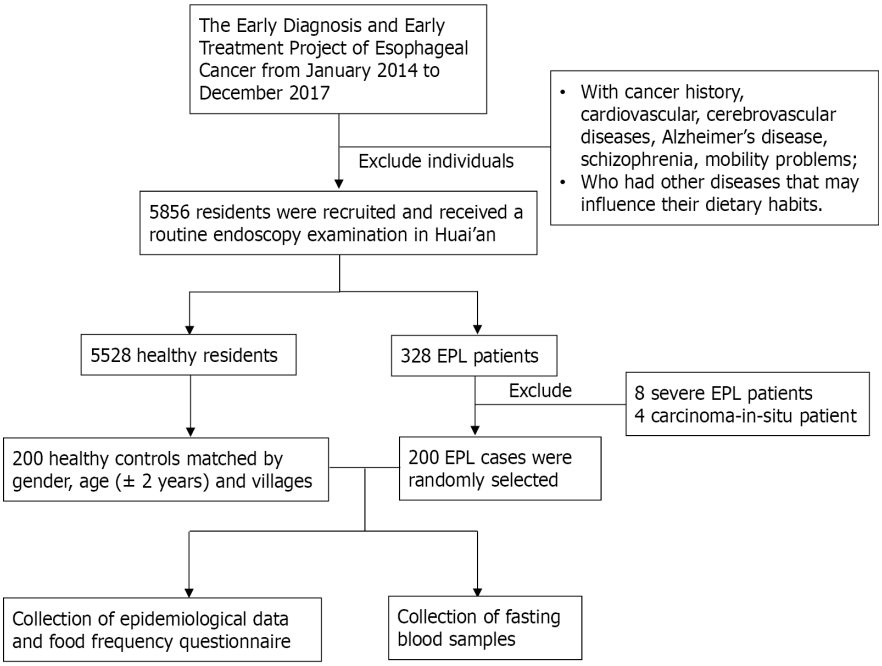
[a]*P* value of Fisher's exact test.
H. pylori: *Helicobacter pylori*.

Table 3 Association between *Helicobacter pylori* infection and esophageal precancerous lesion risk dichotomized by gender, age, cigarette smoking, and alcohol drinking

	Cases	Controls	Crude OR (95%CI)	*P* value	Adjusted OR (95%CI)[1]	*P* value
Male	*n* = 100	*n* = 100				
H. pylori (-)	78 (78.0%)	72 (72.0%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	22 (22.0%)	28 (28.0%)	0.73 (0.38-2.38)	0.328	0.64 (0.32-1.27)	0.200
Female	*n* = 100	*n* = 100				
H. pylori (-)	75 (75.0%)	70 (70.0%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	25 (25.0%)	30 (30.0%)	0.78 (0.42-1.45)	0.429	0.82 (0.42-1.58)	0.548
Age < 65 years	*n* = 107	*n* = 107				
H. pylori (-)	76 (71.0%)	73 (68.2%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	31 (29.0%)	34 (31.8%)	0.88 (0.49-1.57)	0.656	0.89 (0.47-1.67)	0.708
Age ≥ 65 years	*n* = 93	*n* = 93				
H. pylori (-)	77 (82.8%)	69 (74.2%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	16 (17.2%)	24 (25.8%)	0.60 (0.29-1.22)	0.156	0.59 (0.27-1.28)	0.183
Cigarette smoking (-)	*n* = 126	*n* = 134				
H. pylori (-)	97 (77.0%)	93 (69.4%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	29 (23.0%)	41 (30.6%)	0.68 (0.39-1.18)	0.170	0.74 (0.42-1.32)	0.310
Cigarette smoking (+)	*n* = 74	*n* = 66				
H. pylori (-)	56 (75.7%)	49 (74.2%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	18 (24.3%)	17 (25.8%)	0.93 (0.43-1.99)	0.845	0.80 (0.34-1.86)	0.601
Alcohol drinking (-)	*n* = 147	*n* = 151				
H. pylori (-)	109 (74.1%)	109 (72.2%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	38 (25.9%)	42 (27.8%)	0.91 (0.54-1.51)	0.702	0.94 (0.55-1.61)	0.831
Alcohol drinking (+)	*n* = 53	*n* = 49				
H. pylori (-)	44 (83.0%)	33 (67.3%)	1.00 (reference)	-	1.00 (reference)	-
H. pylori (+)	9 (17.0%)	16 (32.7%)	0.42 (0.17-1.07)	0.070	0.32 (0.11-0.95)	0.040

[1]Adjustment for gender, age, BMI, education level, annual income, number of cigarettes per day, and alcohol units consumed per day.
H. pylori: *Helicobacter pylori*; 95%CI: 95% confidence interval; OR: Odds ratio.

carcinogenesis caused by acid reflux. Our results also reported that EPL cases of lower thoracic esophagus had the lowest positive rate of *H. pylori* infection, which may support the hypotheses to some extent, although the difference was not statistically significant. In addition, there is more data indicating the positive role of this bacterium for humans. For example, a recent review considered the data on *H. pylori* and suggested that *H. pylori* may be a latent or opportunistic pathogen rather than a true pathogen



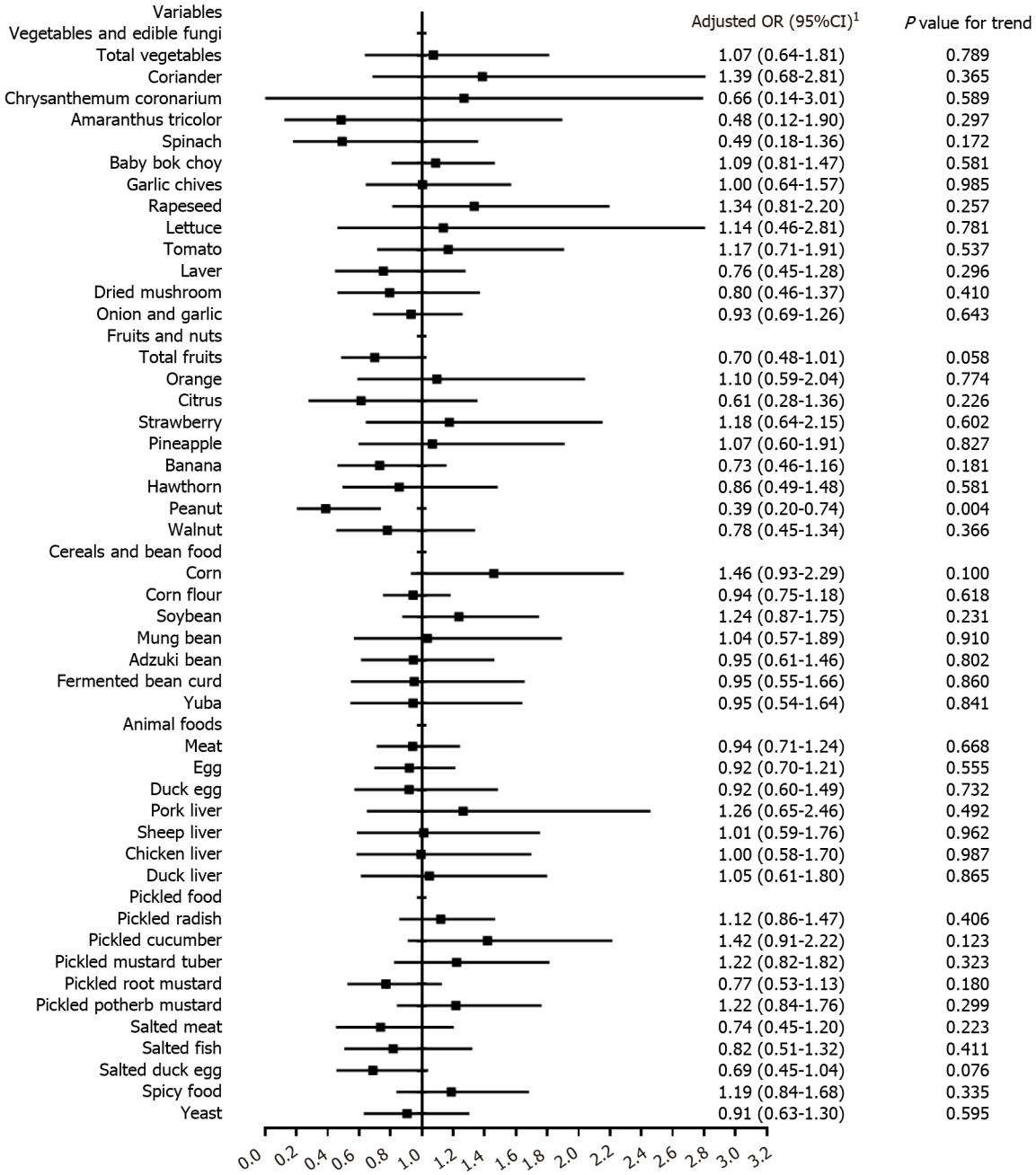
DOI: 10.4251/wjgo.v14.i9.1689

Figure 1 Flowchart of study population and sample collection.

of some diseases, and is possibly part of the normal human microbiome as a commensal or even a symbiont organism[34]. However, it was reported that a regular but moderate alcohol intake could possibly facilitate elimination of *H. pylori* infection[35]. Supplementary material also shows a nonsignificant decreasing trend of *H. pylori* infection rate when alcohol consumption is increasing. This partly supports the hypothesis that there is a possibility that drinkers without *H. pylori* infection could have more alcohol consumption. In other words, the decreased EPL risk in drinkers with *H. pylori* infection is possibly related to a lower alcohol consumption. However, because the result was not statistically significant, and there was no significant association between alcohol consumption and EPL risk in Huai'an in both this study and the previous epidemiological investigation[21], it is hard to address whether the reduced risk of EPL in drinkers with *H. pylori* infection was related to a reduced alcohol intake.

Additionally, the present study reported that the consumption of peanuts may provide protection from *H. pylori* infection. Since peanuts are high in fat, the duodenal mucosa secretes the hormone enterogastrone when fatty food is present in the stomach or small intestine[36]. Enterogastrone inhibits gastric movements and secretion of gastric acid, possibly by blocking the production or activity of gastrin, the hormone that initially leads to these functions[37]. Therefore, the reduced amount of acid produced may influence the growth of *H. pylori*, as *H. pylori* is dependent on acidity to survive for a long time[38]. In addition, in China, people are likely to drink and eat peanuts at the same time, and Supplementary material shows that there was a positive association between peanut consumption and alcohol drinking. Therefore, the inverse association between the consumption of peanut and the risk of *H. pylori* infection may be mediated by alcohol drinking. However, there is still a lack of evidence to verify the above hypotheses, thus further researches are required to evaluate the relationship between peanut consumption and *H. pylori* infection.

At present, about 50% of the global population and more than 70% of the population in some developing countries are infected by *H. pylori*[39]. However, this study reported that the positive rates of *H. pylori* infection were only 23.5% and 29.0% in EPL cases and healthy controls. In an early study conducted by Gao *et al*[40], Huai'an, Jiangsu Province was selected as a high incidence area of upper digestive tract cancers, and Pizhou, Jiangsu Province was selected as a low incidence area. They used ELISA and latex agglutinate test for the detection of *H. pylori* infection, and found that the prevalence of *H. pylori* infection among the gastric cancer group/upper digestive tract cancer group in the low incidence area of Pizhou (66.67%/63.46%) was significantly higher than that in the high incidence area of Huai'an (38.64%/39.33%). However, in the high incidence area of Huai'an, the prevalence of *H. pylori* infection in non-cancer controls and the healthy family members of the cancer cases was higher than that of cases. Therefore, the previous study and our study found that the prevalence of *H. pylori* infection in Huai'an may be much lower than that in other areas, and the prevalence in upper digestive tract cancers or EPL cases can be lower than that in non-cancer population in this region.



DOI: 10.4251/wjgo.v14.i9.1689

Figure 2 Association between dietary intake and the risk of *Helicobacter pylori* infection after adjustment for covariates *via* unconditional logistic regression model. BMI: Body mass index; OR: Odds ratio.

CONCLUSION

In summary, our study suggested that *H. pylori* infection is likely to decrease EPL risk in drinkers for a rural adult Chinese population, and the consumption of peanuts may be related to a reduced risk of *H. pylori* infection. However, the sample size used is a limitation of the study, which may bring difficulties to evaluate statistical significance in some statistical analyses, thus the findings should be framed as preliminary evidence. A case-control study might be difficult to determine causality, so the statement of "protective role" might be overestimated. Hence, it is necessary to design a large-scale prospective cohort study to address the impact of *H. pylori* infection on ESCC, the localization of lesions, and the association with dietary intake and the use of alcohol in the future. Additionally, the low prevalence of *H. pylori* infection in Huai'an is a peculiar finding, which implies that further investigations are recommended.

ARTICLE HIGHLIGHTS

Research background

The role of *Helicobacter pylori* (*H. pylori*) infection in esophageal squamous cell carcinoma (ESCC) remains a topic of much debate.

Research motivation

To assess the relationship between *H. pylori* infection and the risk of precancerous lesions of ESCC, which is an identified early stage of carcinogenesis.

Research objectives

This study aimed to evaluate the association between *H. pylori* infection and the risk of esophageal precancerous lesions (EPL) in a high-incidence area in Huai'an, and further explore the association between dietary factors and the risk of *H. pylori* infection.

Research methods

The study was based on a case-control design. Epidemiological data were collected and *H. pylori* seropositivity was tested. An unconditional logistic regression model was used to analyze the association between *H. pylori* infection and EPL risk with adjustment for confounders, as well as the association between dietary factors and risk of *H. pylori* infection.

Research results

The control group had the highest positive rate of *H. pylori* infection (29.0%), followed by EPL cases of upper and mid thoracic esophagus (24.8%) and EPL cases of lower thoracic esophagus (20.9%). The protective effect of *H. pylori* infection against the risk of EPL was observed in the group of drinkers after adjustment for covariates [odds ratio (OR) = 0.32, 95% confidence interval (95%CI): 0.11-0.95]. Peanut intake was significantly associated with a reduced risk of *H. pylori* infection (OR = 0.39, 95%CI: 0.20-0.74).

Research conclusions

H. pylori infection may decrease the risk of EPL in drinkers for a rural adult Chinese population, and the consumption of peanuts may be related to a reduced risk of *H. pylori* infection.

Research perspectives

A well-designed prospective cohort study is required to address the impact of *H. pylori* infection on ESCC, the localization of lesions, and the association with dietary intake and alcohol drinking. Additionally, the low prevalence of *H. pylori* infection in Huai'an is a peculiar finding, which implies that further investigations are recommended.

ACKNOWLEDGEMENTS

Thanks to all the subjects and researchers for their contributions and hard work. We really appreciate Mr Rob Unwin's assistance in polishing the manuscript.

FOOTNOTES

Author contributions: Pan D, Wang SK, and Sun GJ designed the research; Pan D, Su M, Yan QY, Song G, Wang YY, Xu DF, and Wang NN conducted the research; Wang SK, Su M, Wang X, and Sun GJ provided essential materials; Pan D analyzed the data and wrote the manuscript; Wang SK had primary responsibility for final content; all authors read and approved the final manuscript.

Supported by the Fellowship of China Postdoctoral Science Foundation, No. 2022M710675; the National Natural Science Foundation of China, No. 81673147; and the Danone Dietary Nutrition Research and Education Foundation, No. DIC2020-08.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Southeast University Zhongda Hospital (No. 2016ZDKYSB017).

Informed consent statement: Written informed consent was obtained from all subjects.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: All data generated or analyzed during this study are included. The technical appendix and statistical procedure are available from the corresponding author.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: Wang TQ
P-Editor: Chen YL

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1699-1710

DOI: 10.4251/wjgo.v14.i9.1699

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic impact of tumor deposits on overall survival in colorectal cancer: Based on Surveillance, Epidemiology, and End Results database

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Lunkka P, Finland; Rajagopalan A, Australia; Shinozaki E, Japan

Received: April 19, 2022
Peer-review started: April 19, 2022
First decision: May 11, 2022
Revised: May 18, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

In colorectal cancer, tumor deposits (TDs) are considered to be a prognostic factor in the current staging system, and are only considered in the absence of lymph node metastases (LNMs). However, this definition and the subsequent prognostic value based on it is controversial, with various hypotheses. TDs may play an independent role when it comes to survival and addition of TDs to LNM count may predict the prognosis of patients more accurately.

AIM

To assess the prognostic impact of TDs and evaluate the effect of their addition to the LNM count.

METHODS

The patients are derived from the Surveillance, Epidemiology, and End Results database. A prognostic analysis regarding impact of TDs on overall survival (OS) was performed using Cox regression model, and other covariates associating with OS were adjusted. The effect of addition of TDs to LNM count on N restaging was also evaluated. The subgroup analysis was performed to explore the different profile of risk factors between patients with and without TDs.

RESULTS

Overall, 103755 patients were enrolled with 14131 (13.6%) TD-positive and 89624 (86.4%) TD-negative tumors. TD-positive patients had worse prognosis compared with TD-negative patients, with 3-year OS rates of 47.3% (95%CI, 46.5%-48.1%) and 77.5% (95%CI, 77.2%-77.8%, $P < 0.0001$), respectively. On multivariable

analysis, TDs were associated poorer OS (hazard ratio, 1.35; 95%CI, 1.31-1.38; $P < 0.0001$). Among TD-positive patients, the number of TDs had a linear negative effect on disease-free survival and OS. After reclassifying patients by adding TDs to the LNM count, 885 of 19 965 (4.4%) N1 patients were restaged as pN2, with worse outcomes than patients restaged as pN1 (3-year OS rate: 78.5%, 95%CI, 77.9%-79.1% *vs* 63.2%, 95%CI, 60.1%-66.5%, respectively; $P < 0.0001$).

CONCLUSION

TDs are an independent prognostic factor for OS in colorectal cancer. The addition of TDs to LNM count improved the prognostic accuracy of tumor, node and metastasis staging.

Key Words: Extranodal extension; Colorectal neoplasms; Prognosis; Neoplasm staging; Surveillance, Epidemiology, and End Results program

Core Tip: We evaluated the predictive value of tumor deposits (TDs) for overall survival (OS) in patients with colorectal cancer based on a collection of 103755 patients derived from Surveillance, Epidemiology, and End Results database, including TD-negative and TD-positive subpopulations with Cox proportional hazard model. The sensitivity analyses were performed to detect outcome robustness. TD was an independent prognostic factor for OS. We also performed exploratory analysis to evaluate the effect of TD addition to the lymph node metastases count in tumor, node and metastasis-stage III subpopulations. The outcomes of subgroup analysis investigating the different risk factor profiles indicated that TDs may affect survival through more than one approach.

Citation: Wu WX, Zhang DK, Chen SX, Hou ZY, Sun BL, Yao L, Jie JZ. Prognostic impact of tumor deposits on overall survival in colorectal cancer: Based on Surveillance, Epidemiology, and End Results database. *World J Gastrointest Oncol* 2022; 14(9): 1699-1710
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1699.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1699

INTRODUCTION

Colorectal cancer was the third most common cancer and second leading cause of death among all types of cancer with 1.93 million new cases and 0.94 million deaths in 2020[1]. The key point of treatment for colorectal cancer is to determine the stage on which we depend when carrying out treatment strategies. The American Joint Committee on Cancer (AJCC) tumor, node and metastasis (TNM) staging system is the standard tool for staging. Staging systems for colorectal cancer are evolving as more information regarding predictors of outcome emerges; among which, tumor deposits (TDs) have been debated and investigated. Previous studies have shown that TDs are associated with poor survival and earlier development of metastasis[2-4]. However, the definition and prognostic value of TDs remains controversial. TDs first appeared in the fifth edition of TNM staging system in 1997 and the definition of TDs has been evolving since then. The distinction of a TD from involved lymph nodes (LNs) has progressed from a reliance on size, to contours, to only features of residual LN structure[2,5]. The latest TNM 8th staging system was released in 2016, aiming to exclude any lesion with identifiable structures pointing towards LN metastasis (LNM), extramural venous invasion or perineural invasion[6]. However, some researchers have proposed that nodules with evidence of origin should still be categorized as TDs and the exclusion of lesions of vascular, lymphatic and perineural origin by TNM 8th has no evidence base[7,8]. Another controversial issue is the introduction of a new category of N1c in the TNM staging system. In the 7th edition, if TDs are observed with lesions that would otherwise be classified as T1 or T2, then T classification is not changed but nodules are recorded as N1c in the absence of LN involvement. The prognostic value of N1c remains unclear. Some researchers suggest that TDs should be taken into consideration for N staging, while others propose that N1c is not by definition worse than N1a or N1b and the use of N1c was chosen because the letter c was the subsequent letter in the alphabet[5]. The *post hoc* analyses of the IDEA France and GALGB/SWOG 80702 studies have suggested addition of TDs to the LNM count. The results of these studies require validation, as the potential bias may derive from the *post hoc* analysis and some information related to the analysis was not recorded in the primary clinical trial. Moreover, the outcomes of these study could only represent a part of patients due to the rigorous inclusion criteria. As a result of these controversies and the fact that the TNM stage can affect the therapeutic decision, this analysis aimed to assess the prognostic impact of TDs in colorectal cancer and to evaluate the effect of their addition to the LNM count.

MATERIALS AND METHODS

Patients

The patients in the current study are derived from the Surveillance, Epidemiology, and End Results (SEER) database (November 2020). We enrolled patients diagnosed with colorectal cancer between 2010 and 2015. SEER used a study cutoff date for data submission and the study cutoff was 12/31/2018 for the November 2020 data submission. All deaths up to this point had been recorded in the data through death clearance linkages. The survival time was recorded as the interval between the time of diagnosis and the date of last contact. For cancer registries that did not conduct active patient follow-up, the presumed-alive method was used by which the survival time was calculated based on the assumption that the registry has ascertained all available deaths, and persons not known to be deceased were presumed to be alive on the last date for which complete death ascertainment was available. The inclusion criteria were: histological confirmed colorectal cancer, malignant behavior, known age, without other *in situ* or malignant tumors. Exclusion criteria were: patients without available TNM stage, TDs indeterminate or not documented, last contact date was the date of diagnosis, and survival time not documented.

The histopathological characteristics, including survival months, survival status, race, tumor site, carcinoembryonic antigen (CEA), perineural invasion, sex, age, TNM stage, liver metastasis, lung metastasis and TDs, were derived from the SEER database. Patients were allocated into White, Black and Others according to race. Tumor site was reclassified as colon and rectum. Age was pooled into three groups of < 45, 45-75 and ≥ 75 years. Patients were divided into two categories based on the presence or absence of TDs. The TNM stage for patients was derived from the 7th AJCC TNM staging system. The outcome included OS, defined as the time from diagnosis to any cause of death, and patients lost to follow-up were treated as censored, which is equivalent to the record of survival months derived from the SEER database. This study was based on the public data derived from SEER database in which the private information related to patients was not available. Therefore, this study was exempt from institutional review board approval and informed consent.

Methods

The primary objective of the current study was to assess the association between the presence of TDs and OS. As exploratory outcomes, the impact of number of TDs on OS was investigated in patients with available record for number of tumor deposits and the N stage was reclassified to the novel N category by the addition of TDs to the LNM count. A score of 2 was assigned for the number of LNMs of cases with stage N1b. Finally, survival was estimated according to this reclassification.

Continuous and categorical variables were summarized as median values with interquartile ranges and frequencies with percentages. Proportions were compared using the χ^2 test. Cox proportional hazards models were performed to estimate hazard ratio (HR) and 95%CIs for factors associated with OS. Parameters with $P < 0.1$ in the univariable Cox analysis were entered into a final multivariable Cox regression model including TDs, with stepwise selection for both directions with respect to collinearity among covariates after excluding variables with > 10% missing data. To assess robustness of the association between TDs and OS evaluated in the primary Cox multivariable analysis, multiple imputation was performed to limit the bias as a result of missing data for sensitivity analysis. With regard to potential heterogeneity between patients with and without TDs, a propensity score approach with inverse probability of treatment weighting (IPTW) method was applied. Survival curves were constructed using the Kaplan–Meier method. Curves adjusted for covariates associated with OS in Cox regression model were also performed. The difference of HRs between subgroups was tested[9]. The statistical methods were reviewed by Wen-Quan Niu from the Institute of Clinical Medical Science of the China–Japan Friendship Hospital.

RESULTS

Using data from 18 SEER registries between 2010 and 2015, 162328 patients were diagnosed with colorectal cancer and 103755 patients were enrolled in the current study. Baseline characteristics with respect to the presence or absence of TDs are listed in Table 1: 14131 patients (13.6%) had TDs and 89624 patients (86.4%) had no TDs. Patients with TDs were more likely to have advanced-stage tumors (linear-by-linear association $P < 0.0001$). Similar trends were also observed as for T-stage and N-stage. Patients with TDs had more extensive T-stage and higher nodal stage. In the TD-positive subpopulation, patients had more metastatic disease including liver (25.7% *vs* 7.2% in TD-negative patients; $P < 0.001$) and lungs (6.3% *vs* in 1.7% TD-negative patients; $P < 0.001$), more perineural invasion (33.0% *vs* 8.2% in TD-negative patients; $P < 0.001$) and elevated CEA (40.4% *vs* 23.4% in TD-negative patients; $P < 0.001$). The presence of TDs was associated with tumors in the colon and in younger patients (Table 1).

The median overall follow-up was 68 (31.0-74.0) mo. Median OS was 34.0 (33.0–36.0) mo for TD-positive patients and not reached in the TD-negative patients. According to the presence or absence of TDs, TD-positive patients had a worse prognosis than TD-negative patients. The 3-year OS rates were

Table 1 Characteristics of patients according to the presence and absence of tumor deposits

	Tumor deposits		*P* value
	No (89624)	Yes (14131)	
Sex			0.827
Female	43569 (48.6%)	6855 (48.5%)	
Male	46055 (51.4%)	7276 (51.5%)	
Race			0.420
White	70019 (78.1%)	11015 (77.9%)	
Black	10345 (11.5%)	1681 (11.9%)	
Others	9260 (10.3%)	1435 (10.2%)	
Age group, yr			< 0.001[a]
< 45	5740 (6.40%)	1231 (8.71%)	
45-75	58860 (65.7%)	9387 (66.4%)	
≥ 75	25024 (27.9%)	3513 (24.9%)	
TNM-stage			< 0.001[a]
I	24816 (27.7%)	111 (0.79%)	
II	29374 (32.8%)	825 (5.84%)	
III	26627 (29.7%)	7697 (54.5%)	
IV	8807 (9.83%)	5498 (38.9%)	
T-stage			< 0.001[a]
T1	15480 (17.3%)	181 (1.28%)	
T2	14312 (16.0%)	482 (3.41%)	
T3	47654 (53.2%)	7890 (55.8%)	
T4	12178 (13.6%)	5578 (39.5%)	
N-stage			< 0.001[a]
N0	56512 (63.1%)	1247 (8.82%)	
N1	22127 (24.7%)	6582 (46.6%)	
N2	10985 (12.3%)	6302 (44.6%)	
M-stage			< 0.001[a]
M0	80817 (90.2%)	8633 (61.1%)	
M1	8807 (9.83%)	5498 (38.9%)	
Liver metastasis			< 0.001[a]
No	82821 (92.4%)	10377 (73.4%)	
Yes	6443 (7.19%)	3627 (25.7%)	
Unknown	360 (0.40%)	127 (0.90%)	
Lung metastasis			< 0.001[a]
No	87677 (97.8%)	13053 (92.4%)	
Yes	1526 (1.70%)	893 (6.32%)	
Unknown	421 (0.47%)	185 (1.31%)	
Site			< 0.001[a]
Colon	71779 (80.1%)	11697 (82.8%)	
Rectum	16603 (18.5%)	2148 (15.2%)	
Unknown	1242 (1.39%)	286 (2.02%)	

CEA			< 0.001[a]
Normal	32166 (35.9%)	3543 (25.1%)	
Elevated	20936 (23.4%)	5705 (40.4%)	
Borderline	279 (0.31%)	48 (0.34%)	
Unknown	36243 (40.4%)	4835 (34.2%)	
Perineural invasion			< 0.001[a]
Negative	75025 (83.7%)	8437 (59.7%)	
Positive	7301 (8.15%)	4659 (33.0%)	
Unknown	7298 (8.14%)	1035 (7.32%)	

[a]$P < 0.05$.

TNM: Tumor, node and metastasis; CEA: Carcinoembryonic antigen.

47.3% (95%CI, 46.5%-48.1%) and 77.5% (95%CI, 77.2%-77.8%, log rank $P < 0.0001$), respectively. The negative effect of TDs on OS was observed for both N1 and N2 subgroups. Three-year OS rates for N1a/b patients with or without TDs were 53.5% (95%CI, 52.0%-55.0%) and 73.6% (95%CI, 73.0%-74.2%, log rank $P < 0.0001$), respectively. For N2 patients with or without TDs, 3-year OS rates were 35.5% (95%CI, 34.3%-36.7%) and 54.7% (95%CI, 53.7%-55.6%, log rank $P < 0.0001$) (Figure 1A).

In a univariable Cox model, the presence of TDs was associated with poor OS (HR, 2.73; 95%CI, 2.67-2.80; $P < 0.0001$). Other variables significantly associated with OS were TNM, T, N, M, race, age, tumor site, CEA, perineural invasion, liver metastasis and lung metastasis. In multivariable analysis including TNM-stage, T-stage, N-stage, TDs, liver metastasis, lung metastasis, age, perineural invasion and race, the negative prognostic impact of TD remained significant (HR, 1.35; 95%CI, 1.31-1.38; $P < 0.0001$) (Table 2). Because of unavailable records, 10291 patients were excluded in a multivariable Cox model analysis and the factor CEA with 39.6% missing data was excluded. The analysis outcome for the complete dataset was robust with multiple imputation (HR, 1.39; 95%CI, 1.35-1.42; $P < 0.0001$) and propensity score approach with IPTW method (HR, 1.29; 95%CI, 1.19-1.39; $P < 0.0001$) (Table 3). After adjusting for other covariates, the HR value of TDs was lowered. In the subgroup analysis, T-stage, N-stage, M-stage, CEA, perineural invasion, liver metastasis and lung metastasis were associated with poor OS both in patients with and without TDs, but these risk factors had less impact on survival in patients with than those without TDs, which may partly explain the lower HR value in multivariable analysis (Table 4).

In the exploratory analysis, there were 7860 patients with records of numbers of TDs. Among these, the number of TDs was subdivided into four groups with 1, 2, 3 and ≥ 4 TDs. The 3-year OS rates were 62.8% (95%CI, 61.2%–64.5%), 55.6% (95%CI, 53.2%–58.1%), 51.6%, (95%CI, 48.3%-55.1%), and 39.7% (95%CI, 37.6%–42.0%; $P < 0.0001$), respectively (Figure 1B). The 3-year OS rates were linearly associated with the number of TDs (P for trend < 0.0001).

There were 19965 N1-staged patients with records of numbers of TDs, in TNM-stage III subpopulations. Among these, 885 were restaged as N2 by the addition of TDs to the LNM count (Table 5). Patients with tumors restaged as N2 had a lower 3-year OS rate than those with tumors remaining as N1 despite the addition of TDs to the LNM count (78.5%, 95%CI, 77.9%-79.1% *vs* 63.2%, 95%CI, 60.1%-66.5%, respectively; $P < 0.0001$). OS was not different between patients restaged as N2 and those initially staged as N2 (63.2%, 95%CI, 60.1%-66.5% *vs* 61.7%, 95%CI, 60.8%-62.6%, respectively; $P = 0.8$) (Figure 1C).

DISCUSSION

In the current TNM staging system for colorectal cancer, neither the presence nor the number of TDs is considered in the N staging in case of concomitant LNM, and the N1c category is only used if no LNM is present.

Our study demonstrated that the presence of TDs was associated with significantly poorer survival outcomes and the negative impact of TDs remained significant across all N stages, indicating that TDs should be considered when performing N staging. The number of TDs had a linear effect on OS. Thus, valuable prognostic information is lost when ignoring the number of TDs. Given the prognostic value of TDs both qualitatively and quantitatively, we went further in our analysis by adding the number of TDs to the LNM count. The current study is, to our knowledge, the largest comparative effectiveness research to investigate reclassification of the TNM staging system by incorporation of TDs into the LNM count. We showed that N1-staged patients who were reclassified as N2 through the integration of the number of TDs into LNM count had poorer outcomes than those who remained as N1, despite the

Table 2 Overall survival univariate and multivariate Cox models of baseline characteristics

	Univariate Cox models			Multivariate Cox model		
	Events/total	HR (95%CI)	*P* value	Events/total	HR (95%CI)	*P* value
Sex			0.3665			
Female	19008/50424	Reference				
Male	20358/53331	1.01 (0.99-1.03)	0.3665			
Race			< 0.0001[a]			< 0.0001[a]
White	30958/81034	Reference		27279/73091	Reference	
Black	5049/12026	1.14 (1.11-1.17)	< 0.0001[a]	4440/10821	1.19 (1.16-1.23)	< 0.0001[a]
Others	3359/10695	0.80(0.77-0.83)	<.0001[a]	2926/9552	0.83(0.80-0.86)	<.0001[a]
Age group, yr			< 0.0001[a]			< 0.0001[a]
< 45	1866/6971	Reference		1627/6276	Reference	
45-75	20956/68247	1.17 (1.11-1.22)	< 0.0001[a]	18394/61529	1.49 (1.42-1.57)	< 0.0001[a]
≥ 75	16544/28537	2.78 (2.65-2.92)	< 0.0001[a]	14624/25659	4.40 (4.17-4.63)	< 0.0001[a]
TNM-stage			< 0.0001[a]			< 0.0001[a]
I	4709/24927	Reference		4154/22321	Reference	
II	9334/30199	1.79 (1.73-1.85)	< 0.0001[a]	8362/27688	0.96 (0.89-1.02)	0.2060
III	13498/34324	2.46 (2.38-2.55)	< 0.0001[a]	12086/31183	1.32 (1.21-1.43)	< 0.0001[a]
IV	11825/14305	9.08 (8.77-9.40)	< 0.0001[a]	10043/12272	3.27 (2.99-3.56)	< 0.0001[a]
T-stage			< 0.0001[a]			< 0.0001[a]
T1	2794/15661	Reference		2259/13476	Reference	
T2	3477/14794	1.35 (1.29-1.42)	< 0.0001[a]	3167/13728	1.18 (1.12-1.25)	< 0.0001[a]
T3	21562/55544	2.52 (2.42-2.62)	< 0.0001[a]	19171/50529	1.71 (1.59-1.83)	< 0.0001[a]
T4	11533/17756	5.80 (5.57-6.05)	< 0.0001[a]	10048/15731	2.83 (2.63-3.05)	< 0.0001[a]
N-stage			< 0.0001[a]			< 0.0001[a]
N0	16014/57759	Reference		14081/52146	Reference	
N1	12199/28709	1.73 (1.69-1.78)	< 0.0001[a]	10718/25885	0.96 (0.91-1.02)	0.1880
N2	11153/17287	3.38 (3.30-3.47)	< 0.0001[a]	9846/15433	1.44 (1.36-1.53)	< 0.0001[a]
M-stage			< 0.0001[a]			
M0	27541/89450	Reference				
M1	11825/14305	5.05 (4.94-5.16)	< 0.0001[a]			
Liver metastasis			< 0.0001[a]			< 0.0001[a]
No	30670/93198	Reference		27418/84728	Reference	
Yes	8406/10070	4.67(4.56-4.79)	< 0.0001[a]	7227/8736	1.33(1.28-1.39)	< 0.0001[a]
Lung metastasis			< 0.0001[a]			< 0.0001[a]
No	36847/100730	Reference		32838/91390	Reference	
Yes	2119/2419	4.62 (4.42-4.83)	< 0.0001[a]	1807/2074	1.32 (1.25-1.38)	< 0.0001[a]
Site			< 0.0001[a]			
Colon	32774/83476	Reference				
Rectum	5782/18751	0.71 (0.69-0.73)	< 0.0001[a]			
CEA			< 0.0001[a]			
Normal	9708/35709	Reference				
Elevated	14098/26641	2.46 (2.40-2.53)	< 0.0001[a]			

Borderline	125/327	1.51 (1.26-1.80)	0.0001[a]			
Perineural invasion			< 0.0001[a]			< 0.0001[a]
Negative	28478/83462	Reference		27655/81868	Reference	
Positive	7260/11960	2.32 (2.26-2.38)	< 0.0001[a]	6990/11596	1.22 (1.19-1.26)	< 0.0001[a]
Tumor deposits			< 0.0001[a]			< 0.0001[a]
No	30165/89624	Reference		26523/80813	Reference	
Yes	9201/14131	2.73 (2.67-2.80)	< 0.0001[a]	8122/12651	1.35 (1.31-1.38)	< 0.0001[a]

[a]$P < 0.05$.

TNM: Tumor, node and metastasis; CEA: Carcinoembryonic antigen.

Table 3 Sensitivity analysis for effect of tumor deposits on overall survival

Analysis	HR (95%CI)	*P* value
Univariable analysis	2.73 (2.67-2.80)	< 0.0001[a]
Multivariable analysis	1.35 (1.31-1.38)	< 0.0001[a]
Propensity score analysis (with inverse probability of treatment weighting)	1.29 (1.19-1.39)	< 0.0001[a]
Multiple imputation for missing data analysis	1.39 (1.35-1.42)	< 0.0001[a]

[a]$P < 0.05$.

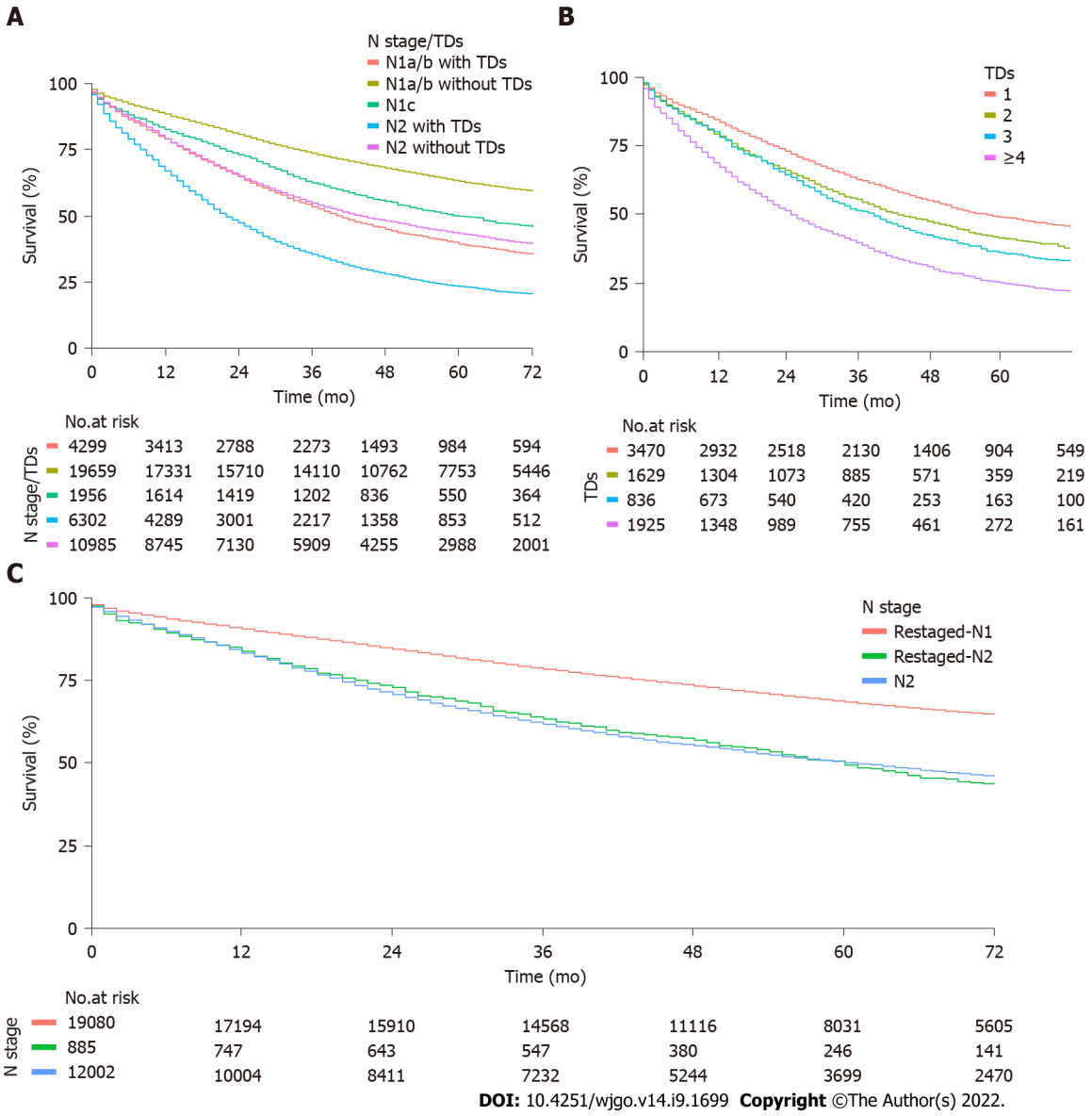
HR: Hazard ratio.

addition of TDs to the LNM count and outcomes similar to those of patients initially staged as N2. Therefore, our results, in agreement with other studies[3,4,10], suggest that both TDs and their numbers should be integrated into N staging and that the N1c category in TNM staging was inappropriate because there were subpopulations with ≥ 4 TDs whose survival was similar to that in patients with ≥ 4 LNMs. Moreover, the results were similar in subgroup analysis when considering the different tumor sites. Our study is, to our knowledge, the first to investigate the outcomes of reclassification in patients with rectal cancer.

Advanced TNM stage, extensive T-stage, higher nodal stage, metastatic disease, perineural invasion and elevated CEA were more often present among TD-positive patients. Although these correlations may partly explain the pejorative prognosis of TD-positive tumors, the poor prognostic value of TDs remains when the imbalance of these covariates is taken into account in the propensity score approach analysis. The different HR values of these covariates between TD-positive and -negative subpopulations remain to be clarified, which may indicate more than one way through which TDs influence survival [10]. In light of these results, we propose that the presence of TDs is an independent prognostic factor for OS in colorectal cancer and the origin and formation of TDs need to be further investigated.

Although there were multiple origins reported in previous studies of TDs, including perineural, perivascular, intravascular and a mixture of them[10-13], the definition of TDs is still ambiguous with regard to the inclusion of recognized structures of vascular, lymphatic and perineural TDs[8]. The hypotheses of mechanisms through which the TDs affect survival are diverse. A previous study demonstrated that TD-positive patients was more likely to present vascular and perineural invasion [14]. Certain groups showed that the prognostic value of TDs and extra nodal extension of which the negative effect towards survival has been demonstrated previously was similar with regard to HR values for OS and DFS. Thus, some researchers suggested that TDs could be complete replacement of an lymph node by metastatic tumor and represent the advanced stage of extra nodal extension[8,15-18]. Some authors hypothesize that TDs may reflect blood-borne spread associated with poor prognosis and may be included in M category[19], while others consider TDs as in-transit metastases, where tumor cells spread through lymphatic channels and form tumors before reaching LNs[20]. In addition, the biological behavior of TDs is considered to be similar to tumor budding in the leading area of colorectal cancer, which represents migration over and crossing through histological boundaries[11]. The TDs may migrate and metastasize after undergoing epithelial-to-mesenchymal transition[21].

There were two major limitations to the current study. First, the results of the exploratory analysis may reflect potential bias due to the missing data of TDs. However, it does lend support to the TD-based staging approach. Second, we did not take into consideration that novel adjuvant therapy has already been the standard regimen in some settings. Further studies are needed to investigate patients with and without novel adjuvant therapy, especially when patients achieve substantial downstaging, to



DOI: 10.4251/wjgo.v14.i9.1699

Figure 1 Overall survival in subpopulations with colorectal cancer. A: N1a/b, N1c and N2 patients with colorectal cancer according to the presence or absence of tumor deposits; B: Patients with 1, 2, 3 and ≥ 4 tumor deposits; C: Patients restaged as N1, N2 after the addition of tumor deposits to lymph node metastases count and patients initially staged as N2. OS: Overall survival; CRC: Colorectal cancer; TDs: Tumor deposits; LNM: Lymph node metastases.

substantiate the definition and demonstrate the pathogenesis of TDs[22]. In the exploratory analysis, we chose a worse-case scenario by assigning a value of 2 for the number of LNs involved for cases with N1b stage, by which some patients were confirmed as N1 who should in fact be restaged as N2. Despite this, the outcome still indicated the addition of TDs to LNM count. Therefore, we do not believe that this compromises the accuracy of our results. Our analysis shows that TDs play an important role in the survival of patients. The N1c category is not optimal in the current staging system and adding the number of TDs to LN count may improve the prognostic accuracy. In addition, more investigations are needed with respect to the origin and pathophysiological mechanism of development of TDs, by which a more reproducible and scientific definition can be developed.

CONCLUSION

Addition of TDs to the LNM count improves the prognostic accuracy of current TNM staging. However, the origin and pathogenesis of TDs remain to be clarified.

Table 4 Univariable Cox analysis in patients with and without tumor deposits

	TDs (no)			TDs (yes)			RHR	*P* value
	Events/total	HR (95%CI)	*P* value	Events/total	HR (95%CI)	*P* value		
Sex			0.1265			0.0528		
Female	14534/43569	Reference		4474/6855	Reference			
Male	15631/46055	1.02 (1.00-1.04)	0.1265	4727/7276	0.96 (0.92-1.00)	0.0528		
Race			< 0.0001[a]			< 0.0001		
White	23736/70019	Reference		7222/11015	Reference			
Black	3904/10345	1.16 (1.12-1.20)	< 0.0001[a]	1145/1681	1.06 (1.00-1.13)	0.0536	0.91 (0.85-0.98)	0.0118[a]
Others	2525/9260	0.79 (0.76-0.82)	< 0.0001[a]	834/1435	0.83 (0.77-0.89)	< 0.0001[a]	1.05 (0.97-1.14)	0.2380
Age group, yr			< 0.0001[a]			< 0.0001[a]		
< 45	1201/5740	Reference		665/1231	Reference			
45-75	15238/58860	1.26 (1.19-1.34)	< 0.0001[a]	5718/9387	1.23 (1.13-1.33)	< 0.0001[a]	0.98 (0.88-1.08)	0.6384
≥ 75	13726/25024	3.37 (3.18-3.58)	< 0.0001[a]	2818/3513	2.34 (2.15-2.55)	< 0.0001[a]	0.69 (0.63-0.77)	< 0.0001[a]
TNM-stage			< 0.0001[a]			< 0.0001[a]		
I	4683/24816	Reference		26/111	Reference			
II	8962/29374	1.77 (1.70-1.83)	< 0.0001[a]	372/825	2.40 (1.61-3.57)	< 0.0001[a]	1.36 (0.91-2.02)	0.1362
III	9545/26627	2.17 (2.09-2.24)	< 0.0001[a]	3953/7697	3.05 (2.08-4.49)	< 0.0001[a]	1.41 (0.96-2.07)	0.0836
IV	6975/8807	8.05 (7.76-8.36)	< 0.0001[a]	4850/5498	9.25 (6.29-13.6)	< 0.0001[a]	1.15 (0.78-1.69)	0.4840
T-stage			< 0.0001[a]			< 0.0001[a]		
T1	2726/15480	Reference		68/181	Reference			
T2	3321/14312	1.35 (1.29-1.42)	< 0.0001[a]	156/482	0.84 (0.63-1.11)	0.2246	0.62 (0.47-0.83)	0.0012[a]
T3	17029/47654	2.30 (2.20-2.39)	< 0.0001[a]	4533/7890	1.84 (1.45-2.34)	< 0.0001[a]	0.80 (0.63-1.02)	0.0602
T4	7089/12178	4.84 (4.63-5.05)	< 0.0001[a]	4444/5578	3.69 (2.90-4.68)	< 0.0001[a]	0.76 (0.60-0.97)	0.0286[a]
N-stage			< 0.0001[a]			< 0.0001[a]		
N0	15350/56512	Reference		664/1247	Reference			
N1	8434/22127	1.53 (1.49-1.57)	< 0.0001[a]	3765/6582	1.14 (1.05-1.24)	0.0014[a]	0.75 (0.68-0.81)	< 0.0001[a]
N2	6381/10985	2.86 (2.77-2.94)	< 0.0001[a]	4772/6302	1.99 (1.83-2.16)	< 0.0001[a]	0.70 (0.64-0.76)	< 0.0001[a]
M-stage			< 0.0001[a]			< 0.0001[a]		
M0	23190/80817	Reference		4351/8633	Reference			
M1	6975/8807	4.90 (4.77-5.04)	< 0.0001[a]	4850/5498	3.13 (3.00-3.27)	< 0.0001[a]	0.64 (0.61-0.67)	< 0.0001[a]
Site			< 0.0001[a]			< 0.0001[a]		
Colon	24983/71779	Reference		7791/11679	Reference			
Rectum	4595/16603	0.74 (0.71-0.76)	< 0.0001[a]	1187/2148	0.66 (0.62-0.71)	< 0.0001[a]	0.89 (0.83-0.96)	0.0030[a]
CEA			< 0.0001[a]			< 0.0001[a]		
Normal	7926/32166	Reference		1782/3543	Reference			
Elevated	9908/20936	2.34 (2.27-2.41)	< 0.0001[a]	4190/5705	1.95 (1.84-2.06)	< 0.0001[a]	0.83 (0.78-0.89)	< 0.0001[a]
Borderline	95/279	1.47 (1.20-1.80)	0.0002[a]	30/48	1.32 (0.92-1.89)	0.1343	0.90 (0.59-1.36)	0.6100
Perineural invasion			< 0.0001[a]			< 0.0001[a]		
Negative	23417/75025	Reference		5061/8437	Reference			
Positive	3845/7301	2.05 (1.98-2.12)	< 0.0001[a]	3415/4659	1.46 (1.40-1.52)	< 0.0001[a]	0.71 (0.68-0.75)	< 0.0001[a]
Liver metastasis			< 0.0001[a]			< 0.0001[a]		
No	24811/82821	Reference		5859/10377	Reference			

Yes	5164/6443	4.73 (4.59-4.88)	< 0.0001[a]	3242/3627	2.66 (2.54-2.78)	< 0.0001[a]	0.56 (0.53-0.59)	< 0.0001[a]
Lung metastasis			< 0.0001[a]			< 0.0001[a]		
No	28622/87677	Reference		8225/13053	Reference			
Yes	1297/1526	4.97 (4.70-5.25)	< 0.0001[a]	822/893	2.40 (2.23-2.58)	< 0.0001[a]	0.48 (0.44-0.53)	< 0.0001[a]

[a]$P < 0.05$.

TNM: Tumor, node and metastasis; CEA: Carcinoembryonic antigen; HR: Hazard ratio; TDs: Tumor deposits.

Table 5 N1 colorectal cancers after being restaged

Initial N stage	Restaged N1	Restaged N2	Total
N1a/b	18077	752	18820
N1c	1003	133	1136
Total	19080	885	19965

ARTICLE HIGHLIGHTS

Research background

Tumor deposits (TDs) plays an important role in The American Joint Committee on Cancer (AJCC) tumor, node and metastasis (TNM) staging system. However, the definition of TDs as well as N1c remains controversial. Just taking the quantitative information of TDs into consideration may be suboptimal in the current staging system while adding TDs into lymph node metastases (LNMs) count may improve accuracy and N1c category may represents patients with heterogeneous survival.

Research motivation

AJCC TNM staging system is the standard tool for tumor staging and the treatment strategies for patients mostly depend on tumor stage. To guarantee more appropriate treatment strategies can be received by patients and to predict prognosis of patients better, developing an optimal staging system is crucial.

Research objectives

The main objective of this study is to assess the association between the presence of TDs and overall survival (OS). As exploratory outcomes, the impact of number of TDs on OS was investigated and the N stage was reclassified to the novel N category by the addition of TDs to the LNM count. The outcome indicated that TDs are an independent prognostic factor for OS in colorectal cancer and the addition of TDs to LNM count improved the prognostic accuracy of TNM staging. Therefore, a part of patients staged as N1 previously would be N2 after the addition of TDs to LNM count and the prognosis would change subsequently.

Research methods

Patients with colorectal cancer including TD-negative and TD-positive subpopulations were derived from Surveillance, Epidemiology, and End Results database (SEER). Cox proportional hazard model was used for survival analysis and the sensitivity analyses were performed to detect outcome robustness. The subgroup analysis was also performed to explore the different profile of risk factors between patients with and without TDs. Comparative effectiveness research was used in current study.

Research results

The presence of TDs is an independent prognostic factor for OS in colorectal cancer and there may be more than one way through which TDs influence survival. Both TDs and their numbers should be integrated into N staging and the N1c category in TNM staging was inappropriate. Given that novel adjuvant therapy has already been the standard regimen in some settings and there is no evidence whether TDs in patients with novel adjuvant therapy should be regarded the same as patients without novel adjuvant therapy, further investigations need to be conducted.

Research conclusions

The presence of TDs is an independent prognostic factor for OS in colorectal cancer and addition of TDs to the LNM count improves the prognostic accuracy of current TNM staging.

Research perspectives

The origin as well as formation of TDs remains ambiguous and further studies are needed to substantiate the definition and demonstrate the pathogenesis of TDs. Patients with and without novel adjuvant therapy need to be investigated separately, especially when patients achieve substantial downstaging.

ACKNOWLEDGEMENTS

We wish to thank the useful suggestions given by Professor Wen-Quan Niu from the Institute of Clinical Medical Science of the China–Japan Friendship Hospital.

FOOTNOTES

Author contributions: Jie JZ and Wu WX designed the study; Wu WX, Zhang DK and Chen SX acquired data; Wu WX and Hou ZY analyzed and interpreted data; Wu WX, Sun BL and Yao L drafted the manuscript.

Supported by the Scientific and Technological Project of Qinghai Province, China, No. 2015-ZJ-742.

Institutional review board statement: The current study relied on the SEER cancer registry, which did not require further approval of institutional review aboard.

Informed consent statement: This study was exempt from informed consent.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

Country/Territory of origin: China

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1711-1726

DOI: 10.4251/wjgo.v14.i9.1711

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Cohort Study

Consolidation chemotherapy with capecitabine after neoadjuvant chemoradiotherapy in high-risk patients with locally advanced rectal cancer: Propensity score study

Xue-Qing Sheng, Hong-Zhi Wang, Shuai Li, Yang-Zi Zhang, Jian-Hao Geng, Xiang-Gao Zhu, Ji-Zhong Quan, Yong-Heng Li, Yong Cai, Wei-Hu Wang

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bairwa DBL, India; Cabezuelo AS, Spain; Preziosi F, Italy

Received: April 29, 2022
Peer-review started: April 29, 2022
First decision: July 6, 2022
Revised: July 14, 2022
Accepted: August 9, 2022
Article in press: August 9, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

The effects of consolidation chemotherapy (CC) in neoadjuvant therapy in locally advanced rectal cancer (LARC) have been explored. However, the optimal neoadjuvant chemoradiotherapy (NCRT) and surgery interval, regimen, and cycles of chemotherapy remains unclear.

AIM

To evaluate the effects of one to two cycles of CC with capecitabine on high-risk patients with LARC without extending NCRT and surgery interval.

METHODS

We retrospectively evaluated high-risk patients with LARC, who were defined as having at least one of the following factors by magnetic resonance imaging: depth of invasion beyond the muscularis propria of more than 5 mm (cT3c-cT3d), T4, meso-rectal fascia or extramural vascular invasion positive, and treatment date between January 2015 and July 2019 in our center. Patients were divided into the CC and non-CC group according to whether they received CC (capecitabine 1000 mg/m^2 twice daily from days 1 to 14 every 21 d) after NCRT. Propensity score

matching (PSM) and inverse probability of treatment weight (IPTW) were used to balance the differences between the two groups. The main outcome was the complete response (CR) rate.

RESULTS

A total of 265 patients were enrolled: 136 patients in the CC group and 129 patients in the non-CC group. The median interval was 70 d (range, 37-168). The CR rate was 24.3% and 16.3% (P = 0.107) in the CC and non-CC groups' original samples, respectively. After PSM and IPTW, the CR rate in the CC group was higher than that in non-CC group (27.6% *vs* 16.2%, P = 0.045; 25.9% *vs* 16.3%, P = 0.045). The median follow-up was 39.8 mo (range, 2.9-74.8), and there were no differences in 3-year non-regrowth disease-free survival nor overall survival in the original samples (73.2% *vs* 71.9%, P = 0.913; 92.3% *vs* 86.7%, P = 0.294), PSM (73.2% *vs* 73.5%, P = 0.865; 92.5% *vs* 89.3%, P = 0.612), and IPTW (73.8% *vs* 72.1%, P = 0.913; 92.4% *vs* 87.4%, P = 0.294). There was also no difference in grade 2 or higher acute toxicity during neoadjuvant therapy in the two groups (49.3% *vs* 53.5%, P = 0.492).

CONCLUSION

One to two cycles of CC with capecitabine after NCRT was safe and increased the CR rate in high-risk LARC but failed to improve the long-term outcomes.

Key Words: High-risk locally advanced rectal cancer; Neoadjuvant chemoradiotherapy; Capecitabine; Consolidation chemotherapy; Complete response

Core Tip: This is the first study to explore the effects of one to two cycles of consolidation chemotherapy with capecitabine after neoadjuvant chemoradiotherapy (NCRT) in magnetic resonance imaging-defined high-risk patients with locally advanced rectal cancer without extending NCRT and surgery interval. After propensity score-matching and inverse probability of treatment weighting, the complete response rate increased. Although it showed no significant difference in long-term results, this relatively low-toxicity program deserves further exploration.

Citation: Sheng XQ, Wang HZ, Li S, Zhang YZ, Geng JH, Zhu XG, Quan JZ, Li YH, Cai Y, Wang WH. Consolidation chemotherapy with capecitabine after neoadjuvant chemoradiotherapy in high-risk patients with locally advanced rectal cancer: Propensity score study. *World J Gastrointest Oncol* 2022; 14(9): 1711-1726
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1711.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1711

INTRODUCTION

Neoadjuvant chemoradiotherapy (NCRT) followed by total mesorectal excision (TME) was the standard treatment for patients with locally advanced rectal cancer (LARC)[1,2]. After NCRT, approximately 50% to 60% of LARC patients were downstaged, and nearly 20% achieved pathologic complete response (pCR)[3,4]. Patients with pCR had better prognosis than those with worse regression[4-6]. In addition, the "watch-and-wait" approach was feasible for patients who achieved clinical complete response (cCR) after neoadjuvant therapy, which significantly improved their quality of life[7-10].

Accurate staging before treatment is extremely important, and magnetic resonance imaging (MRI) has unique advantages compared with other radiology methods for rectal cancers[11]. Although the current American Joint Committee on Cancer (AJCC) tumor node metastasis staging system stratifies patients with rectal cancer, some rectal MRI-based parameters, such as the extramuscular invasion distance, mesorectal fascia (MRF), and extramural venous invasion (EMVI) statuses are strongly related to the prognosis[12]. On the basis of the MERCURY series study[13], the European Society for Medical Oncology (ESMO) clinical practice guidelines recommend treatments after stratifying rectal cancer by using pelvic MRI[11]. Previous studies showed that the complete response (CR) rate after NCRT of low-risk patients with rectal cancer was more than 30%[14-16]. However, that of high-risk patients with rectal cancer were approximately 10%-20%[5,17]. Increasing the CR rate, especially in high-risk patients, is a current research target for neoadjuvant therapy in LARC.

Several studies have explored the effects of additional induction or consolidation chemotherapy (CC) [18-22] in neoadjuvant therapy in LARC. However, the optimal timing, regimen, and number of cycles in chemotherapy remained unknown. Compared with induction chemotherapy, CC seemed to improve

CR rate, but the increase in CR rate might also be related to the prolonged interval between NCRT and TME surgery[23-27]. The extended time could also aggravate pelvic fibrosis, thus making surgery more difficult[28] and potentially offsetting the tumor reduction benefit. In addition, most of the regimens in neoadjuvant chemotherapy consisted of double or triple drugs that increased the toxicity induced by treatment[21,22]. The additional oxaliplatin in concurrent chemotherapy not only increased toxicity but also failed to improve the efficacy[29-31]. Previous studies have also explored CC with capecitabine monotherapy in LARC[32,33]. However, patients in these studies were not stratified by pelvic MRI before treatment. This retrospective study explored the effects of one to two cycles of CC with capecitabine after NCRT in high-risk LARC patients without extending the time between the end of NCRT and surgery by considering the efficacy and low toxicity of capecitabine in the treatment of rectal cancer and the convenience of oral therapy.

MATERIALS AND METHODS

Patients

From January 2015 to July 2019, all patients with histologically confirmed, newly diagnosed locally advanced rectal adenocarcinoma with tumors within 15 cm of the anal verge were included in the screening. The inclusion criteria included: (1) High-risk patients with LARC defined by MRI, including at least one of the following high-risk factors: depth of invasion beyond the muscularis propria of more than 5 mm (cT3c-T3d), T4, EMVI (+), or MRF (+); (2) patients who had not received induction chemotherapy; (3) patients who achieved cCR or underwent surgery after NCRT in our center; (4) patients older than 18 years old; and (5) patients with an Eastern Cooperative Oncology Group score of ≤ 2 points and with no medical comorbidities or other tumors with a poor prognosis. Patients were divided into two groups, namely, the CC and non-CC groups, on the basis of CC administration during the interval between NCRT and surgery.

MRI assessment

A high-resolution, diagnostic, or simulation 3D T2-weighted sequence MRI was performed before NCRT. The scanning layer thickness was 3-5 mm, with mandatory axial scanning perpendicular to the long axis of the rectal tumor[34,35]. The tumor stage, T3 substage, lymph node metastases, EMVI, MRF, and tumor length and thickness were evaluated in primary MRI on the basis of the ESMO and the European Society of Gastrointestinal and Abdominal Radiology consensus meeting guidelines[11,35]. Evaluating tumor regression by MRI is still strongly recommended after NCRT, especially to diagnose cCR.

Neoadjuvant treatment

Computed tomography (CT) simulations were performed with a thermoplastic film with patients in the supine position by using contrast-enhanced CT with a 5 mm slice thickness. An empty rectum and a filled bladder were required to ensure consistency in the rectal tumor positioning and protect the intestine from radiation. MRI simulation was mandatory to obtain a more accurate tumor location. The target contour details were described previously[36]. The Simultaneous Integrated Boost-Intensity Modulated Radiation Therapy was delivered during radiotherapy. The prescription doses for the planning gross tumor volume and planning target volume were 50-50.6 Gy and 41.8-45 Gy, respectively, in 22-25 fractions. Chemotherapy with capecitabine at 825 mg/m^2 was administered orally twice daily and concomitantly with radiotherapy. One to two weeks after NCRT, one to two cycles of capecitabine (1000 mg/m^2 twice daily, d1-d14/q21d) were administered.

Patients underwent detailed and comprehensive restaging, including tumor marker, digital rectal examination, rectal endoscopy, and pelvic MRI six to eight weeks after NCRT. CT scans of the chest and abdomen were also performed to assess distant metastases. All patients received a multi-disciplinary team evaluation to develop a further treatment strategy. For patients who achieved cCR, a non-operative "watch-and-wait" strategy with rigorous and meticulous follow-up was feasible. The cCR diagnostic criteria included the following: (1) The absence of a viable tumor on MRI; (2) negative biopsies from the scar; (3) normal carcinoembryonic antigen (CEA) levels (< 5 ng/mL); and (4) no signs of distant metastasis. Patients who did not achieve cCR were highly recommended with surgery based on the TME principles. The pathology reports were based on the AJCC/College of American Pathologists standards[37]. R0 resection was defined as a longitudinal margin and circumferential resection margin of no more than 1 mm.

Adjuvant CapeOX chemotherapy (oxaliplatin 130 mg/m^2, d1; capecitabine 1000 mg/m^2 twice daily, d1-d14/q21d) was recommended for every patient, and capecitabine monotherapy was the alternative. Full-dose adjuvant chemotherapy was defined as capecitabine for six months or CapeOX for more than six cycles.

Follow-up and outcome measures

Toxicities during neoadjuvant treatment were evaluated on the basis of the Common Terminology Criteria for Adverse Events (version 3.0). After completing primary treatment, the patients were followed up at three-month intervals for the first two years, six-month intervals until five years, and annually thereafter by evaluating the symptoms, tumor markers, chest and abdominal CT, pelvic CT or MRI, and physical examination results.

The primary outcome was CR rate, including the pCR and cCR rate. Other outcomes included pCR, TRG classification, non-regrowth disease-free survival (NR-DFS), overall survival (OS), and acute toxicity during neoadjuvant treatment. TRG classification was based on the NCCN standard. NR-DFS was measured from the first day of NCRT to any type of recurrence or death for any reason. OS was calculated from the first day of NCRT to death for any reason.

Statistical analysis

Data were collected and analyzed using the Statistical Package for the Social Sciences (IBM Corp. SPSS Statistics for Windows, version 22.0, Armonk, NY, United States) and R statistical software package (R Project for Statistical Computing, version 4.1.2, Vienna, Austria). The chi-square test and independent sample t-test/Wilcoxon test were used to compare the differences in the two groups. Propensity score (PS) analysis, including PS matching (PSM) and inverse probability of treatment weighting (IPTW), were applied to balance the baseline characteristics of the two groups. The PS was developed with a logistic regression model, and variables including gender, age, tumor location, pathology, CEA, T stage, tumor length, thickness, MRF, EMVI, and interval were included. Patients in CC and non-CC groups were randomly matched 1:1 on the basis of PS by using the nearest neighbor method (maximum caliper distance, 0.2). IPTW was then calculated with PS by using IPTWs, and the number of observations is the sum of the weights[38]. The CR rates of the two groups in the original samples after PSM and IPTW were compared. The proportions of pCR, TRG, pT0-2, and pN0 were compared in the original samples and after PSM. The Kaplan-Meier method was used to plot NG-DFS and OS and was compared with the log-rank test. After PSM, subgroup analysis and interaction were conducted to assess the heterogeneity of treatment effects. A P value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

During the study period, 265 patients who met the screening criteria were included in the analysis. The median age was 59 years (range, 25-82). In total, 183 (69.1%) were males, 130 (49.1%) were categorized as a low location LARC, and 130 (49.4%) had normal CEA levels. There were 168 (63.4%) patients with stage > T3b disease, 206 (77.7%) patients who were MRF positive, and 170 (64.2%) patients with clinical EMVI positivity. Overall, 136 patients (51.3%) received CC after NCRT (CC group), of whom 79 (56.8%) received 1 cycle of capecitabine, and the remaining 129 patients were classified as the non-CC group.

Patients in the CC group had a longer interval between the end of NCRT and surgery (or the time of diagnosis of distant metastasis or cCR) than those in the non-CC group (P = 0.04). All other factors did not differ between the two groups (Table 1). PS analysis with PSM and IPTW achieved balance for all variables between the two groups (Table 2). Histograms and density graphs description comparisons of the original, PSM, and IPTW distributions of each group are shown in Figure 1.

Surgical and pathological outcomes

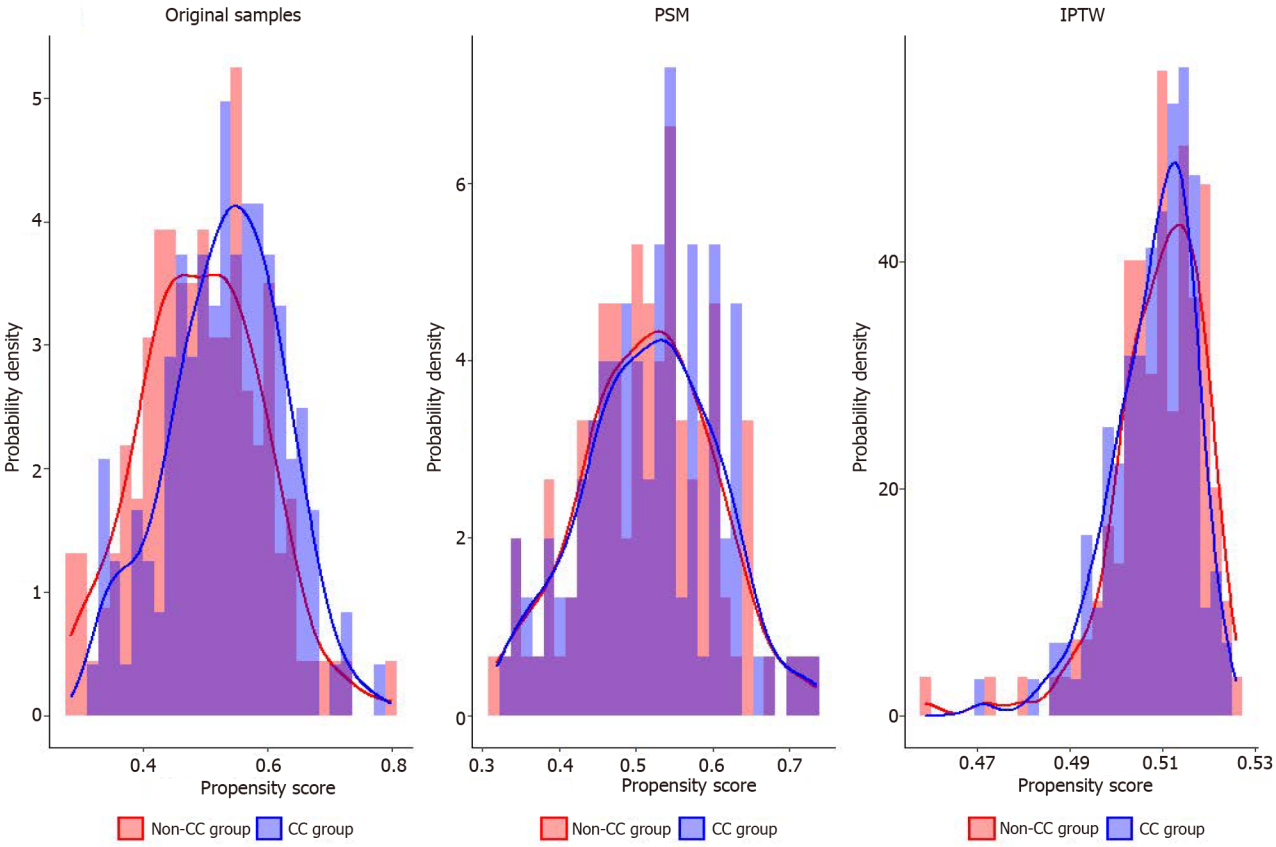
In the original samples before matching, 6 patients (2.3%) developed distant metastasis, 9 (3.4%) achieved cCR and received the "watch-and-wait" approach, and the remaining 250 (94.3%) underwent surgery after neoadjuvant therapy. Among patients who received surgery, 126 were in the CC group, and 124 were in the non-CC group. The mean interval in the CC and non-CC groups were 77.9 and 71.7 days (P = 0.015). The rates of pCR and TRG0 were 21.4% *vs* 14.5% (P = 0.155) and 24.6% *vs* 16.9% (P = 0.123) in the CC and non-CC group, respectively. The proportion of pN0 and pT0-2N0 was 78.6% *vs* 72.6% (P = 0.541) and 52.4% *vs* 46.0% (P = 0.311).

After PSM, each group had 105 patients: 6 (2.9%) developed distant metastasis, 8 (3.8%) achieved cCR, and the remaining 196 (93.3%) underwent surgery after neoadjuvant therapy. Among patients who received surgery, 96 were in the CC group, and 100 were in the non-CC group. The mean interval in the CC and non-CC groups were 76.8 and 74.5 days (P = 0.410). The rate of TRG 0 in the CC group was higher than that in the non-CC group (29.1% *vs* 17.0%, P = 0.015). The pCR rate was 25.0% (24/96) in the CC group, and 14.0% (14/100) in the non-CC group (P = 0.051). The proportions of pT0-2N0 and ypN0 in CC and non-CC groups were 59.4% *vs* 46.0% (P = 0.061) and 77.1% *vs* 72.0% (P = 0.712), respectively. Table 3 shows the details of surgery and pathology in the two groups in the original samples before matching and after PSM.

Table 1 The clinical characteristics between the two groups

	CC group (*n* = 129)	non-CC group (*n* = 136)	*P* value
Gender, *n* (%)			0.177
Male	84 (65.1)	99 (72.8)	
Female	45 (34.9)	37 (27.2)	
Age, yr			0.446
mean (SD)	57.5 (11.4)	58.5 (9.8)	
Primary location, *n* (%)			0.812
Up	4 (3.1)	6 (4.4)	
Middle	60 (46.5)	65 (47.8)	
Low	65 (50.4)	65 (47.8)	
Pathology, *n* (%)			0.996
Well differentiated	6 (4.7)	6 (4.4)	
Moderately differentiated	95 (73.6)	102 (75.0)	
Poorly differentiated	16 (12.4)	16 (11.8)	
Others	12 (9.3)	12 (8.8)	
CEA, *n* (%)			0.307
Normal	67 (51.9)	64 (47.1)	
Unnormal	49 (38.0)	63 (46.3)	
Unidentified	13 (10.1)	9 (6.6)	
T stage, *n* (%)			0.650
< T3c	49 (38.0)	48 (35.3)	
> T3b	80 (62.0)	88 (64.7)	
N stage, *n* (%)			0.190
N0	12 (9.3)	7 (5.1)	
N+	117 (90.7)	129 (94.9)	
Tumor length (mm)			0.916
mean (SD)	49.0 (12.7)	49.1 (13.7)	
Tumor thickness (mm)			0.838
mean (SD)	16.4 (5.0)	16.5 (7.2)	
MRF, *n* (%)			0.501
Negative	31 (24.0)	28 (20.6)	
Positive	98 (76.0)	108 (79.4)	
EMVI, *n* (%)			0.565
Negative	44 (34.1)	51 (37.5)	
Positive	85 (65.9)	85 (62.5)	
Numbers of high-risk factor, *n* (%)			0.557
1	38 (29.5)	34 (25.0)	
2	48 (37.2)	59 (43.4)	
3	43 (33.3)	43 (31.6)	
Interval time (d)			0.040
mean (SD)	71.7 (21.7)	76.8 (18.5)	

CC: Consolidation chemotherapy; SD: Standard deviation; CEA: carcinoembryonic antigen; MRF: Mesorectal fascia; EMVI: Extralmural venous invasion.



DOI: 10.4251/wjgo.v14.i9.1711

Figure 1 Histograms and density graphs description comparisons of the original, propensity score match and inverse probability of treatment weighting distributions in the consolidation chemotherapy and non-consolidation chemotherapy groups. PSM: Propensity score match; IPTW: Inverse probability of treatment weighting; CC: Consolidation chemotherapy.

Complete response rate and subgroup analysis

In the original samples before matching, there were 24.3% (33/136, 6 cCR and 27 pCR) of patients in the CC group, and 16.3% (21/129, 3 cCR and 18 pCR) of patients in the non-CC group obtained CR (P = 0.107). After PSM, 5 and 24 patients achieved cCR and pCR in the CC group, respectively, and 3 and 14 patients achieved cCR and pCR in the non-CC group, respectively. The CR rate in the CC group was higher than that in the non-CC group (27.6% *vs* 16.2%, P = 0.045). After IPTW, the CR rate in the CC group and the non-CC group was 25.9% (35/135) and 16.2% (21/130), respectively (P = 0.045). Table 4 shows the CR rates and univariate regression of CC in the original samples before matching and after PSM and IPTW.

In the exploratory subgroup analysis of the PSM cohort, the median of continuous variables was used for grouping. The results showed that CC could improve the CR rate in patients with MRF positive and intervals < 70 d. After the interaction test, the heterogeneity of the CC effect remained in the subgroup with interval (Figure 2).

Adjuvant chemotherapy

Adjuvant chemotherapy was collected for patients who underwent surgery. In the original samples before matching, 146 patients (58.4%) received adjuvant chemotherapy: 73 (57.9%) in the CC group, and 73 (58.9%) in the non-CC group (P = 0.881). Among them, 38 (30.2%) patients in the CC group and 34 (27.4%) patients in the non-CC group completed the full dose of adjuvant chemotherapy (P = 0.632). After PSM, 117 patients (59.7%) received adjuvant chemotherapy: 56 (58.3%) in the CC group, and 61 (61.0%) in the non-CC group (P = 0.704). A total of 28 patients (29.2%) in the CC group and 27 (27.0%) patients in the non-CC group completed the full dose of adjuvant chemotherapy (P = 0.736).

Long-term outcomes

The median follow-up time was 39.8 mo (range, 2.9-74.8). In the original samples before matching, three

Table 2 The clinical parameters between the two groups after propensity score match and inverse probability of treatment weighting

	PSM			IPTW		
	non-CC group (*n* = 105)	CC-group (*n* = 105)	*P* value	non-CC group (*n* = 130)	CC-group (*n* = 135)	*P* value
Gender, *n* (%)			0.762			0.970
Male	75 (71.4)	73 (69.5)		89.4 (68.5)	92.6 (68.7)	
Female	30 (28.6)	32 (30.5)		41.1 (31.5)	42.2 (31.3)	
Age			0.692			0.993
mean (SD)	57.7 (11.8)	58.3 (9.7)		58.2 (11.2)	58.2 (9.7)	
Primary location, *n* (%)			0.849			0.996
Up	3 (2.9)	2 (1.9)		4.9 (3.8)	4.9 (3.7)	
Middle	52 (49.5)	50 (47.6)		61.0 (46.7)	63.8 (47.3)	
Low	50 (47.6)	53 (50.5)		64.6 (49.5)	66.1 (49.0)	
Pathology, *n* (%)			0.903			0.999
Well-differentiated	5 (4.8)	5 (4.8)		6.3 (4.9)	6.6 (4.9)	
Moderately-differentiated	79 (75.2)	75 (71.4)		94.0 (72.0)	97.9 (72.6)	
Poorly-differentiated	12 (11.4)	13 (12.4)		16.6 (12.7)	17.0 (12.6)	
Others	9 (8.6)	12 (11.4)		13.6 (10.4)	13.3 (9.9)	
CEA, *n* (%)			0.428			0.997
Normal	51 (48.6)	58 (55.2)		64.1 (49.1)	66.8 (49.5)	
Unnormal	45 (42.9)	42 (40.0)		55.2 (42.3)	56.7 (42.1)	
unidentified	9 (8.6)	5 (4.8)		11.2 (8.6)	11.3 (8.4)	
T stage, *n* (%)			0.568			0.992
< T3c	41 (39.0)	37 (35.2)		48.0 (36.8)	49.7 (36.9)	
> T3b	64 (61.0)	68 (64.8)		82.5 (63.2)	85.1 (63.1)	
N stage, *n* (%)			0.097			0.176
N0	10 (9.5)	4 (3.8)		12.1 (9.3)	6.7 (5.0)	
N+	95 (90.5)	101 (96.2)		118.4 (90.7)	128.1 (95.0)	
Tumor length (mm)			0.916			0.983
mean (SD)	48.6 (13.0)	48.4 (13.2)		48.9 (12.5)	48.9 (13.5)	
Tumor thickness (mm)			0.484			0.999
mean (SD)	16.6 (5.0)	16.0 (7.0)		16.4 (4.9)	16.4 (7.2)	
MRF, *n* (%)			> 0.99			0.865
Negative	23 (21.9)	23 (21.9)		29.7 (22.8)	29.5 (21.9)	
Positive	82 (78.1)	82 (78.1)		100.7 (77.2)	105.3 (78.1)	
EMVI, *n* (%)			0.771			0.998
Negative	35 (33.3)	37 (35.2)		46.4 (35.6)	48.0 (35.6)	
Positive	70 (66.7)	68 (64.8)		84.0 (64.4)	86.8 (64.4)	
Numbers of high-risk factor, *n* (%)			0.510			0.883
1	31 (29.5)	26 (24.8)		36.5 (28.0)	35.1 (26.0)	
2	37 (35.2)	45 (42.9)		51.2 (39.2)	56.9 (42.2)	
3	37 (35.2)	34 (32.4)		42.8 (32.8)	42.8 (31.7)	

Interval time (d)			0.659		0.819
mean (SD)	74.4 (20.0)	75.6 (18.4)		75.5 (25.1)	74.8 (17.7)

PSM: Propensity score match; IPTW: Inverse probability of treatment weighting; CC: Consolidation chemotherapy; SD: Standard deviation; CEA: Carcinoembryonic antigen; MRF: Mesorectal fascia; EMVI: Extralmural venous invasion.

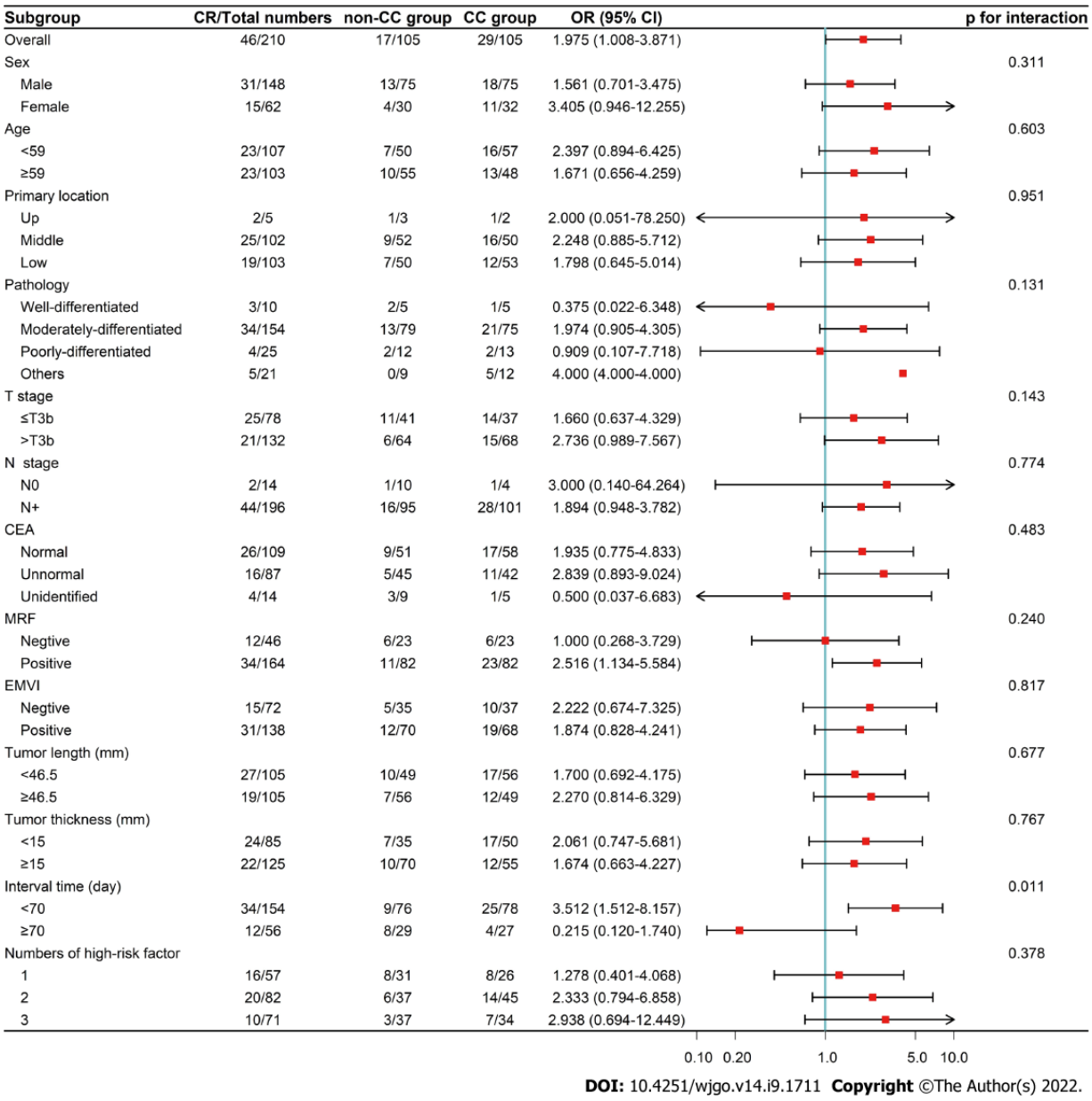


Figure 2 Forest plot of the subgroup analysis for complete response rate in the propensity score match cohort. Odds rate (OR) > 1 favors consolidation chemotherapy (CC) group, and OR < 1 favors non-CC group. CR: complete response; PSM: Propensity score match; CC: Consolidation chemotherapy; OR: Odds rate; CI: Confident interval; CEA: Carcinoma embryonic antigen; MRF: Mesorectal fascia; EMVI: Extralmural venous invasion.

(33.3%) of nine cCR patients developed local regrowth: two patients within one year and one patient after two years; all three patients received radical surgery. Furthermore, one (11.11%) of the nine patients developed distant metastasis after one year. The three-year NR-DFS and OS were 73.2% *vs* 71.9% (P = 0.913) and 92.3% *vs* 86.7% (P = 0.294) in the CC and non-CC groups, respectively. After PSM, the three-years NR-DFS and OS were 73.2% *vs* 73.5% (P = 0.865) and 92.5% *vs* 89.3% (P = 0.612). After IPTW, the three-year NR-DFS and OS in the CC group and non-CC groups were 73.8% *vs* 72.1% (P = 0.913) and 92.4% *vs* 87.4% (P = 0.294), respectively (Figure 3).

Table 3 Details of surgical and pathological results in the original samples before matching and after propensity score match in the two groups

	Original samples			PSM		
	non-CC group (*n* = 124)	CC group (*n* = 126)	*P* value	non-CC group (*n* = 100)	CC group (*n* = 96)	*P* value
Interval time (d)			0.015			0.410
mean (SD)	71.7 (21.9)	77.9 (18.6)		74.5 (20.1)	76.8 (18.7)	
Surgical method, *n* (%)			0.232			0.990
APR	42 (33.9)	31 (24.6)		30 (30.0)	29 (30.2)	
LAR	77 (62.1)	91 (72.2)		66 (66.0)	63 (65.6)	
Hartmann	5 (4.0)	4 (3.2)		4 (4.0)	4 (4.2)	
Surgery time (h)			0.684			0.953
mean (SD)	3.0 (1.3)	3.1 (1.4)		3.0 (1.3)	3.0 (1.4)	
Blood loss (mL)			0.345			0.407
mean (SD)	75.4 (51.4)	105.4 (145.5)		74.5 (47.8)	99.3 (105.0)	
R0, *n* (%)	123 (99.2)	124 (98.4)	0.571	99 (99.0)	94 (97.9)	0.537
Numbers of dissected lymph nodes			0.194			0.502
mean (SD)	9.1 (4.9)	8.3 (5.0)		9 (4.8)	8.54 (5.0)	
pT satge, *n* (%)			0.400			0.136
T0	21 (16.9)	31 (24.6)		17 (17.0)	28 (29.2)	
T1	6 (4.8)	10 (7.0)		5 (5.0)	9 (9.4)	
T2	41 (33.1)	34 (27.0)		32 (32.0)	28 (29.2)	
T3	54 (43.5)	50 (39.7)		44 (44.0)	30 (31.2)	
T4	2 (1.6)	1 (0.8)		2 (2.0)	1 (1.0)	
pN stage, *n* (%)			0.541			0.712
N0	90 (72.6)	99 (78.6)		72 (72.0)	74 (77.1)	
N1	26 (21.0)	21 (16.7)		22 (22.0)	17 (17.7)	
N2	8 (6.5)	6 (4.8)		6 (6.0)	5 (5.2)	
TRG, *n* (%)			0.123			0.015
0	21 (16.9)	31 (24.6)		17 (17.0)	28 (29.1)	
1	43 (34.7)	51 (40.5)		33 (33.0)	41 (42.7)	
2	59 (47.6)	42 (33.3)		49 (49.0)	26 (27.1)	0.176
3	1 (0.8)	2 (1.6)		1 (1.0)	1 (1.1)	
pT0-2N0, *n* (%)	57 (46.0)	66 (52.4)	0.311	46 (46.0)	57 (59.4)	0.061
pCR, *n* (%)	18 (14.5)	27 (21.4)	0.155	14 (14.0)	24 (25.0)	0.051

PSM: Propensity score match; CC: Consolidation chemotherapy; APR: Abdominoperineal resection; LAR: Low anterior resection; TRG: Tumor regression grade; pCR: Pathological complete response; SD: Standard deviation.

Treatment-related toxicity

Treatment-related toxicity during neoadjuvant treatment was collected for all 265 patients. In total, 136 (51.3%) patients showed grade ≥ 2 toxicity; 67 (49.3%) patients were in the CC group, and 69 (53.5%) patients were in the non-CC group (P = 0.492). Proctitis/diarrhea (28.3%) was the most common grade ≥ 2 acute toxicity, followed by leukopenia (21.9%). Nine (3.4%) patients developed grade 3 acute toxicity; 4 (2.9%) patients were in the CC group, and 5 (3.9%) patients were in the non-CC group. There was no grade 4 toxicity, as well as toxicity-related deaths, in the two groups (Table 5).

Table 4 The complete response rate and univariate regression of consolidation chemotherapy in the original samples before matching, after propensity score match and inverse probability of treatment weighting in the two groups

	CR			Univariate regression	
	non-CC group, *n* (%)	CC group, *n* (%)	*P* value	OR (95%CI)	*P* value
Original samples	21 (16.3)	33 (24.3)	0.107	1.648 (0.895-3.033)	0.109
PSM	17 (16.2)	29 (27.6)	0.045	1.975 (1.008-3.871)	0.047
IPTW	21 (16.3)	35 (25.9)	0.045	1.185 (1.008-3.395)	0.047

CR: Complete response; PSM: Propensity score match; IPTW: Inverse probability of treatment weighting; CC: Consolidation chemotherapy; OR: Odds rate; CI: Confident interval.

Table 5 Toxicities during neoadjuvant treatment in the two groups

	non-CC group (*n* = 129), *n* (%)				CC group (*n* = 136), *n* (%)			
	Grade 1	Grade 2	Grade 3	Grade 4-5	Grade 1	Grade 2	Grade 3	Grade 4-5
Total	59 (45.7)	64 (49.6)	5 (3.9)	0	66 (48.5)	63 (46.3)	4 (2.9)	0
Leukopenia	47 (36.4)	29 (22.5)	2 (1.6)	0	51 (37.5)	27 (19.9)	0	0
Neutropenia	22 (17.1)	9 (7.0)	0	0	22 (16.2)	9 (6.6)	0	0
Anemia	5 (3.9)	6 (4.7)	2 (1.6)	0	14 (10.3)	5 (3.7)	0	0
Thrombocytopenia	9 (7.0)	0	1 (0.8)	0	5 (3.7)	0	0	0
Aminotransferase increased	0 (0.0)	1 (0.8)	0	0	6 (4.4)	0	0	0
Bilirubin increased	19 (14.7)	2 (3.1)	0	0	18 (13.2)	2 (1.5)	1 (0.7)	0
Nausea	39 (30.2)	0	0	0	30 (22.1)	1 (0.7)	0	0
Fatigue	58 (45.0)	3 (2.3)	0	0	66 (44.9)	2 (1.5)	0	0
Proctitis/diarrhea	66 (51.2)	36 (27.9)	1 (0.8)	0	66 (48.5)	39 (28.7)	2 (1.5)	0
Cystitis	38 (29.5)	0	0	0	42 (30.9)	0	0	0
Radiodermatitis	75 (58.1)	6 (4.7)	0	0	70 (51.5)	3 (2.2)	0	0

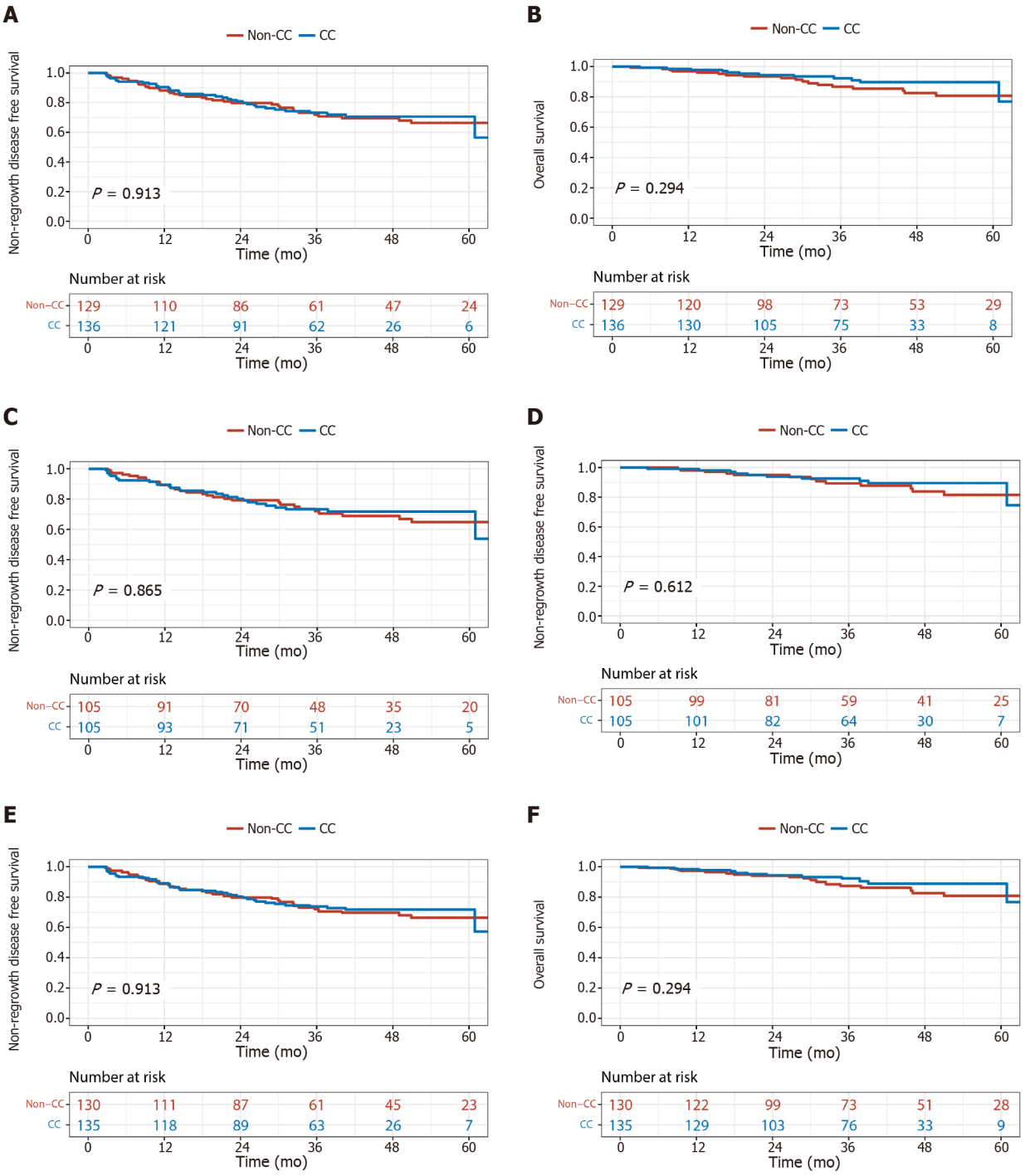
CC: Consolidation chemotherapy.

DISCUSSION

To our knowledge, this is the first study to explore the effects of one to two cycles of CC with capecitabine after NCRT for high-risk LARC patients. The results showed that without extending the interval between the end of NCRT and surgery, this regimen increased CR rates, but did not improve the three-year NR-DFS and OS.

Pelvic MRI has been widely used to evaluate rectal cancer. It could evaluate the primary tumor and pelvic lymph node stage and accurately determine the depth of invasion beyond the muscularis propria, MRF, and EMVI status that affected the prognosis of patients. In 2001, Merkel *et al*[39] analyzed the postoperative pathology of 853 patients with rectal cancer and found that patients with tumor invasion distance ≤ 5 mm had a better 5-year local recurrence rate and tumor-specific survival than those with > 5 mm (10.4% *vs* 26.3%, $P < 0.0001$; 85.4% *vs* 54.1%, $P < 0.0001$). In the MERCURY study, patients who were MRF negative had better three-year DFS and OS than those who were MRF positive (47.3% *vs* 67.2%, $P < 0.05$; 42.2% *vs* 62.2%, $P < 0.01$)[40]. A meta-analysis that included 6 studies of 1262 rectal cancer found that patients with EMVI-positive were 3.91 times more likely to develop distant metastases than EMVI-negative patients[41]. According to the depth of invasion beyond muscularis propria, MRF, EMVI status and other factors, ESMO guidelines stratified the risk groups in rectal cancer and recommended treatment options within the risk category[11]. For patients with high-risk rectal cancer, neoadjuvant chemoradiotherapy was still the standard treatment[11].

After neoadjuvant treatment, patients with pCR had good long-term prognosis[4,5], and patients with cCR could receive the "'watch-and-wait" strategy, which improved the quality of life[7-10]. Maas *et al*[5] analyzed 3105 LARC, and the results showed that patients with pCR had significantly better five-year DFS (83.3% *vs* 65.6%, $P < 0.0001$), local recurrence (2.8% *vs* 9.7%, $P < 0.0001$), and distant metastases



DOI: 10.4251/wjgo.v14.i9.1711

Figure 3 Non-regrowth disease free survival and overall survival of consolidation chemotherapy and non-consolidation chemotherapy groups. A: Non-regrowth disease free survival (NR-DFS) before matching; B: Overall survival (OS) before matching; C: NR-DFS after propensity score match (PSM); D: OS after PSM; E: NR-DFS after inverse probability of treatment weighting (IPTW); F: OS after IPTW. CC: Consolidation chemotherapy.

(11.2% *vs* 25.1%, $P < 0.0001$) rates than those who did not achieve pCR. The International Watch and Wait Database and OnCoRe project showed that cCR patients had stable biological behavior and good prognosis with a local regrowth rate of 20%-25.2%, distant metastasis of 7%-9%, and a five-year OS of 73%-97%[7-10]. In our study, 33.3% (3/9) patients had local tumor growth, and 11.1% (1/9) had distant metastasis; these findings were higher than those in published data. This might be related to the small size of the cCR patients, and all patients enrolled in the study were at high-risk with LARC. Therefore, this result deserved further exploration.

Although patients with pCR had good prognosis, the pCR rate after NCRT was approximately 20%, and it was even lower in patients with high-risk LARC[5,17]. To increase the CR rate, some studies explored the effect of CC. Garcia-Aguilar *et al*[20] analyzed zero, two, four, and six cycles of FOLFOX after NCRT in LARC, and the pCR rates increased (18% for zero cycles, 25% for two cycles, 30% for four

cycles, and 38% for six cycles). The CAO/ARO/AIO-12 study analyzed three additional chemotherapy cycles before and after NCRT in MRI-defined high-risk LARC. The results demonstrated that the pCR rate in the CC group was better than that in the induction chemotherapy group (25% *vs* 17%)[19]. However, increasing the cycles of CC also prolonged the interval between NCRT and surgery, and current research indicates that the extended intervals increase the pCR rate[28,42]. When the time was 10-11 wk, the pCR rate was the highest[23]. In the original samples before matching, the interval in the CC group was longer than that in the non-CC group. After PSM and IPTW, the interval was balanced in the 2 groups with a median of 70 days, and the CR rate in the CC group was higher than that in the non-CC group. The subgroup analysis showed that the CR rates increased when the interval was < 70 d. This may be because all the patients enrolled in this study were at high-risk with LARC, and the standard dose of NCRT was not enough to get the best regression. When the interval was < 70 d, both low-intensity CC and extending time could increase the tumor regression.

Several studies have also explored the effect of CC with capecitabine after NCRT. Zampino *et al*[32] evaluated the effect of NCRT followed by 2 cycles of capecitabine in 51 patients. The interval between the end of NCRT and surgery was less than eight weeks. The results showed that the pCR rate was 18%, and the five-year DFS was 85.4%, with no increase in acute toxicity or postoperative complications. The OIGIT-01 trial was designed with 1 cycle of induction chemotherapy with capecitabine followed by NCRT and 2 cycles of CC with capecitabine in 66 patients. The median interval was eight weeks, and this regimen was well-tolerated. The pCR rate was 17.5%, and the 5-year DFS was 64%[33]. However, these two studies were single-arm studies with a small sample size, and the patients were not stratified by pelvic MRI before treatment. In a previous study, we analyzed the efficacy of one to two cycles of CC with capecitabine in low-risk patients with LARC, which did not improve the CR rate and three-year NR-DFS[16]. In the current study, we included high-risk patients with LARC. After PSM and IPTW, the CR rate in the CC group was higher than that in the non-CC group. Data after PSM also showed that the CC increased the rate of TRG 0. In addition, subgroup analysis after PSM showed that MRF-positive patients were more likely to benefit from CC. These results suggest that one to two cycles of CC with capecitabine can increase tumor regression in high-risk patients with LARC, thus providing new evidence for the individualized treatment of patients with LARC.

The PRODIGE 23 trial explored the intensification of chemotherapy by using triple drugs before NCRT, and the results showed that it significantly improved three-year DFS (76% *vs* 69%, $P = 0.034$) compared with NCRT in patients with LARC[22]. In the CAO/ARO/AIO-12 study, there were no difference in the three-year DFS of patients in the induction chemotherapy and CC groups (73% *vs* 73%, $P = 0.82$)[43]. In the current study, one to two cycles of CC with capecitabine did not increase the three-year NR-DFS in high-risk patients with LARC (73.2% *vs* 71.9%, $P = 0.913$). Intensified systemic therapy should be implemented to improve long-term outcomes.

As a single-center retrospective study, this study had some inherent limitations. First, despite applying the PSM and IPTW analysis to balance differences between the two groups, bias might still exist in the study. Second, the sample size was small, and the follow-up time was short. Prospective studies with more participants and a longer follow-up period need to be performed to confirm these findings.

CONCLUSION

Without extending the interval between the end of NCRT and surgery, one to two cycles of CC with capecitabine after NCRT was safe and increased the CR rate in high-risk patients with LARC. However, it failed to improve long-term outcomes. This study provides a powerful rationale for further exploration in phase 3, multicenter, randomized trails.

ARTICLE HIGHLIGHTS

Research background

Patients with locally advanced rectal cancer (LARC) who achieved complete response (CR) after neoadjuvant therapy had a better prognosis, but the optimal neoadjuvant therapy regimen remained unclear.

Research motivation

Several studies have suggested that consolidation chemotherapy (CC) after neoadjuvant chemoradiotherapy (NCRT) seemed to improve CR rate, however it also prolonged interval between NCRT and surgery, making surgery more difficult. Besides, in the concurrent chemotherapy, the additional oxaliplatin not only increased toxicity but also failed to improve the efficacy. Further, high-risk patients with LARC were less likely to achieve CR, and had worse prognosis than patients in low-risk. Considering the efficacy and low toxicity of capecitabine in the treatment of rectal cancer and the

convenience of oral therapy, we designed this retrospective study.

Research objectives

To evaluate the effects of one to two cycles of CC with capecitabine in high-risk patients with LARC without extending NCRT and surgery interval.

Research methods

From January 2015 to July 2019, high-risk patients with LARC were divided into the CC and non-CC group according to whether they received CC after NCRT. Propensity score matching (PSM) and inverse probability of treatment weight (IPTW) were used to balance the differences between the two groups.

Research results

After PSM and IPTW, the CR rate in the CC group was higher than that in the non-CC group. The median follow-up was over three years, and there were no differences in 3-year non-regrowth disease-free survival nor overall survival in the two groups. There was also no increase in acute toxicity in the CC group.

Research conclusions

Our study first confirmed without extending the interval between the end of NCRT and surgery, one to two cycles of CC with capecitabine after NCRT was safe and increased the CR rate in high-risk patients with LARC. However, it failed to improve long-term outcomes.

Research perspectives

Further studies with more participants and a longer follow-up period need to be investigated to confirm these findings.

ACKNOWLEDGEMENTS

We thank all the patients and staff of Peking University Cancer Hospital who participated in this study for their valuable contributions.

FOOTNOTES

Author contributions: Wang WH and Cai Y are responsible for the manuscript conceptualization; Sheng XQ, Wang HZ, Zhang YZ and Geng JH are responsible for the methodology; Sheng XQ and Wang HZ are responsible for the formal analysis; Sheng XQ, Li S and Wang HZ are responsible for the investigation; Wang WH, YL, Cai Y and Zhu XG collect the resources; Sheng XQ, Li S, Zhang YZ and Geng JH do the data curation; Sheng XQ write the original draft; Wang WH and Cai Y are responsible for the reviewing and editing; Li YH and Quan JZ are responsible for the supervision; Li S, Wang HZ, Geng JH, and Zhu XG do the project administration.

Supported by Beijing Municipal Science and Technology Commission, No. Z181100001718192; Capital's Funds for Health Improvement and Research, No. 2020-2-1027 and No. 2020-1-4021; National Natural Science Foundation, No. 82073333.

Institutional review board statement: The study was approved by the Ethics Committee of Peking University Cancer Hospital review board, No. 2021YJZ62.

Informed consent statement: Informed consent from patients was waived by the Ethics Committee of Peking University Cancer Hospital review board.

Conflict-of-interest statement: All authors report no relevant conflict of interest for this article.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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S-Editor: Wu YXJ
L-Editor: A
P-Editor: Wu YXJ

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1727-1738

DOI: 10.4251/wjgo.v14.i9.1727

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Efficacy and safety of computed tomography-guided microwave ablation with fine needle-assisted puncture positioning technique for hepatocellular carcinoma

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): D, D
Grade E (Poor): 0

P-Reviewer: Giacomelli L, Italy; Nakano M, Japan; Nakano M, Japan; Raissi D, United States

Received: March 14, 2022
Peer-review started: March 14, 2022
First decision: April 11, 2022
Revised: April 18, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

In microwave ablation (MWA), although computed tomography (CT) scanning can overcome gas interference, it cannot achieve real-time localization. Therefore, the puncture technique is more important in CT-guided ablation.

AIM

To compare the fine needle-assisted puncture (FNP) positioning technique and the conventional puncture (CP) technique for the safety and efficacy of CT-guided MWA in treating hepatocellular carcinoma (HCC).

METHODS

This retrospective study included 124 patients with 166 tumor nodules from February 2018 and June 2021. Seventy patients received CT-guided MWA under the FNP technique (FNP group), and 54 patients received MWA under the CP technique (CP group). Intergroup comparisons were made regarding local tumor progression (LTP), recurrence-free survival (RFS), overall survival (OS), and complications. The influencing variables of LTP and RFS were analyzed through univariate and multivariate regressions.

RESULTS

The 1-, 2-, and 3-year cumulative incidences of LTP in the FNP group were significantly lower than those in the CP group (7.4%, 12.7%, 21.3% *vs* 13.7%, 32.9%, 36.4%; $P = 0.038$). The 1-, 2-, and 3-year RFS rates in the FNP group were significantly higher than those in the CP group (80.6%, 73.3%, 64.0% *vs* 83.3%,

39.4%, and 32.5%, respectively; $P = 0.008$). The FNP technique independently predicted LTP and RFS. Minor complications in the FNP group were lower than those in the CP group ($P < 0.001$). The difference in median OS was insignificant between the FNP and CP groups ($P = 0.229$).

CONCLUSION

The FNP technique used in CT-guided MWA may improve outcomes in terms of LTP, RFS, and procedure-related complications for HCC.

Key Words: Hepatocellular carcinoma; Fine needle puncture; Microwave ablation; Recurrence-free survival; Local tumor progression

Core Tip: This was a retrospective study that compared the fine needle-assisted puncture positioning (FNP) technique and conventional puncture (CP) technique for the safety and efficacy of computed tomography (CT)-guided microwave ablation (MWA) in treating hepatocellular carcinoma (HCC). In total, 124 patients were divided into two groups by the puncture technique. Seventy patients received CT-guided MWA under the FNP technique (FNP group), and 54 patients received MWA under the CP technique (CP group). The FNP technique used in CT-guided MWA may improve outcomes in terms of local tumor progression, recurrence-free survival, and procedure-related complications for HCC.

Citation: Hao MZ, Hu YB, Chen QZ, Chen ZX, Lin HL. Efficacy and safety of computed tomography-guided microwave ablation with fine needle-assisted puncture positioning technique for hepatocellular carcinoma. *World J Gastrointest Oncol* 2022; 14(9): 1727-1738
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1727.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1727

INTRODUCTION

Apart from being the world's sixth most frequently diagnosed malignancy, hepatocellular carcinoma (HCC) is also the third primary reason for cancer-associated mortality on a global scale. In 2018, about 841080 new incidences and 781631 mortalities were caused by HCC[1]. For nearly 20 years since the 1990s, HCC has been managed by percutaneous radiofrequency ablation (RFA) and microwave ablation (MWA). The development of ablation technology is of extensive value in the treatment of HCC[2]. It is considered the third major treatment for HCC, following surgical resection and liver transplantation[3].

Tumors located ≤ 5 mm from large vessels, gallbladder, gastrointestinal tract, diaphragm, or liver capsule have been defined as high-risk locations (HRLs), which are contraindicated for RFA treatment [4,5]. The RFA of tumors lying close to large vessels with at least 3 mm in diameter is often incomplete due to the heat sink effect[6]. Compared with RFA, MWA can shorten the ablation time, increase the local temperature faster, reduce the heat sink effect of adjacent vessels, and simultaneously use multiple therapeutic probes[7]. Due to these advantages, MWA is more attractive in the ablation of HCC[8].

Ultrasound (US) and computed tomography (CT) are the most commonly applied image guidance methods for MWA. Due to the influence of the gas, the subphrenic area is one of the most difficult places for ultrasound guidance[9]. A CT scan can compensate for this shortcoming. CT-guided puncture, in contrast, does not allow for real-time positioning. Thus, puncture skills are more important in CT-guided ablation[10].

Although a CT-guided stereotactic navigation system can provide accurate puncture path planning, the equipment is still not popularized due to economic reasons[11,12]. The fine needle-assisted puncture (FNP) positioning technique is to insert a separate fine needle (21G) near the tumor nodule as the positioning and marking of the microwave antenna insertion to improve the success rate of microwave needle puncture. Although Wu *et al*[13] recently confirmed that the FNP technique is a safe and effective puncture auxiliary technique for CT-guided biopsy or MWA of small tumor nodules near the diaphragm by a retrospective study. To date, no study has compared the efficacy of CT-guided MWA using the FNP and conventional puncture (CP) techniques in the treatment of HCC.

The current work investigated the efficacy and safety of CT-guided MWA for HCC under the FNP and CP techniques.

MATERIALS AND METHODS

Patients

We retrospectively analyzed 170 patients with consecutive primary HCC, who received CT-guided MWA or transarterial chemoembolization (TACE) combined with MWA at our hospital from February 2018 to June 2021. These patients were either inappropriate for or rejected surgery. The diagnosis of HCC was verified by imaging and serum alpha-fetoprotein (AFP) assay or hepatic needle biopsy as per the Chinese guidelines for the primary liver cancer diagnosis and treatment (2017 edition).

The inclusion criteria were: Child-Pugh class A or B, single tumor with the largest diameter ≤ 5 cm before MWA, 2-3 tumors with the largest diameter ≤ 5 cm, Eastern Cooperative Oncology Group performance status (ECOG PS) 0-1, and patients with platelet count > 50 × 10^9/L. The exclusion criteria were: Patients with a known additional malignancy that is progressing concurrently; patients with portal vein thrombosis or extrahepatic metastases; patients with ≥ 4 HCC nodules; patients with ablation to reduce tumor burden; and recurrent HCC, except for recurrence after resection.

Figure 1 shows 4 patients with a known additional malignancy that is progressing concurrently, 6 patients with ≥ 4 HCC nodules, 15 patients with extrahepatic metastases, 3 patients with portal vein thrombosis, 6 patients with ablation to reduce tumor burden, and 12 patients with recurrent HCC after treatment other than surgery. For 17 patients who had intrahepatic distal recurrence (IDR) and 4 patients with local tumor progression (LTP) who underwent MWA twice or more, the second or subsequent MWA procedure was excluded from the study to avoid statistical bias caused by the repetition of patient data twice.

Thus, 124 patients with 166 nodules were incorporated in the current study. Patients were divided into two groups according to the microwave needle puncture methods. Seventy patients received CT-guided MWA under the FNP technique and were categorized into the FNP group, whereas 54 patients received CT-guided MWA supported with a conventional puncture and were categorized into the CP group. The operators of all TACE and MWA procedures belonged to the same attending physician team, and the chief operator in the CT-guided MWA procedures of both groups had at least 15 years of experience in RFA. The choice of puncture method was not based on the tumor size, tumor number, and tumor location. The corresponding author and the assistant performed CT-guided MWA under the FNP technique, whereas another chief operator and assistant performed CT-guided MWA under the CP technique.

Study protocol approval was obtained from the corresponding ethics committee (2018-022-02). The experimental procedures conformed to the principles of the Declaration of Helsinki. Before the retrospective study, each patient provided written informed consent.

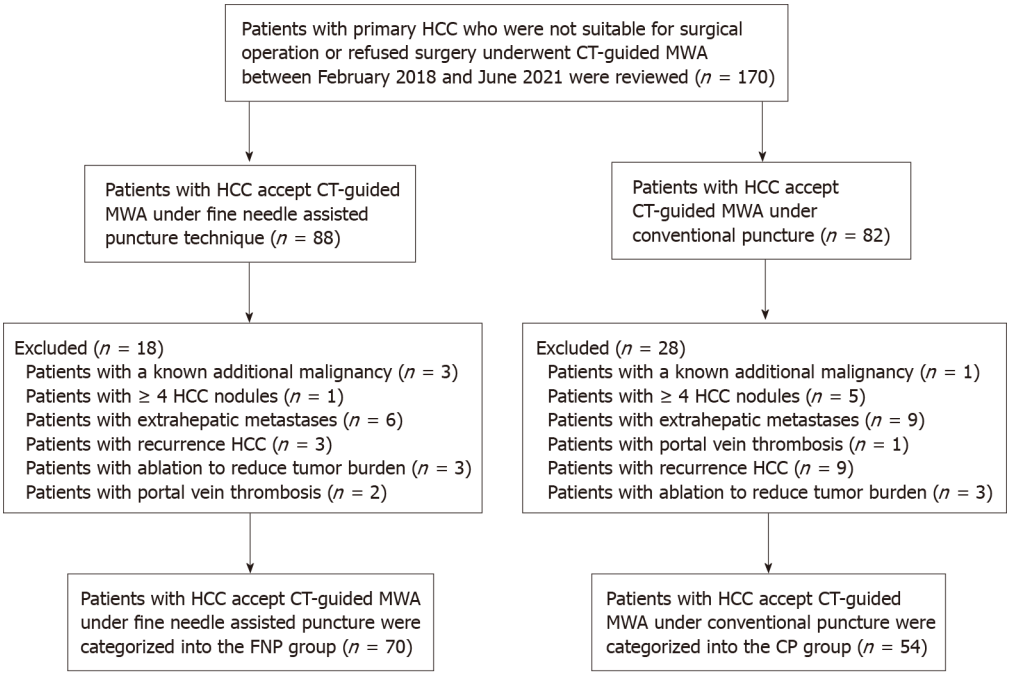
Methods

MWA procedure: MTC-3 C MWA equipment (Yigao Microwave System Engineering Co. Ltd., Nanjing, Jiangsu Province, China) was used at 2450 MHz (± 10%) in a continuous wave mode and 5-120 W ± 30% power output. The MWA antenna was 1.8 mm in diameter with a surface coating. Before MWA, the tumor size, number, site, and relationship with important structures were evaluated by contrast-enhanced magnetic resonance imaging (MRI) and/or helical CT scan. A multidisciplinary team comprising a liver surgeon, radiologist, sonographer, oncologist, and interventional radiologist created the treatment plans for patients with HCC.

Percutaneous CT-guided MWA was conducted on an inpatient basis under local anesthesia and analgesics. The patient was awake during the MWA procedures. Before performing MWA, each tumor's antenna layout, power output setup, and emission duration were meticulously planned. A single MWA antenna was used in the nodules ≤ 1.7 cm; if > 1.7 cm, then a double-needle was used, keeping a space of 2.0 cm between the two needles. The ablation margin was kept between 5 and 10 mm at 60 and 70 W for 5 to 10 min. After treatment, the needle was gradually withdrawn with a parallel needle tract ablation. For tumors under the liver capsule attached to the diaphragm, intestinal tube, or gallbladder, saline was injected between the target lesion and the adjacent organs, a process called hydrodissection to protect them from possible heat damage if a safe distance could not be maintained. Adjuvant hydrodissection techniques were performed on 2 patients.

A CT scan was acquired immediately after treatment and again 24 h after operation to evaluate the margin of ablation and complications, such as bleeding and pneumothorax. Nearly 2 mo post-operation, treatment response was evaluated through contrast-enhanced MRI or helical CT examination. Complete ablation was verified based on the absence of enhanced areas. In the case of incomplete ablation, a second ablation was considered. An ablation failure was indicated if complete ablation was not achieved even after two ablations, and other treatment methods were applied.

The MWA puncture technique: Before the operation, the ablation needle electrode was selected based on the size and location of the tumor, and the puncture angle and depth were set under CT guidance. The percutaneous transhepatic puncture was performed by free hands following a detailed procedure. In the CP group, the needle path had to pass through the normal liver tissue at > 1.5 cm, avoiding large blood vessels and bile ducts. CT scanning was repeated at half the depth of needle insertion to observe the relationship between the electrode needle or tumor and the surrounding tissue structure. Next, the



DOI: 10.4251/wjgo.v14.i9.1727

Figure 1 Flow chart showing the selection process of participants for this study. CT: Computed tomography; CP: Conventional puncture; FNP: Fine needle-assisted puncture positioning; HCC: Hepatocellular carcinoma; MWA: Microwave ablation.

puncture angle was adjusted, and the needle was inserted gradually inside the tumor and approximately 0.5 to 1 cm beyond the tumor margin. In the FNP group, a 21-gauge fine needle of 15 cm in length was inserted near the tumor nodule before ablation needle puncture as a marker (Figure 2A). Following that, CT scanning was used to determine the angle and direction of the MWA electrode needle puncture based on fine-needle marking. Subsequently, the microwave electrode needle was step by step gradually inserted inside the tumor and approximately 0.5 to 1 cm beyond the tumor margin, and each step was confirmed by a CT scan (Figure 2B). Afterward, the fine needle was pulled out after the electrode needle was consistent with the plan confirmed by CT scanning (Figure 2C). Figure 3 shows a sequence of images of a patient with an HCC nodule in segment 5 treated with CT-guided MWA by the FNP technique after TACE.

Assessment of the outcome: The patients were followed up 2 mo after MWA and then every 3 to 6 mo. Follow-up included general, physical, imaging, and laboratory examinations such as biochemistry and tumor marker levels. LTP was indicated when following thorough tumor ablation, any new lesions connected to ablation focus were seen at the focus rim[14]. IDR was defined as the appearance of any new lesions distant from the ablation zone (excluding extrahepatic metastasis)[15,16]. Recurrent-free survival (RFS) refers to the duration between the first ablation and the final follow-up or the tumor recurrence including LTP and IDR, whereas overall survival (OS) refers to the duration between the time of diagnosis and the time of death or date of the last follow-up. Under the Society of Interventional Radiology Classification System, we classified the complications into major and minor types[17]. In addition, we monitored the hospital stay of patients after treatment completion. Follow-up was continued through December 25, 2021, and the median follow-up time was 22.6 mo (range: 6.0-43.4 mo).

Statistical analysis

Student's *t*-test was used to assess the mean difference, whereas the χ^2 test was adopted for frequency distribution comparison. The LTP, RFS, and OS were tested using the Kaplan–Meier technique and log-rank tests. The multifactor influences on LTP and RFS were examined through Cox regression analysis. Differences were regarded as significant when the two-tailed $P < 0.05$. SPSS v24 was used for data processing and analysis.

RESULTS

Patient characteristics

The patient information and tumor characteristics are detailed in Table 1. Between the two groups, there

Table 1 Baseline characteristics of patient and tumor between the two groups

Variables	FNP group, *n* = 70	CP group, *n* = 54	*P* value
Age (yr)[1]	58.6 ± 1.7	59.4 ± 1.4	0.728
Sex (M/F)	60/10	48/6	0.601
ECOG PS			0.200
0	22	23	
1	48	31	
AFP (ng/mL)			0.530
< 400	61	49	
≥ 400	9	5	
No. of nodules in each patient			0.871
1	47	37	
2-3	23	17	
Total no. of nodules	93	73	
Tumor size before MWA (cm)			0.922
< 3	50	39	
3-5	20	15	
Mean tumor diameter (cm) pre-MWA	2.3 ± 0.1	2.2 ± 0.2	0.711
Proportion of TACE prior to MWA	50 (71.4%)	41 (75.9%)	0.574
Post-MWA hospital stay (d)[1]	3.3 ± 0.3	3.9 ± 0.2	0.130
Tumor location			
HRL	49	34	0.409
Liver subcapsular region	30	16	
Diaphragmatic surface	7	4	
Adjacent to large vessel	8	7	
Adjacent to gallbladder	3	4	
Adjacent to gastrointestinal tract	1	3	
LRL	21	20	
LTP	11 (15.7%)	16 (29.6%)	0.063

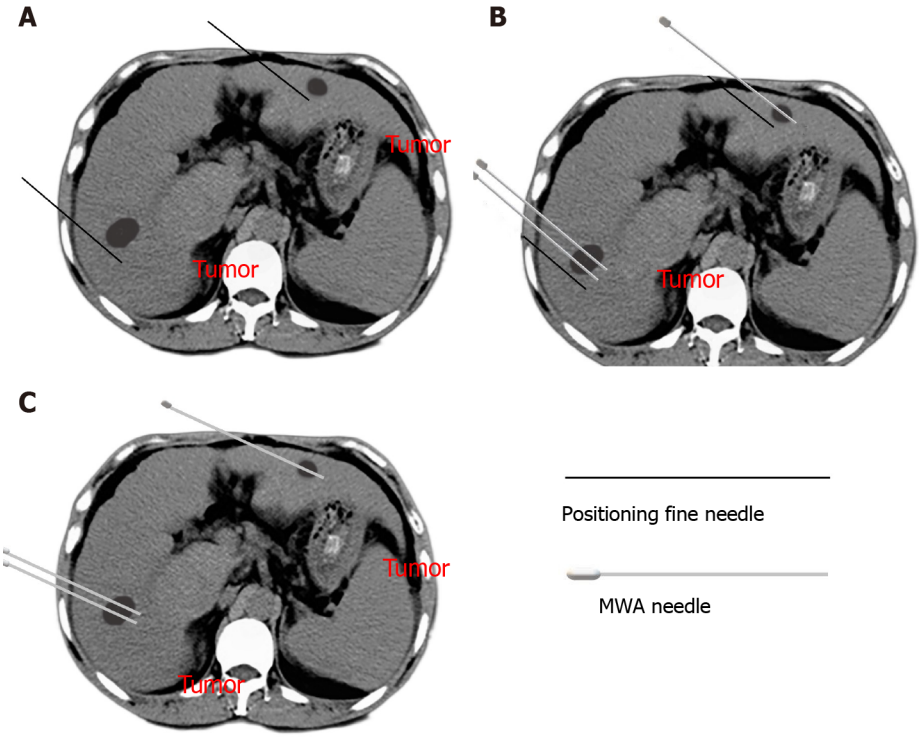
[1]Values are mean ± SE. $P < 0.05$ is significant; $P > 0.05$ is non-significant.

FNP: Fine needle-assisted puncture; CP: Conventional puncture; ECOG PS: Eastern Cooperative Oncology Group performance status rating; AFP: Alpha-fetoprotein; HRL: High-risk location; LRL: Low-risk location; LTP: Local tumor progression; MWA: Microwave ablation; TACE: Transarterial chemoembolization.

were insignificant differences in age, sex, ECOG PS, AFP, tumor number, tumor size, tumor location, and the application rate of combined TACE therapy before MWA. At the end of the follow-up, no significant difference was observed in the detection rate of LTP between the two groups ($P = 0.063$). The liver function of all patients was Child-Pugh class A before MWA. A total of 91 patients were treated with TACE combined with MWA in 124 patients. MWA was conducted approximately 4 wk after TACE. No patient achieved complete remission before ablation after TACE as per the Response Evaluation Criteria in Solid Tumors criteria. There was an insignificant difference in post-MWA hospital stay between both groups ($P = 0.130$).

Survival and recurrence outcome

One patient in the FNP group and 2 patients in the CP group were detected to have residual tumor by MRI scan 2 mo post-MWA, which was completely ablated by MWA again. The one-stage ablation success rate was 98.6% in the FNP group and 96.3% in the CP group ($P = 0.820$). During the follow-up, LTP was detected in 11 (15.7%) patients in the FNP group and 16 (29.6%) patients in the CP group. In the last follow-up, 6 patients were dead, 5 were lost to the follow-up, and 113 were alive.



DOI: 10.4251/wjgo.v14.i9.1727

Figure 2 Fine needle-assisted puncture positioning technique in computed tomography-guided microwave ablation. A: The patient was trained to hold his breath, and then a 21-gauge fine needle was inserted near the tumor nodules; B: A microwave electrode needle was gradually inserted inside the tumor according to the mark of the fine needle while the fine needle served as a breathing indicator; C: The fine needle was pulled out after the electrode needle, consistent with the plan confirmed by computed tomography scanning.

The 1-, 2-, and 3-year cumulative incidences of LTP in the FNP group were significantly lower than those in the CP group (7.4%, 12.7%, 21.3% *vs* 13.7%, 32.9%, 36.4%, respectively; $P = 0.038$ *via* log-rank test, Figure 4A).

The 1-, 2-, and 3-year RFS rates in the FNP group were significantly higher than those in the CP group (80.6%, 73.3%, 64.0% *vs* 83.3%, 39.4%, and 32.5%, respectively; $P = 0.008$ *via* log-rank test, Figure 4B).

The 1- and 2-year OS rates were 98.4% and 96.0% in the FNP group and 98.1% and 88.8% in the CP group, with a median OS of 45.8 mo [95% confidence interval (CI): 44.1-47.4] and 40.2 mo (95%CI: 37.6-42.8) ($P = 0.229$ *via* log-rank test, Figure 4C).

Univariate and multivariate analyses

According to univariate analysis, ECOG PS, tumor number, and puncture method were significantly related to LTP. According to multivariate analysis, tumor number (≥ 2) was independently related to a poor LTP and superior ECOG PS, and the FNP technique was independently related to a good LTP (Table 2).

Univariate analysis indicated that ECOG PS, tumor number, and puncture method were significantly related to RFS. Multivariate analysis indicated that tumor number (≥ 2) was independently associated with a poor RFS, and the FNP technique was independently associated with a good RFS (Table 3).

Subgroup analysis

In the subgroup analysis stratified by tumor number, the median time to LTP in the FNP group was significantly longer than that in the CP group for patients with a single tumor (44.9 ± 1.2 *vs* 33.7 ± 2.4 mo, respectively; $P = 0.005$ *via* log-rank test).

Furthermore, in the subgroup analysis stratified by tumor number, the median RFS time in the FNP group was considerably higher than that in the CP group for patients with a single tumor (37.3 ± 1.8 *vs* 28.5 ± 2.5 mo, respectively; $P = 0.013$ *via* log-rank test).

Complications

No deaths were directly related to the early complications of MWA. Table 4 shows the frequency of complications in all patients. The FNP group had two cases of major complications. One patient diagnosed with bacteremia recovered with anti-infective therapy, whereas another patient developed massive pneumothorax and recovered by thoracic drainage. The CP group had 4 cases of massive pneumothorax recovered by thoracic drainage. The intergroup differences were insignificant regarding

Table 2 Factors associated with local tumor progression

Factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Sex	0.706 (0.166, 2.966)	0.637		
Age (yr): > 60; 60	1.041 (0.486, 2.229)	0.918		
ECOG PS: 0; 1	3.169 (1.197, 8.392)	0.020	2.979 (1.108, 8.014)	0.031
Tumor number: 1; 2-3	3.370 (1.561, 7.277)	0.002	3.008 (1.383, 6.546)	0.005
AFP (ng/mL): < 400; ≥ 400	0.601 (0.142, 2.538)	0.488		
Tumor size (cm): < 3; 3-5	1.649 (0.754, 3.603)	0.210		
Auxiliary TACE pre-MWA: Yes; No	1.232 (0.497, 3.055)	0.653		
Tumor location: HRL; LRL	1.523 (0.937, 2.476)	0.090		
Puncture method: FNP technique; CP technique	2.205 (1.021, 4.761)	0.044	2.596 (1.197, 5.631)	0.016

AFP: Alpha-fetoprotein; CP: Conventional puncture; ECOG PS: Eastern Cooperative Oncology Group performance status rating; FNP: Fine needle-assisted puncture positioning; HRL: High-risk location; LRL: Low-risk location; MWA: Microwave ablation; TACE: Transarterial chemoembolization.

Table 3 Factors associated with recurrence-free survival

Factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Sex	0.547 (0.170, 1.764)	0.313		
Age (yr): > 60; 60	1.021 (0.590, 1.767)	0.942		
ECOG PS: 0; 1	1.831 (1.008, 3.325)	0.047	1.609 (0.878, 2.948)	0.124
Tumor number: 1; 2-3	3.692 (2.112, 6.456)	< 0.001	3.910 (2.195, 6.966)	< 0.001
AFP (ng/mL): < 400; ≥ 400	0.606 (0.218, 1.682)	0.336		
Tumor size (cm): < 3; 3-5	1.491 (0.841, 2.642)	0.171		
Auxiliary TACE pre-MWA: Yes; No	0.947 (0.504, 1.780)	0.867		
Tumor location: HRL; LRL	1.060 (0.789, 1.424)	0.699		
Puncture method: FNP technique; CP technique	2.078 (1.196, 3.612)	0.009	2.484 (1.415, 4.359)	0.002

AFP: Alpha-fetoprotein; CP: Conventional puncture; ECOG PS: Eastern Cooperative Oncology Group performance status rating; FNP: Fine needle-assisted puncture positioning; HRL: High-risk location; LRL: Low-risk location; MWA: Microwave ablation; TACE: Transarterial chemoembolization.

major complications (P = 0.454). The CP group exhibited more minor complications, including postoperative pain and fever compared to those in the FNP group (P < 0.001).

DISCUSSION

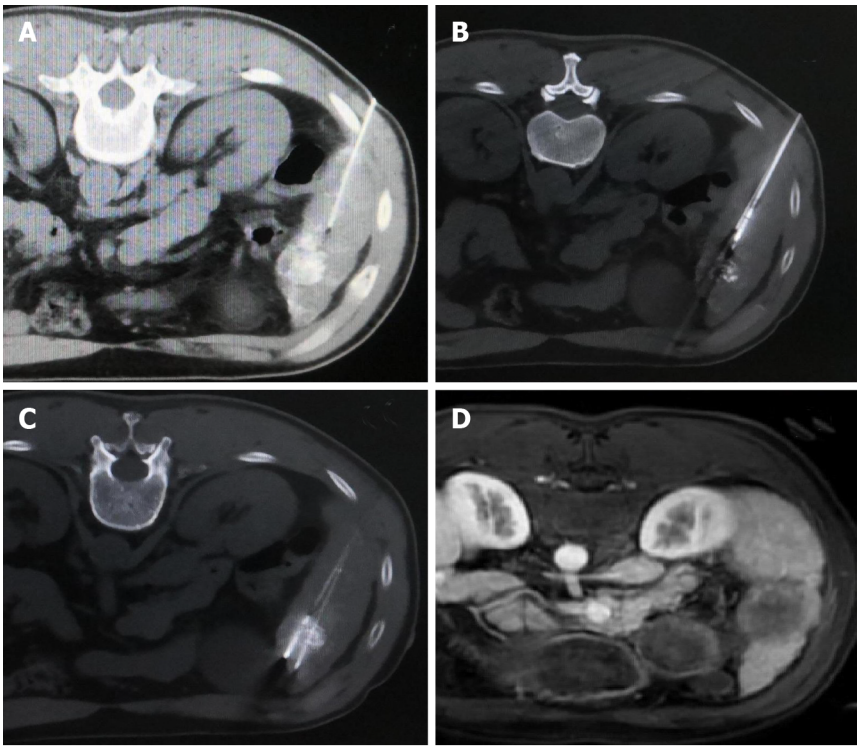
According to the outcomes of this study, although no significant difference was observed in 1- and 2-year OS rates and median OS between the FNP and CP groups, the FNP technique may improve outcomes in terms of LTP, RFS, and procedure-related complications for HCC treated with CT-guided MWA. The FNP technique was independently associated with good LTP and RFS. In the subgroup analysis, the FNP technique may improve the median time to LTP and median RFS time for patients with a single HCC nodule. The results of this study are clinically important considering that the FNP technique has better efficacy and safety in CT-guided MWA compared to the CP technique.

Image guidance techniques play a critical role in MWA. Although no reports are available that compare the efficacies of US-guided and CT-guided MWA, several studies have demonstrated that both US-guided and CT-guided RFA are similar in terms of LTP and complete ablation[18,19]. In US-guided

Table 4 Complications of computed tomography-guided microwave ablation

Type of reactions	FNP group, *n* = 70	CP group, *n* = 54	χ^2	*P* value
Major complications			0.561	0.454
Bacteremia	1	0		
Pneumothorax	1	4		
Total	2	4		
Minor complications			12.345	< 0.001
Postoperative pain	15	11		
Postoperative fever	2	7		
Self-limiting pneumothorax	2	1		
Self-limiting pleural effusion	3	0		
Transient elevation of aminotransferase	18	27		
Bleeding at the probe-inserting point	1	2		
Total	41	48		

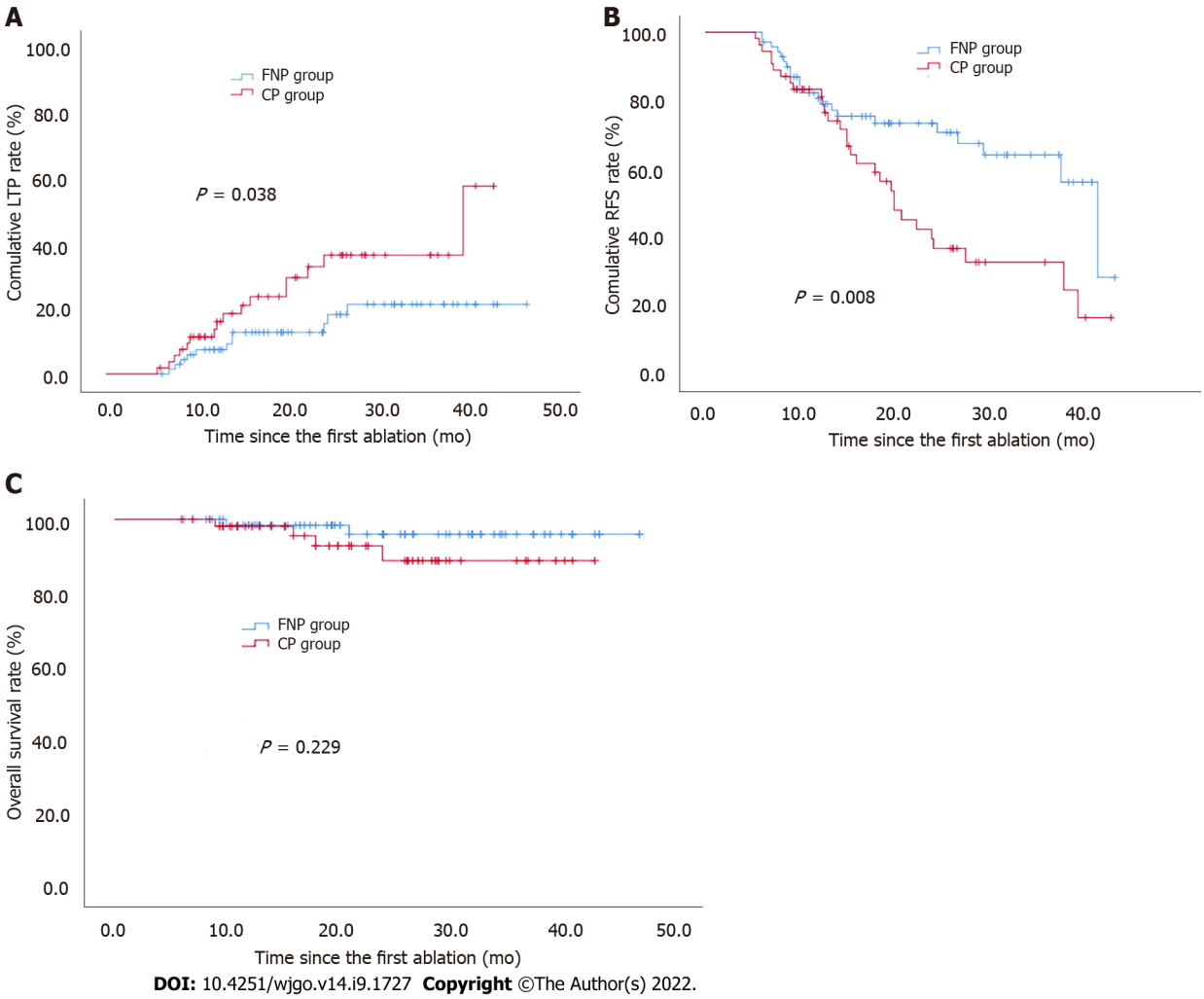
CP: Conventional puncture; FNP: Fine needle-assisted puncture positioning.



DOI: 10.4251/wjgo.v14.i9.1727

Figure 3 Computed tomography-guided microwave ablation under fine needle-assisted puncture technique for a patient with hepatocellular carcinoma nodule in segment 5 accepted transarterial chemoembolization pre-microwave ablation. A: A 21-gauge fine needle of 15 cm length was inserted near the tumor nodule after computed tomography scanning before ablation needle puncture; B: A microwave electrode needle was gradually inserted inside the tumor according to the mark of the fine needle; C: The second microwave electrode needle was gradually inserted inside the tumor needle and approximately 1 cm beyond the tumor margin according to the mark of the fine needle; D: The complete ablation was confirmed by magnetic resonance imaging 2 mo post-microwave ablation.

ablation, there are blind spots and vaporization interferences[20]; these disadvantages can be overcome by CT. However, it is a non-real-time image guidance technology. Repeated CT scans caused by unskilled puncture techniques can significantly increase radiation exposure. Thus, it has higher requirements for puncture technology under CT guidance.



DOI: 10.4251/wjgo.v14.i9.1727

Figure 4 Comparison of cumulative incidence of local tumor progression, recurrence-free survival, and overall survival following post-computed tomography-guided microwave ablation between fine needle-assisted puncture positioning and conventional puncture groups. A: Local tumor progression (P = 0.038 based on log-rank statistics); B: Recurrence-free survival (P = 0.008 based on log-rank statistics); C: Overall survival (P = 0.229 based on log-rank statistics). CP: Conventional puncture; FNP: Fine needle-assisted puncture positioning; LTP: Local tumor progression; OS: Overall survival; RFS: Recurrence-free survival.

A recent study reported that the FNP technique used in biopsy or MWA for small nodules near the diaphragm offered an improved puncture success rate and a low reduced radiation dose[13]. In this method, a fine needle puncture is made, which reduces the radiation exposure because the biopsy needle or MWA electrode needle can be inserted easily in the subsequent process. Repeated puncture in ablation procedure may lead to complications of needle bleeding, tumor implantation, and pneumothorax, *etc*[4,21]. Moreover, electrode needle placement is associated with complications such as bleeding, vascular injuries, and pneumothorax[22]. We used the FNP technique in CT-guided MWA procedures to treat HCC without limiting tumors near the diaphragm. The fine needle inserted near the tumor nodule could fix the liver and thoracoabdominal wall. The fine needle, liver, thoracoabdominal wall, and diaphragm are moved as a whole unit during breathing. The fine needle inserted near the tumor nodule can be used as a sign for subsequent electrode needle insertion and also help judge the patient's respiratory movement and reduce the error of subsequent puncture and puncture times. Due to the artifact of the microwave electrode needle in CT scanning, it is difficult to judge whether the position of the end of the electrode needle is consistent with the pre-designed position. The fine needle without artifact in CT scanning as a mark is helpful for the proper placement of the MWA electrode needle. The primary reason for the better performance of the FNP than CP in CT-guided MWA may be the reduction of puncture times and the appropriate MWA electrode needle placement. Univariate and multivariate analyses showed that the FNP technique was significantly related to good RFS and LTP, which further supported the advantages of the FNP technique.

Tumor number, tumor size, and performance status are important factors affecting tumor recurrence and survival[23-26]. In this study, tumor number (≥ 2) was independently associated with a poor LTP and RFS, as in previous studies. Nevertheless, tumor size was not an independent prognostic factor for LTP and RFS, and it may be attributed to the application of adjuvant TACE before MWA in 73.4% of

patients. According to a meta-analysis, TACE + MWA contributed to prominently higher rates of local control and objective remission[27,28]. TACE + MWA achieved better efficacy than TACE or MWA monotherapy for managing HCC of 3 to 5 cm or even > 5 cm in size[29,30]. In this study, no significant difference was observed in the proportion of TACE before MWA between the two groups, which does not affect the primary results of the study.

This study had a few limitations, including the possibility of bias due to the retrospective analysis of a single-center small sample. Another limitation is the lack of comparison between FNP and CT-guided stereotactic navigation systems. Prospective multicenter studies must be conducted in the future to gain further insight.

CONCLUSION

The FNP technique used in CT-guided MWA in the current study may improve outcomes in terms of LTP, RFS, and procedure-related complications for HCC. The FNP technique was independently associated with good LTP and RFS. The results of this study have important clinical value in CT-guided MWA for HCC with the FNP technique.

ARTICLE HIGHLIGHTS

Research background

Due to the influence of the gas, the subphrenic area is one of the most difficult places for ultrasound guidance. A computed tomography (CT) scan can compensate for this shortcoming. CT-guided ablation is a commonly used ablation image-guided method in our department.

Research motivation

CT-guided puncture does not allow for real-time positioning and the microwave electrode needle will produce artifacts in CT scanning, which is different from our previous application of radiofrequency ablation.

Research objectives

To compare fine needle-assisted puncture (FNP) positioning technique and conventional puncture technique for the safety and efficacy of CT-guided microwave ablation (MWA) in treating hepatocellular carcinoma (HCC).

Research methods

The efficacy and safety were compared between the patients received CT-guided MWA under FNP technique and patients received MWA under conventional puncture technique.

Research results

The 1-, 2-, and 3-year cumulative incidences of local tumor progression (LTP) in the FNP group were significantly lower than those in the conventional puncture (CP) technique (CP group). The 1-, 2-, and 3-year RFS rates in the FNP group were significantly higher than those in the CP group. The FNP technique independently predicted LTP and recurrence-free survival (RFS). The minor complications in the FNP group were lower those in the CP group.

Research conclusions

The FNP technique used in CT-guided MWA may improve outcomes in terms of LTP, RFS, and procedure-related complications for HCC.

Research perspectives

Prospective multicenter randomized controlled studies must be conducted in the future to obtain further insights.

FOOTNOTES

Author contributions: Hao MZ, Chen QZ, Hu YB, and Lin HL participated in the microwave ablation operation; Hao MZ participated in the data analysis and interpretation, manuscript writing, and final approval of the manuscript; Chen ZX contributed to the data collection and assembly; Lin HL participated in the conception and design.

Supported by the Fujian Key Laboratory of Translational Cancer Medicine and Fujian Provincial Clinical Research

Center for Cancer Radiotherapy and Immunotherapy, China, No. 2020Y2012.

Institutional review board statement: The protocol was approved by the ethics committee of Fujian Provincial Tumor Hospital (approval No. 2018-022-02).

Informed consent statement: Written informed consent was obtained from each patient before study initiation.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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Corresponding Author's Membership in Professional Societies: Anti-Cancer Association of China, No. M166300908s.

S-Editor: Yan JP
L-Editor: Filipodia
P-Editor: Yan JP

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1739-1757

DOI: 10.4251/wjgo.v14.i9.1739

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Clinicopathological characterization of ten patients with primary malignant melanoma of the esophagus and literature review

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Corvino A, Italy; Dambrauskas Z, Lithuania; Gupta R, India

Received: December 2, 2021
Peer-review started: December 2, 2021
First decision: March 11, 2022
Revised: April 24, 2022
Accepted: July 26, 2022
Article in press: July 26, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Primary malignant melanoma of the esophagus (PMME) is a rare malignant disease and has not been well characterized in terms of clinicopathology and

survival.

AIM
To investigate the clinical features and survival factors in Chinese patients with PMME.

METHODS
The clinicopathological findings of ten cases with PMME treated at Henan Provincial People's Hospital were summarized. Moreover, the English- and Chinese-language literature that focused on Chinese patients with PMME from 1980 to September 2021 was reviewed and analyzed. Univariate and multivariate analyses were employed to investigate the clinicopathologic factors that might be associated with survival.

RESULTS
A total of 290 Chinese patients with PMME, including ten from our hospital and 280 from the literature were enrolled in the present study. Only about half of the patients (55.8%) were accurately diagnosed before surgery. Additionally, 91.1% of the patients received esophagectomy, and 88 patients (36.5%) received adjuvant therapy after surgery. The frequency of lymph node metastasis (LNM) was 51.2% (107/209), and LNM had a positive rate of 45.3% even when the tumor was confined to the submucosal layer. The risk of LNM increased significantly with the pT stage [$P < 0.001$, odds ratio (OR): 2.47, 95% confidence interval (CI): 1.72-3.56] and larger tumor size ($P = 0.006$, OR: 1.21, 95%CI: 1.05-1.38). The median overall survival (OS) was 11.0 mo (range: 1-204 mo). The multivariate Cox analysis showed both the pT stage [$P = 0.005$, hazard ratio (HR): 1.70, 95%CI: 1.17-2.47] and LNM ($P = 0.009$, HR: 1.78, 95%CI: 1.15-2.74) were independent prognostic factors for OS. The median disease-free survival (DFS) was 5.3 mo (range: 0.8-114.1 mo). The multivariate analysis indicated that only the advanced pT stage ($P = 0.02$, HR: 1.93, 95%CI: 1.09-3.42) was a significant independent indicator of poor RFS in patients with PMME.

CONCLUSION
The correct diagnosis of PMME before surgery is low, and physicians should pay more attention to avoid a misdiagnosis or missed diagnosis. Extended lymph node dissection should be emphasized in surgery for PMME even though the tumor is confined to the submucosal layer. Both the LNM and pT stage are independent prognosis factors for OS, and the pT stage is the prognosis factor for DFS in patients with PMME.

Key Words: Primary malignant melanoma of the esophagus; Clinicopathological characteristics; Treatment; Recurrence; Survival

Core Tip: Primary malignant melanoma of esophagus (PMME) is a rare malignant disease. We comprehensively analyzed the clinicopathological characteristics of 290 Chinese patients with PMME. Only about half of the patients were accurately diagnosed before surgery. The positive rate of lymph node metastasis (LNM) was 45.3% even the tumor confined to the submucosal layer. The median overall survival (OS) and disease-free survival were 11.0 mo and 5.3 mo, respectively. Cox analysis showed that both pT stage and LNM were the independent prognostic factors for OS, while only advanced pTNM stage was a significant independent indicator of poor RFS in patients with PMME.

Citation: Zhou SL, Zhang LQ, Zhao XK, Wu Y, Liu QY, Li B, Wang JJ, Zhao RJ, Wang XJ, Chen Y, Wang LD, Kong LF. Clinicopathological characterization of ten patients with primary malignant melanoma of the esophagus and literature review. *World J Gastrointest Oncol* 2022; 14(9): 1739-1757
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1739.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1739

INTRODUCTION

Primary malignant melanoma of the esophagus (PMME) is the most common non-epithelial malignancy in the esophagus[1], which comprises approximately 0.2% of all tumors of the esophagus[2]. Until now, only several hundred cases of PMME have been reported in the literature, most as case reports[3]. The limited sample size restricted research on the malignancy. Reports on Chinese PMME are limited,

although some areas of China have a high incidence of esophageal cancer. Large studies on Chinese PMME were reported by Wang *et al*[4] (n = 76), Dai *et al*[5] (n = 70), Sun *et al*[6] (n = 21), and Chen *et al*[7] (n = 20). PMME has the following characteristics: Difficult to diagnosis, rapid progression, high rate of recurrence and metastasis, and poor prognosis. The median survival of PMME in China is 13.5 mo[5]. To date, the diagnosis, treatment, and pathological staging of PMME follow the guidelines for esophageal cancer[5]. Systematically analyzing the clinicopathologic features and the possible prognostic factors of PMME will improve the effectiveness of its diagnosis and treatment.

In this retrospective study, we presented ten cases of PMME encountered at Henan Provincial People's Hospital, together with a systematic analysis of 280 Chinese patients with PMME collected from both the English- and Chinese-language literature, with the aim of analyzing the clinicopathological and prognostic characteristics of Chinese patients with PMME.

MATERIALS AND METHODS

Summary of ten cases in our hospital

The records of 12 patients with PMME were retrieved at Henan Provincial People's Hospital from January 1990 to September 2021. Two patients were excluded because of a history of cutaneous melanoma. The clinical data of the remaining ten patients, including gender, age, symptoms, endoscopic and radiographic examination, tumor location, tumor size, operative time, tumor node metastasis (TNM) stage, and others were collected. All of the ten patients were confirmed by endoscopic biopsy and four of them received surgical treatment. None of them had a history of melanoma in the skin or other malignancy history.

The tumor diagnostic evaluation was reviewed and confirmed by two independent pathologists. In order to be consistent with the published literature, the clinical and pathological stages were reassessed according to the 7th edition of the Union for International Cancer Control (UICC) TNM classification system. Follow-ups were performed by telephone and the outpatient medical record system, and the complete follow-up data should include survival status, cause of death, and time of death.

The present study was approved by the Institutional Review Board of Henan Provincial People's Hospital, and it conformed to the provisions of the Declaration of Helsinki. Written informed consent was obtained from all individuals before biopsy or surgery.

Review of the literature

A systematic literature review was performed in databases of China BioMedical Literature on Disc (CBMdisc), and Medical Literature Analysis and Retrieval System online (MEDLINE). Relevant publications were identified using the following terms and keywords: "Malignant melanoma of the esophagus" or "Malignant melanoma" and "Esophagus". The last search was updated on September 1, 2021. References of the retrieved articles were further reviewed to find other potential eligible studies. The title and abstract were first screened, followed by full text assessing for eligibility. Each step was independently conducted by two researchers, results were compared, and differences were resolved by consensus.

Inclusion and exclusion criteria

To be eligible for inclusion in this meta-analysis, the article must meet the following criteria: (1) Describing studies on PMME in Chinese population; (2) Providing detailed information of each patients, including gender and age; and (3) Providing pathology diagnosis. Articles were excluded due to the following reasons: (1) Studies were not focused on Chinese population; (2) Meta-analysis or reviews; (3) There was no detailed information of each patients; (4) Content repeats in different articles; and (5) Accompanied with other malignancies, including melanoma in other body parts simultaneously or heterogeneously.

Data extraction

Data from retrieved articles were independently collected by two reviewers. The following information was extracted from each study: First author, year of publication, and detailed information of each patients. In event of inconsistent evaluations, a third investigator was consulted to resolve the dispute and made the final decision.

Statistical analysis

Descriptive or frequency analysis was used for basic information analysis. Numerical variables are expressed as the mean ± SD. Statistical differences were evaluated by χ^2 test or t test. The effects of the clinicopathologic factors on lymph node metastasis (LNM) was evaluated using univariate and multivariate logistic-regression models. The Kaplan- Meier method was used to assess associations between clinicopathological characteristics and survival outcome. Univariate and multivariate analyses were performed using Cox regression. Hazard ratios (HRs) and 95% confidence intervals (CIs) were

calculated. The log-rank test was used to compare survival curves. All statistical tests were two-sided. *P* values less than 0.05 were considered statistically significant. All statistical analyses were conducted using SPSS 21 (IBM Corporation, Waltham, NY, United States).

RESULTS

Clinicopathological characteristics of ten PMME patients at our hospital

The clinicopathological characteristics of ten PMME patients are summarized in Table 1. There were six men and four women. The ages ranged from 47 years to 80 years with a mean age of 62.2 ± 9.9 years. Although the mean age of female patients (68.3 ± 10.4 years) was much older than that of the male patients (58.0 ± 7.8 years), there was no statistical difference (P = 0.111). Eight of them presented with dysphagia as the main symptom (80%, 8/10), and the other two had retrosternal pain or bellyache. Six of them also had an esophagography and computerized tomography (CT) scan. The esophagography revealed mucosa destruction and an irregular filling defect of the esophageal lumen (Figure 1A). The CT scan showed polypoid masses in the esophagus (Figure 1B). There were one, six, and three patients having the masses located at the upper, middle, and lower portion of the esophagus, respectively.

All of the ten patients had a preoperative esophagoscopy and biopsy pathology. The endoscopy manifestations were polypoid or a protuberant mass (n = 7), ulcerative mass (n = 1), and superficial lesion (n = 2). About half of the patients had pigment deposition on the surface of the tumors (Figure 1C and D). Nine patients had an accurate preoperative diagnosis of PMME, but the remaining one who was initially diagnosed with poor differentiated carcinoma by biopsy pathology, was eventually diagnosed with PMME by postoperative pathology (Figure 1E and F). There were four patients who received surgery and two who received chemoradiotherapy only. A postoperative pathological examination of the four patients showed that the lesions of two cases were confined to the submucous layer (T1b), and two had lesions extended to the muscularis propria (T2). The mean number of lymph nodes dissected in surgery was 14.5 ± 6.1 (range: 6-19). Notably, none of the four patients had LNM.

Five of the six patients who received treatments at our hospital were successfully followed up. One was still alive until the last follow up, but the remaining four died because of recurrence or metastasis. The median survival time was 24.5 mo (range: 3-31.9 mo).

Characteristics of selected studies

The literature flowchart (Supplementary Figure 1) exhibits the entire selection process from the eligible studies. The search can be traced using the publication date from 1980 to September 2021. A total of 122 studies were collected using the inclusion and exclusion criteria, including 98 articles in Chinese and 24 in English. Finally, a total of 280 patients diagnosed with PMME were enrolled in the study. The main characteristics of the included studies[4,6-126] as well as the corresponding clinicopathological features are summarized in the Supplementary Table 1. Finally, a total of 290 patients, including the ten cases recruited from our hospital and the 280 cases collected from the literature, were subjected to subsequent analysis. The clinicopathological characteristics are shown in Table 2.

Gender, age, and tumor location

Each case of the 290 cases had gender, age, tumor location, and pathology documents. There were 200 males and 90 females with a male-to-female ratio of 2.2:1. Their ages ranged from 26 to 84 years, with a mean age of 58.5 ± 9.7 years. No significant difference was found in age between male and female patients (male: 58.6 ± 9.1 years; female: 58.3 ± 11.1 years).

Most of the tumors (274/290, 94.6%) were located in the middle (n = 138) or lower (n = 136) of the esophagus, and only 16 cases (5.4%) had the tumors located in the upper esophagus. Interestingly, the tumors in female patients were prone to being located in the upper esophagus (62.5%, 10/16), and conversely, tumors in male patients were more often located in the both middle and lower esophagus (72.3%, 198/274, P = 0.003).

Symptoms and duration

There were 277 patients who had their main symptoms documented. The most common symptom was dysphagia (219, 79.1%), followed by retrosternal pain (31, 11.2%), bellyache (11, 4.0%), poor food intake with no obvious incentive (6, 2.2%), and hematemesis or melena (2, 0.7%), respectively. Eight (2.9%) patients were asymptomatic and had the tumors detected in the physical examination. The interval between the diagnosis of the disease and the onset of symptom occurrence was documented in 188 patients. The symptom duration ranged from 0.2-36 mo, with a median of 2.0 mo.

Imaging examination

Notably, there were 147 patients who had detailed information of upper gastrointestinal barium esophagogram and CT. For most of them, the esophagography revealed mucosa destruction, irregular filling defect, and narrowness of the esophageal lumen. The CT examination mainly showed bulky or

Table 1 Clinicopathological features of ten cases of primary malignant melanomas of the esophagus from Henan Provincial People's Hospital

Case No.	Gender	Age (yr)	Chief complaint	Location	Gross classification	Tumor number	Preoperative diagnosis	Tumor length	Deep in depth	LNM	Treatment	Survival (mo)
1	Male	61	Dysphagia	Middle	NA	1	PMME	NA	NA	NA	NA	FU loss
2	Female	59	Dysphagia	Middle	NA	1	PMME	NA	NA	NA	NA	FU loss
3	Male	47	Dysphagia	Lower	NA	1	PMME	NA	NA	NA	NA	FU loss
4	Female	60	Dysphagia	Lower	NA	1	PMME	NA	NA	NA	NA	FU loss
5	Female	80	Dysphagia	Middle	NA	1	PMME	NA	NA	NA	R + C	31
6	Male	69	Dysphagia	Middle	NA	1	PMME	NA	NA	NA	C	51
7	Male	57	Dysphagia	Upper	Ulcering	1	Poor differentiated carcinoma	5	DP	No	S	18
8	Female	74	Retrosternal pain	Lower	Polypoid	2	PMME	5	SM	No	S	FU loss
9	Male	62	bellyache	Middle	Polypoid	1	PMME	2.5	DP	No	S	3
10	Male	52	Dysphagia and retrosternal pain	Middle	Polypoid	1	PMME	4	SM	No	S	22 alive

PMME: Primary malignant melanoma of esophagus; NA: Not applicable; SM: Submucosal layer; MP: Muscularis propria; FU: Follow up; S: Surgery; C: Chemotherapy; R: Radiotherapy.

polypoid and intraluminal obstructive masses in the esophagus.

Endoscopic biopsy and treatment

About 181 patients had preoperative endoscopy documents. The most common manifestation of the endoscopy was an irregular segmented, lobular, polypoid, or segmented intraluminal tumor mass. Half of the tumors had a rough, eroded, and friable and easily bleeding surface (87/181, 48.1%). Six patients failed to have the mucosa biopsy taken because it bled readily.

The detailed pathological results of the preoperative biopsy were described in 206 patients. Only 115 (55.8%) of the 206 patients were accurately diagnosed as having PMME. Biopsy pathology of the remaining cases were as follows: Poorly differentiated carcinoma (39/206, 18.9%), squamous cell carcinoma (15.5%, 32/206), adenocarcinoma (4.9%, 10/206), and high-grade dysplasia or nonneoplastic lesions (4.9%, 10/206).

Treatment was documented in 257 of the 290 patients (88.6%). The majority of the cases (234/257, 91.1%) accepted esophagogastrostomy or subtotal esophagectomy, and seven (2.7%) patients accepted endoscopic submucosal dissection (ESD). Besides surgery or ESD, 88 (88/241, 36.5%) patients also received adjuvant therapy, including radiotherapy, chemotherapy, and immunotherapy. There were 16 (6.2%) cases that only received adjuvant therapy without surgery.

Tumor number and size

Multiple tumors were defined as there was at least one satellite nodule or it was scattered with a black pigmented spot near the primary tumor. The tumor size of multiple tumors was calculated as the size of the primary tumor instead of the sum of multiple tumors. There were 71.9% of PMME masses that had a pigmented surface. Seventy-four (61.8%) cases had single tumors, and 46 (38.2%) had multiple tumors. The mean size was 5.2 ± 2.9 cm (range: 0.3-17.0 cm). The mean tumor size in males was significantly longer than that in female patients ($P < 0.001$, Figure 2A). Additionally, the tumor size was significantly correlated with tumor location ($P < 0.001$), and the mean tumor size was much shorter when the tumor was located in the upper thoracic esophagus (Figure 2B). No difference was found in tumor size between single and multiple tumors (single: 5.2 ± 2.8 cm; multiple: 5.3 ± 3.1 cm; $P = 0.895$).

Gross classification and TNM stage

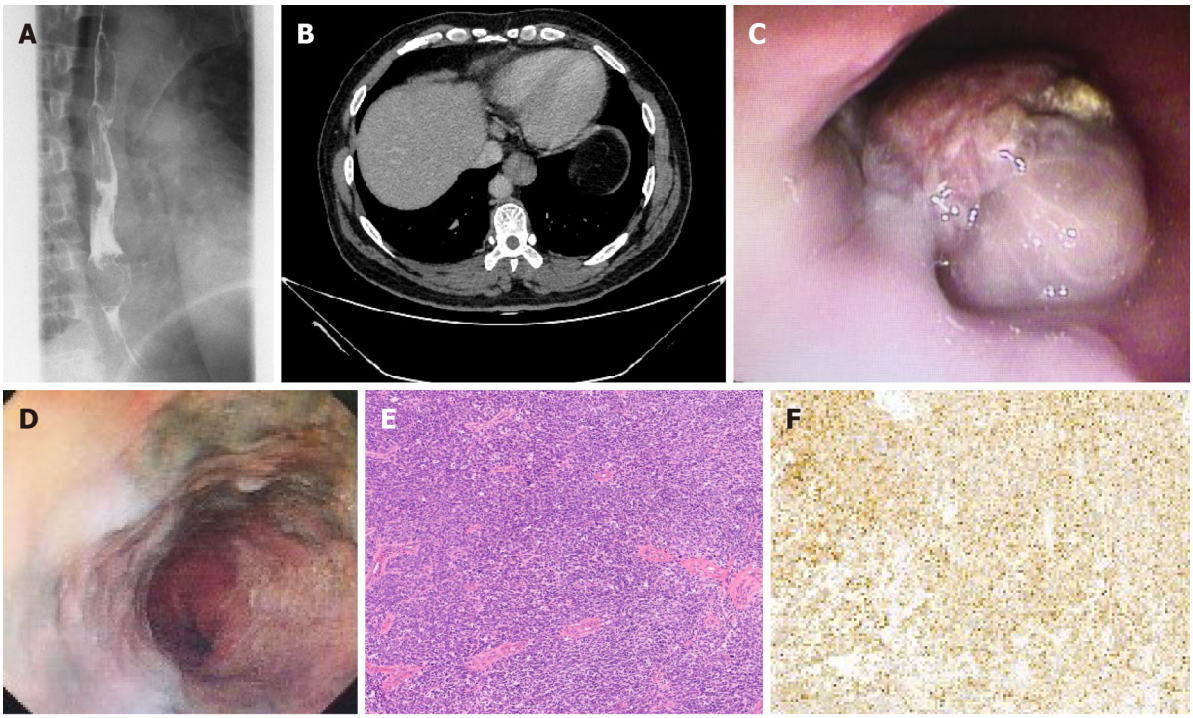
There were 244 patients who had gross classification documents. The most common subtype was polypoid (194/244, 79.5%), followed by ulcerative ($n = 29$, 11.9%), superficial ($n = 14$, 5.7%), medullary ($n = 6$, 2.5%), and constrictive subtypes ($n = 1$, 0.4%).

There were 213 patients who had depth of tumor invasion documents. Pathological examination revealed that the tumors in 45.6% of the PMME patients were limited to submucosal layer, including 14

Table 2 Clinicopathologic characteristics of 290 primary malignant melanoma of esophagus patients

Characteristic	*n*	%
Gender		
Male	200	69.0
Female	90	31.0
Age	58.4 ± 9.7 yr	
Symptoms		
Dysphagia	219	79.1
Restrosternal pain	13	4.7
Dysphagia and restrosternal pain	18	6.5
Bellyache	11	4.0
No symptom found by physical examination	8	2.9
Loss of appetite	6	2.2
Hoematemesis or melena	2	0.7
Censored	13	
Location		
Upper	16	5.4
Middle	137	47.3
Lower	137	47.3
Pigmentation		
Yes	141	71.9
No	55	28.1
Censored	94	
Pathological diagnosis of biopsy		
PMME	115	55.8
ESCC	32	15.5
Poorly differentiated carcinoma	39	18.9
Esophageal adenocarcinoma	10	4.9
High-grade dysplasia or non-neoplastic lesions	10	4.9
Censored	84	
Treatment		
Surgery	153	58.8
Surgery and adjuvant treatment	88	35.1
Adjuvant treatment	16	6.1
Censored	33	
Tumor size (censored: n = 77)	5.2 ± 2.9 cm	
Tumor number		
Single	74	61.7
Multiple	46	38.3
Censored	170	
Gross classification		
Superficial elevated	14	5.7
Polypoid	194	77.9

Ulcerative and others	36	14.4
Censored	46	
Depth of invasion		
T1	97	45.6
T2	67	31.4
T3 and T4	49	23.0
Censored	77	
Lymph node metastasis		
Yes	107	51.2
No	102	48.8
Censored	81	



DOI: 10.4251/wjgo.v14.i9.1739

Figure 1 Imaging and microphotograph of primary malignant melanoma of the esophagus. A: Barium swallow examination showed an irregular filling defect on the lower third of the esophagus, causing mucosa destruction; B: Computed tomography showed an eccentric thickening in the lower third of the esophagus wall, with enhancement; C and D: Esophagoscopy revealed a nonpigmented polypoid tumor with hyperemia and erosion in the lower esophagus, and black lesion scattered on the wall of esophagus; E: Hematoxylin-eosin staining identified malignant melanoma cells in the lamina propria of the esophagus (× 100); F: Immunohistochemical staining with HMB45 (human melanoma black 45) antibody revealed positive tumor cells (× 100).

(6.6%) cases restricted to the mucosa (T1a) and 83 (39.0%) restricted to the submucosal layer (T1b). The number of patients with tumor extension to the muscularis propria (T2), fibrous membrane (T3), and outer membrane (T4) was 67 (31.4%), 40 (18.8%), and 9 (4.2%), respectively. No correlation was found between the tumor infiltration depth and clinical characteristics ($P > 0.05$; data not shown).

Totally, 209 patients had LNM documents. The mean number of lymph nodes dissected in surgery was 11.7 ± 8.9 (range: 1 to 43). The positive rate of LNM was 51.2% (102/209). The correlation between LNM and clinicopathological features is shown in Table 3. Significantly, no LNM was found when the tumor was confined to the mucous layer (T1a). The risk of LNM was significantly increased with the progression of the pT stage [$P < 0.001$, odds ratio (OR): 2.47, 95%CI: 1.72-3.56]. The size for the tumors with LNM was significantly larger than that of tumors without ($P < 0.001$, OR: 1.24, 95%CI: 1.09-1.42). A regression analysis found that the risk of LNM was associated with both the pT stage and tumor size (pT stage: $P < 0.001$, OR: 2.22, 95%CI: 1.47-3.33; tumor size: $P = 0.006$, OR: 1.21, 95%CI: 1.05-1.38).

Table 3 Correlation between lymph node metastasis and clinicopathological features

Feature	LNM-		LNM+		*P* valve	Logistic regression analysis	
	n	%	*n*	%		*P* valve	OR (95%CI)
Gender							
Male	77	51.0	74	49.0	0.307		
Female	25	43.1	33	56.9			
Age (yr)	59.4 ± 8.9		57.2 ± 10.3		0.109		
Location							
Upper	3	75.0	1	25.0	0.202		
Middle	52	53.6	45	46.4			
Lower	47	43.5	61	56.5			
Tumor size (cm)	4.6 ± 2.4		6.0 ± 3.0		**< 0.001**	**0.006**	1.21 (1.05-1.38)
Tumor number							
Single	23	41.1	33	58.9	0.919		
Multiple	14	40.0	21	60.0			
Censored	67	54.5	56	46.5			
Gross classification							
Superficial elevated	11	91.7	1	8.3	**0.01**	0.261	
Polypoid	68	46.6	78	53.4			
Ulcerative and others	14	45.2	17	54.8			
Censored	9	45	11	55			
Infiltration depth							
T1a	14	100	0	0	**< 0.001**	**< 0.001**	2.22 (1.47-3.33)
T1b	40	54.8	33	45.2			
T2	30	48.4	32	51.6			
T3 + T4	9	20.0	36	80.0			
Censored	9	60	6	40			

LNM: Lymph node metastases; OR: Odds ratio; CI: Confidence interval.

Local recurrence and distant metastasis

Eighty-four patients had records for local recurrence and distant metastasis, and 16 cases were combined with distant metastasis in addition to local recurrence. The precise sites of the distant metastasis were well documented in 74 cases. A total of 94 PMME metastatic sites were affected in the 74 patients; 19 cases had two sites involved, and 5 had three sites involved synchronously or metachronously. Both the lung (n = 26, 27.7%) and liver (n = 24, 25.5%) were the sites most frequently involved, followed by the lymph nodes (including those of the enterocoelia, neck, mediastinum, and axilla, n = 19, 20.2%), brain (n = 8, 8.5%), bone (n = 6, 6.4%), and other locations. The detailed distant metastasis locations are shown in Figure 3.

Overall and disease-free survival

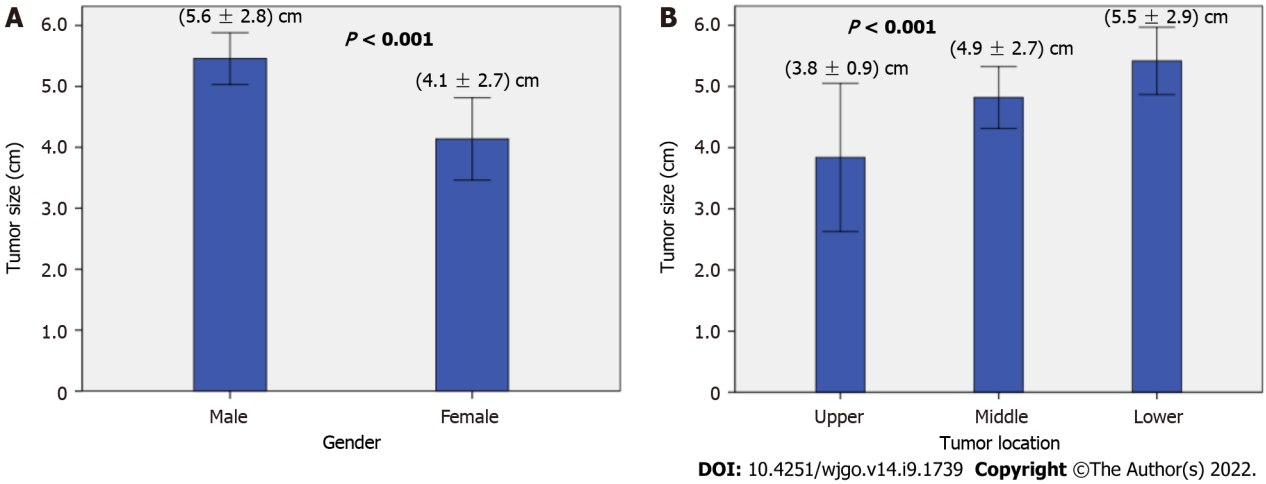
The follow-up data were documented in 179 patients. Three patients died of serious complications during the preoperative period. Two cases were lost after surgery at 12 and 33 mo, respectively. After excluding the five patients, the survival analysis was performed on the remaining 174 patients. There were 116 cases (65.9%) with cancer-specific deaths and 58 (32.9%) were still alive at the time that the articles were published. The median overall survival (OS) of 174 patients was 11.0 mo (range: 1-204 mo), and the 1-, 3-, and 5-year survival rates were 57%, 25%, and 12%, respectively (Figure 4A).

We compared the OS rate between the different clinicopathological characteristics of the PMME patients (Table 4). As shown in Figure 5A, patients at pT1b (n = 60) or advanced pT stages (n = 79) had a significantly worse prognosis than patients at T1a stage (n = 12, P = 0.01 and P = 0.001, respectively).

Table 4 Univariate and multivariate analyses of predictive factors for overall survival and disease-free survival in patients with primary malignant melanoma of esophagus

Variable	Overall survival				Disease-free survival			
	Uni-*P*	Multi-*P*	HR	95%CI	Uni-*P*	Multi-*P*	HR	95%CI
Gender								
Male *vs* female	0.08				0.450			
Age (yr)								
< 55 *vs* ≥ 55	0.348				0.353			
Tumor location								
Upper *vs* middle *vs* lower	0.647				0.385			
Tumor number								
Single *vs* multiple	0.200				0.227			
Tumor size (cm)								
< 5.5 *vs* ≥ 5	0.282				0.124			
Gross classification								
Superficial *vs* polypoid *vs* ulcerative and others	**0.04**	0.249			**0.007**	0.893		
Depth of invasion								
T1a *vs* T1b *vs* T2 and T3 and T4	**0.001**	**0.005**	1.70	1.17-2.47	**0.02**	**0.02**	1.93	1.09-3.42
LNM								
No *vs* yes	**< 0.001**	**0.009**	1.78	1.15-2.74	0.07			
pTNM stage								
I *vs* II *vs* III and IV	**< 0.001**	0.349			**0.02**	0.540		
Treatment								
Surgery *vs* surgery plus adjuvant therapy	0.433				0.02	0.698		

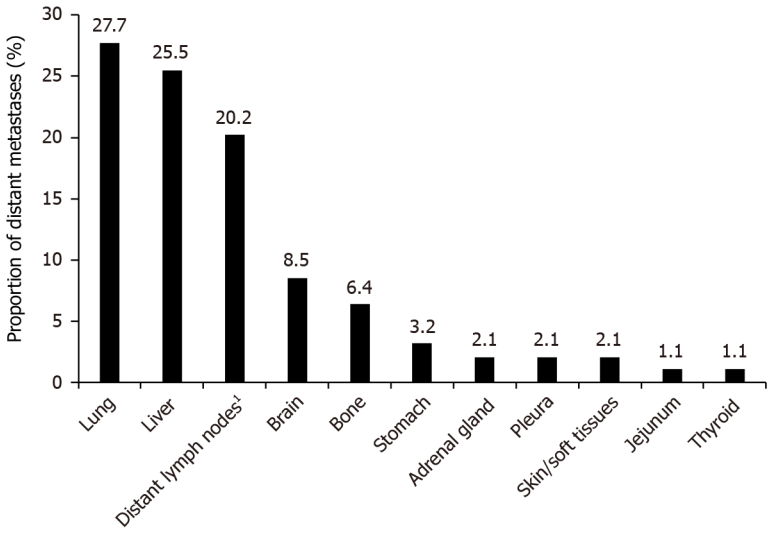
LNM: Lymph node metastases; pTNM: Pathological tumor node metastasis; HR: Hazard ratio; CI: Confidence interval.



DOI: 10.4251/wjgo.v14.i9.1739

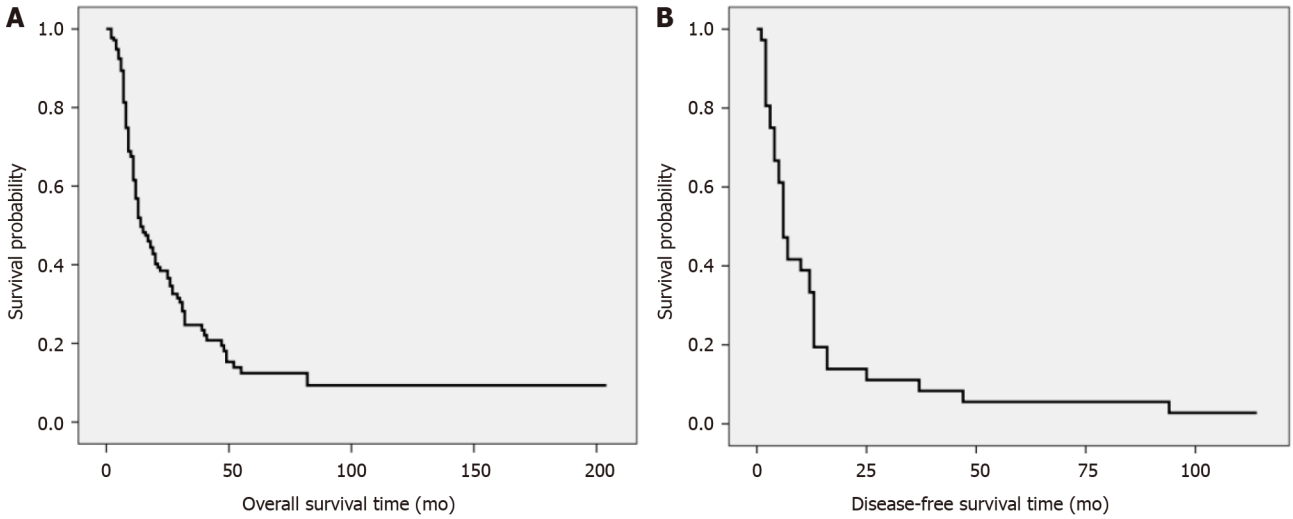
Figure 2 Correlation of tumor size with gender and tumor location. A: Gender; B: Tumor location.

Moreover, the prognosis of patients at the pT1b stage was much better compared with patients at advanced pT stage (P = 0.03, Figure 5A). In addition, the LNM-positive group had a significantly poorer prognosis compared with the LNM-negative group (P < 0.001, Figure 5C). As for the pathological tumor node metastasis (pTNM) stages, both the stage II and stage III/IV groups had a worse prognosis than



DOI: 10.4251/wjgo.v14.i9.1739

Figure 3 Site of metastasis in the study cohort. [1]Distant lymph nodes including those in the enterocoelia, neck, mediastinum, and axilla.



DOI: 10.4251/wjgo.v14.i9.1739

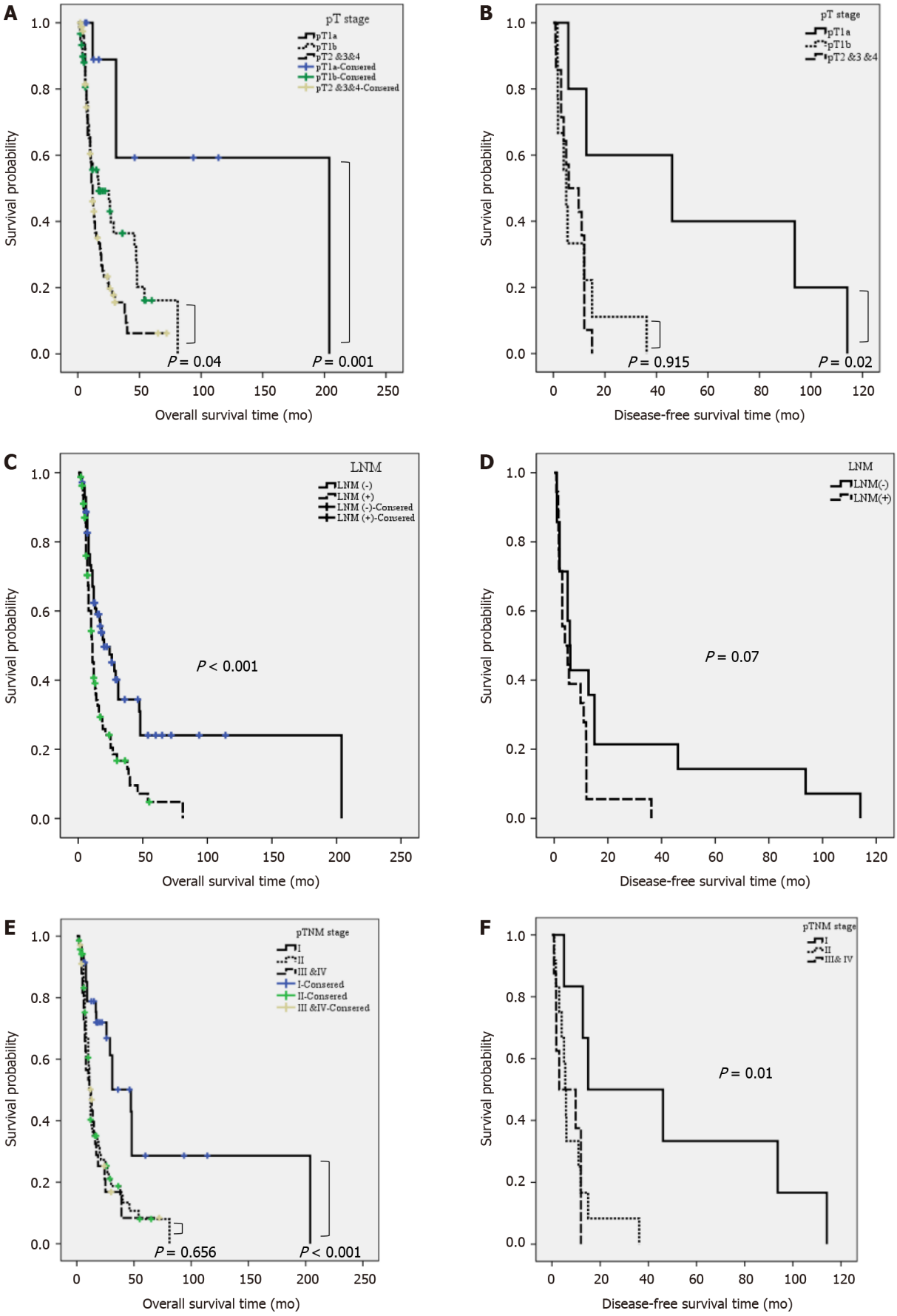
Figure 4 Survival of patients with primary malignant melanoma of the esophagus. A: Overall survival; B: Disease-free survival.

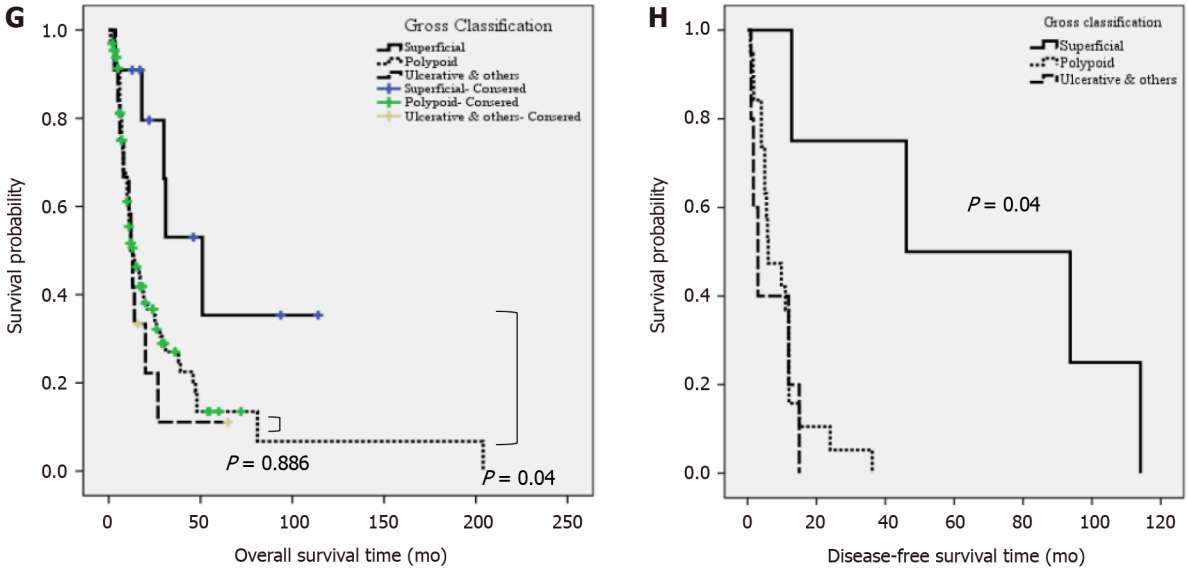
the stage I group ($P < 0.001$, Figure 5E). Furthermore, patients with a superficial subtype had a significantly longer OS time than patients with other gross classifications ($P = 0.02$, Figure 5G). Male patients tended to have a worse prognosis compared with female patients ($P = 0.08$). A multivariate analysis demonstrated that both pT and LNM were independent prognostic factors for PMME patients (pT stage: $P = 0.005$, HR: 1.70, 95%CI: 1.17-2.47; LNM: $P = 0.009$, HR: 1.78, 95%CI: 1.15-2.74).

For disease-free survival (DFS), only 36 cases had detailed documents. The median DFS was 5.3 mo (range: 0.8-114.1 mo), and the 1-, 3-, and 5-year survival rates were 33%, 11%, and 6%, respectively (Figure 4B). Similar to the OS, the DFS of the patients at T1a was significantly better than that of patients at advanced pT stages ($P = 0.01$, Figure 5B). Patients at pTNM I had a better RFS compared with patients at pTNM II-IV ($P = 0.02$, Figure 5F). Furthermore, the DFS of patients with superficial subtype was significantly longer than patients with other gross classifications ($P = 0.007$, Figure 5H). Moreover, LNM-positive patients also tended to had a worse DFS than LNM-negative patients ($P = 0.07$; Figure 5D). Multivariate analysis demonstrated that only pT stage was the independent DFS prognostic factor for patients with PMME ($P = 0.02$, HR: 1.93, 95%CI: 1.09-3.42) (Table 4).

DISCUSSION

Primary mucosal melanomas can be found in the mucosal membranes of the respiratory, gastroin-


A
pT stage
pT1a
pT1b
pT2 &3&4
pT1a-Consered
pT1b-Consered
pT2 &3&4-Consered
Survival probability
P = 0.04
P = 0.001
Overall survival time (mo)
B
pT stage
pT1a
pT1b
pT2 &3 &4
Survival probability
P = 0.915
P = 0.02
Disease-free survival time (mo)
C
LNM
LNM (-)
LNM (+)
LNM (-)-Consered
LNM (+)-Consered
Survival probability
P < 0.001
Overall survival time (mo)
D
LNM
LNM(-)
LNM(+)
Survival probability
P = 0.07
Disease-free survival time (mo)
E
pTNM stage
I
II
III &IV
I-Consered
II-Consered
III &IV-Consered
Survival probability
P = 0.656
P < 0.001
Overall survival time (mo)
F
pTNM stage
I
II
III& IV
Survival probability
P = 0.01
Disease-free survival time (mo)



DOI: 10.4251/wjgo.v14.i9.1739

Figure 5 Kaplan-Meier curves for patients with primary malignant melanoma of the esophagus. A and B: Comparison between the cases with pT1a, pT1b, and pT2, 3, and 4. Patients at pT1a showed a much better overall survival (OS) and disease-free survival (DFS) than those at pT1b or pT2, 3, and 4. No significant difference was observed between groups at pT1b and pT2, 3, and 4 for OS or DFS; C and D: Comparison between the cases with (+) and without (-) lymph node metastasis (LNM). Patients with LNM (+) showed a lower OS than those with LNM (-); for DFS, the difference was only marginal (P = 0.07); E and F: Comparison between the cases with pTNM I, II, and III/IV. Patients at pTNM I showed a much better OS and DFS than those at II and III-IV. No significant difference was noted between groups at pTNM II and III-IV for OS or DFS; G and H: Comparison between the cases with superficial, polypoid, and other gross classifications. Groups with superficial subtype showed a better OS and DFS than those with other subtypes. No significant difference was noted between groups with polypoid and other subtypes for OS or DFS.

testinal, and genitourinary tracts[127-129]. Distant metastasis is not uncommon in mucosal melanomas [127-129]. PMME is a rare disease with aggressive behavior and poor prognosis. To date, the majority of the existing studies were case reports on the Asian population. It is difficult to conduct a comprehensive retrospective study of patients with PMME. In this study, we tried to investigate the present status of PMME in China by systematically analyzing the clinicopathologic and prognostic characteristics of 290 Chinese patients with PMME.

The male-to-female ratio of PMME was 2.2:1, and the mean age was 58.5 ± 9.7 years. The most common site was the middle and lower thoracic esophagus, which accounted for 94.5%. All of the features resembled those of esophageal squamous cell carcinoma (ESCC), a major form of esophageal malignancies in China. The male-to-female ratio of Japanese patients with PMME was 3.5:1, and the median age was 64.5 years[130], which was much higher than that of Chinese patients. In Western populations, male patients were only a little more than female ones with a male-to-female ratio of 1:3.1, and the mean age was 71.8 ± 13.6 years[131]. This distinction suggested that there might be different tumorigeneses between the Asian and Western populations with PMME. Both the middle and lower esophagus were the most common location of PMME for the Asian and Western populations[130,131]. Additionally, our results showed that the tumor masses of female patients were prone to being located in the upper esophagus compared with males, which prompted that an endoscopist should pay more attention to the upper thoracic esophagus of female patients to avoid missing an early lesion even though PMME is rare in the upper of the esophagus.

Polypoid lesions (79.5%) were the predominant gross classification of PMME, many of which are relatively soft, friable, and easily bleed. Sometimes, it was mistaken for phlebangioma under endoscopy [36,86,105]. There were only 5% of patients with PMME who had superficial lesions. The physician and endoscopist might be unfamiliar with the manifestations of PMME at early stage. In one patient from Kunming City, China, who presented with retrosternal pain after eating for 7 d, the first endoscopy showed several black lesions scattered throughout the middle esophagus. He was misdiagnosed because the doctor was unfamiliar with PMME. After 8 mo, the second endoscopy showed a polypoid lesion. The patient died 3 mo after surgery because of systemic metastasis[48].

The pathognomonic endoscopic finding of PMME is pigmentation. Our results showed that about 71.9% of PMME masses had a pigmented surface, which was similar to a previous study[131] showing that 26.9% of the lesions were amelanotic. These results suggested that the absence of pigmentation does not necessarily exclude PMME[3,132]. PMME is always surrounded by satellite lesions. Our results showed that one third of patients had multiple lesions, which was a little higher than that in Japanese patients[3], perhaps because one third of Japanese patients had superficial lesions. Physicians and endoscopists should enhance their awareness of rare diseases of the esophagus, paying particular attention to early lesion, to avoid missed diagnosis and misdiagnosis.

In our study, only 55.8% of patients were clearly diagnosed by biopsy before surgery, which was similar to previous studies[5-7]. The possible reasons for PMME misdiagnosis were as follows[5,6]: (1) Limited biopsy tissue without enough immunohistochemical analysis; (2) Lacking experience in the diagnosis of PMME in clinical practice; (3) Some tumors had no pigmented surface or no melanin granules in the cytoplasm; and (4) The lesion tissue was not biopsied by endoscopy because it bled readily. An accurate diagnosis could be obtained by immunohistochemical analysis. Human melanoma black antibody 45 (HMB45), melanoma antigen protein (Melan-A), and S100 are the specific diagnostic indicators for melanoma.

Melanoma might be associated with cancer predisposition syndromes[133]. In addition, a history of melanoma approximately increase the risk of subsequent melanoma[134]. Thus, multiple imaging diagnostics were employed in PMME and other mucosal melanoma to evaluate primary tumor, metastasis, and treatment responses[127]. Ultrasonography, endoscopic ultrasound, CT, magnetic resonance imaging, and positron emission tomography (PET) contribute to the information for diagnosis and management[127]. PET/CT improves the diagnosis, staging, treatment evaluation, and surveillance of tumors. It is currently considered to be the most sensitive method for the identification of metastatic lesions of solid tumors and has a huge impact on patient management[127].

The tumor size of PMME had a wide range, and the mean value was 5.2 ± 2.7 cm, which was similar to Japanese patients[130]. Previous studies[3,135,136] considered that PMME is prone to spread longitudinally, and local recurrence is frequently found soon after surgery. Thus, PMME should be resected with adequate margins. Masses in male patients had a significantly larger tumor size than that in female patients. Men might endure symptoms longer than women before seeking medical care[137].

The overall LNM-positive rate in our study was 51.2%. Our results showed that nearly half of PMME were at early pT stage, which was different from ESCC - mainly at the advanced pT stage. There were 52.2% of Japanese patients with PMME limited to the submucosal layer[130]. No LNM was found in patients at the pT1a stage in the present study and a previous study[50]. Interestingly, the frequency of LNM increased sharply to 45.3% in our study when the primary tumor was at the pT1b stage. Dai *et al*[5] found that the rate of LNM was as high as 54.2% among patients with pT1 tumors. The risk of LNM increased about 2.5 times along with the deeper depth of the tumor invasion. Previous studies[5,130] also indicated that with a deeper tumor invasion, the probability of LNM was higher. PMME might metastasize through blood or lymph vessels at early stage. Extended lymph node dissection combined with radical esophagectomy should be emphasized even when the tumor is at the pT1b stage.

The median OS of patients with PMME was 11 mo and the 5-year OS was 12%, which were similar to those of the previous studies[5,7]. Japanese patients with PMME have a relatively better survival with a 5-year OS of 25.3%[130]. For the Western population with PMME, the 3-year OS was only 7.3%[131]. It seems the Western population with PMME has a worse survival rate compared with the Asian population, which might be related to elder age of the diagnosed Western patients. Furthermore, PMME patients had poorer outcomes compared with common malignancies of the esophagus (ESCC, adenocarcinoma, and small cell carcinoma)[131]. It is necessary to employ a multidisciplinary team to improve treatments and outcomes for patients with PMME[5].

Multivariate analysis showed that pT (depth of tumor invasion) is an independent prognostic factor for both OS and DFS in patients with PMME. Patients at pT1 had better OS, which was also found in previous studies focused on the Chinese[5] and Japanese[130] populations. As mentioned previously, LNM was extremely rare for the tumor at pT1a, and it increased rapidly for tumors at pT1b or the advanced pT stage.

LNM was also an independent prognostic factor for OS. Previous studies on Chinese[5,7,50] and Japanese[130] patients also suggested that LNM was strongly associated with a poor prognosis. However, no influence of LNM on prognosis was found in the Western population[131]. Furthermore, Dai *et al*[5] showed that ≥ 12 lymph nodes dissected was an independent factor for OS and DFS. A thorough lymph node dissection should be emphasized in the surgical treatment of PMME.

Patients at an advanced pTNM stage, including II-IV, had a significantly worse OS and DFS compared with patients at pTNM I. Similar results were also found in previous studies[4,5]. Our results and others[4,5] suggested that TNM stage of PMME according to the AJCC classification for esophageal cancer might discriminate the prognosis of patients with PMME. Although the TNM stage in accordance with the mucosal melanoma classification could also separate the survival curves, the difference was not statistically significant[6]. Further study is needed to confirm the standard staging system of PMME[6].

Until now, treatment consensus on PMME had not been established because of its low prevalence. Surgery is still the primary option for resectable tumors. The median OS for patients who received immunotherapy besides surgery and chemoradiotherapy tended to be longer than patients who received surgery plus chemoradiotherapy or patients who only received surgery. However, there was no apparent difference in DFS between patients who received adjuvant therapy in addition to surgery and those who only received surgery. A comparison of the prognosis between surgery and adjuvant therapy was not conducted because there were only four patients successfully followed who only received adjuvant treatments. Many studies tried to seek optional treatments for patients with PMME. Dai *et al*[5] indicated that adjuvant therapy could improve both DFS and OS of patients with PMME. Wang *et al*[4] also suggested that postoperative chemotherapy could improve DFS. Additionally, PD-1 inhibitors might be a viable option for patients with PMME because the tumor has a dramatically high

response rate to PD-1 checkpoint inhibitor monotherapy[4]. Systemic treatment of PMME, including surgery, chemoradiotherapy, and immunotherapy, should be used to improve multidisciplinary treatments and outcomes for patients with PMME.

Male patients tend to have a worse prognosis compared with female patients. Previous studies indicated that male gender was an independent prognostic predictor of PMME[5,6,128]. Our results also found that male patients had a larger tumor size compared with female patients. The serum estradiol significantly decreased in both male and female patients with ESCC or precancerous lesions[138]; moreover, the expression of estrogen receptor in precursor lesions of the esophagus changed during the multistage process of esophageal carcinogenesis[139]. All those phenomena suggested that estrogen might play an important role in esophageal malignancy.

CONCLUSION

PMME is a rare esophageal malignancy with a poor prognosis. Because of the low rate of correct diagnosis before surgery, physicians and endoscopists should develop their awareness of rare diseases of the esophagus, paying particular attention to early lesions. Extended lymph node dissection combined with radical esophagectomy should be stressed because of multifocality and high frequency of LNM — even the depth of the tumor invasion is limited to within the submucosal layer. Both the LNM and pT stage are independent prognostic factors for the OS, while only pT stage was identified to be an independent prognostic factor for the DFS of patients with PMME. Adjuvant treatment, particularly immunotherapy, might be used in clinical practice to improve multidisciplinary treatments and the prognosis of patients with PMME.

ARTICLE HIGHLIGHTS

Research background

Primary malignant melanoma of the esophagus (PMME) is a rare malignant disease. It has not been well characterized in terms of clinicopathology and survival.

Research motivation

The clinical features, survival, and prognostic factors of Chinese patients with PMME are not comprehensively analyzed until now.

Research objectives

This study aimed to investigate the clinical features, survival, and prognostic factors of Chinese patients with PMME.

Research methods

The clinicopathological findings of ten cases with PMME treated at our hospital and 280 cases from both the English- and Chinese-language literature which focused on Chinese patients with PMME were analyzed.

Research results

Only about half of the patients (55.8%) were accurately diagnosed before surgery. Lymph node metastasis (LNM) was easy to be found with a positive rate of 45.3% even when the tumor was confined in the submucosal layer. The risk of LNM was significantly raised along with the increase of pT stage ($P < 0.001$) and larger tumor size ($P = 0.006$). The median overall survival (OS) and disease-free survival (DFS) were 11 mo and 5.3 mo, respectively. Multivariate Cox analysis showed that both pT stage ($P = 0.005$) and LNM ($P = 0.009$) were independent prognostic factors for OS, but only advanced pT stage ($P = 0.02$) was identified to be a significant independent indicator of poor RFS in patients with PMME.

Research conclusions

Correct diagnosis of PMME before surgery is low. Both LNM and pT stage are the independent prognosis factors for OS, but only pT stage was identified to be an independent indicator for DFS of patients with PMME.

Research perspectives

Physicians and endoscopists should develop their awareness of rare diseases of the esophagus, paying particular attention to early lesions. Extended lymph node dissection combined with a radical esophagectomy should be stressed because of multifocality and a high frequency of LNM. Adjuvant treatment, particularly immunotherapy, might be used in clinical practice to improve multidisciplinary

treatments and the prognosis of patients with PMME.

FOOTNOTES

Author contributions: Wang LD, Kong LF, and Zhou SL designed and wrote the paper; Li B, Zhang LQ, and Wang JJ performed data collection and interpretation and follow-up; Zhao XK and Wu Y contributed to data analysis; Wang XJ and Chen Y revised the manuscript; Liu QY and Zhao RJ reviewed the pathology results; all authors read and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by the Institute Research Ethics Committee of Henan Provincial People's Hospital.

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

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: Dataset available from the corresponding author at lfkong9@163.com.

Country/Territory of origin: China

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S-Editor: Gao CC
L-Editor: Wang TQ
P-Editor: Yuan YY

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1758-1770

DOI: 10.4251/wjgo.v14.i9.1758

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Endoscopic debulking resection with additive chemoradiotherapy: Optimal management of advanced inoperable esophageal squamous cell carcinoma

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Bove A, Italy; Elsayed MO, United Kingdom; Man-i M, Japan; Oguma J, Japan

Received: February 18, 2022
Peer-review started: February 18, 2022
First decision: May 9, 2022
Revised: May 22, 2022
Accepted: July 27, 2022
Article in press: July 27, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

There is no remedial strategy other than definitive chemoradiotherapy for patients with advanced esophageal squamous cell carcinoma (ESCC) who are not eligible to undergo surgical treatment.

AIM

To introduce a novel therapy called endoscopic debulking resection (EdR) followed by additive chemoradiotherapy (CRT) and evaluate its efficacy and safety.

METHODS

Advanced, inoperable ESCC patients between 1 January 2015 and 30 December

2019 were investigated retrospectively. Patients who received EdR followed by CRT were deemed the EdR + CRT group and those without CRT were deemed the EdR group. Overall survival (OS), progression-free survival (PFS), and adverse events were evaluated.

RESULTS

A total of 41 patients were enrolled. At a median follow-up of 36 mo (range: 1-83), the estimated 1-, 2-, and 3-year cumulative OS rates of patients who underwent EdR plus additive CRT were 92.6%, 85.2%, and 79.5%, respectively, which were higher than those of patients who underwent EdR alone (1-year OS, 83.3%; 2-year OS, 58.3%; 3-year OS, 50%; P = 0.05). The estimated 2-year cumulative PFS rate after EdR + CRT was 85.7%, while it was 61.5% after EdR (P = 0.043). According to the univariate and multivariate Cox regression analyses, early clinical stage (stage ≤ IIB) and additive CRT were potential protective factors for cumulative OS. No severe adverse events were observed during the EdR procedure, and only mild to moderate myelosuppression and radiation pneumonia were observed in patients who underwent additive CRT after EdR.

CONCLUSION

EdR plus CRT is an alternative strategy for selective advanced inoperable ESCC patients.

Key Words: Esophageal squamous cell carcinoma; Endoscopic resection; Chemoradiotherapy; Overall survival; Progression-free survival

Core Tip: Forty-one advanced esophageal squamous cell carcinoma (ESCC) patients were retrospectively enrolled, including 28 patients who underwent endoscopic debulking resection (EdR) plus chemoradiotherapy (CRT) and 13 who received EdR without CRT. Clinicopathological characteristics, perioperative outcomes, cumulative overall survival (OS), and progression-free survival (PFS) rates were analyzed. Our results confirm that EdR is safe and feasible for advanced ESCC patients and that EdR + CRT showed better OS and PFS than EdR alone.

Citation: Ren LH, Zhu Y, Chen R, Shrestha Sachin M, Lu Q, Xie WH, Lu T, Wei XY, Shi RH. Endoscopic debulking resection with additive chemoradiotherapy: Optimal management of advanced inoperable esophageal squamous cell carcinoma. *World J Gastrointest Oncol* 2022; 14(9): 1758-1770

URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1758.htm

DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1758

INTRODUCTION

Esophageal carcinoma (EC) is the sixth leading cause of cancer-related death worldwide[1]. The incidence of esophageal squamous cell carcinoma (ESCC), the main type of EC in China, ranks sixth, while its mortality ranks fourth[2]. Over the past decades, clinicians have made great efforts to improve the therapeutic outcomes of ESCC. Early ESCC with stage T1a (mucosal invasion) can be completely cured by endoscopic resection (ER), including endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR)[3]. With regard to T1b (submucosal invasion), studies have reported moderate rates of metastasis in SM1 and high rates of metastatic lymph nodes in SM2 and SM3. For those patients with deeper than SM2 invasion or who undergo noncurative ER (R1 resection), additional treatments such as esophagectomy are always recommended.

Neoadjuvant chemoradiotherapy (nCRT) followed by esophagectomy is currently recommended as the standard therapy for advanced ESCC[4-6]. Advanced ESCC patients who decline to receive surgical treatment or have high surgical risks must choose definitive CRT (dCRT)[7-9]. However, locoregional failure of dCRT is usually unavoidable[10,11]. Patients undergoing dCRT who develop recurrent cancer often have a poor prognosis, with a reported median survival of 4 mo to 28 mo[11]. A series of studies have reported that salvage ER is a promising strategy for locally recurrent lesions after dCRT[12-15], which is good news for recurrent patients. However, salvage ER was deemed to be applicable to superficial lesions only. Furthermore, radiation-induced fibrosis in the submucosa increases the incidence of perforation and bleeding during ER. Therefore, novel strategies that are minimally invasive for advanced inoperable ESCC are urgently needed.

A single-arm prospective study reported by Minashi *et al*[16] concluded that the combination of ER and selective CRT was comparable to surgery, being regarded as a minimally invasive therapy for

T1b(SM1-2)N0M0 patients[16]. Subsequent studies also showed that ER plus CRT had equivalent OS potential to that of esophagectomy for early ESCC patients[16-18], further confirming its high therapeutic value for noncurative ER. However, there are no reports on whether ER plus CRT is suitable for patients with deeper than SM3 invasion.

In this study, we used a new therapy called endoscopic debulking resection (EdR) to treat selected patients diagnosed with advanced ESCC who were unable to undergo surgery, and we extended this treatment option to patients with deeper than T1b (≥ SM3) invasion who were unwilling to receive additional esophagectomy in an attempt to evaluate its efficacy and safety when performed along with additive CRT.

MATERIALS AND METHODS

Patients

From 1 January 2015 to 30 December 2019, patients diagnosed with clinical stage T1b (SM3)-T4N0/+M0/+ inoperable ESCC in our institution were retrospectively included. The inclusion criteria of patients who underwent EdR were as follows: (1) Protruding tumor growth; (2) Tumor invasion ≥ SM3; and (3) Cervical inoperable ESCC or unwillingness to or unable to receive esophagectomy. Patients who had other concurrent malignancies and needed extra therapies were excluded. Patients who received EdR in our study were all suggested to undergo additional selective CRT. The choice of different CRT strategies was made based on the pathological diagnosis and the patients' physical tolerance.

All patients were staged with ^{18}fludeoxyglucose positron emission tomography combined with computed tomography (^{18}FDG-PET/CT) or computed tomography (CT). Magnification endoscopy (ME) and endoscopic ultrasound (EUS) were used to assess the T- and N-stage of each patient. The grading of tumors was performed according to the 2010 WHO classification of tumors of the digestive system. The TNM stage of the tumor was determined according to the American Joint Commission on Cancer (AJCC) and Union of International Cancer Control (UICC), 8th edition.

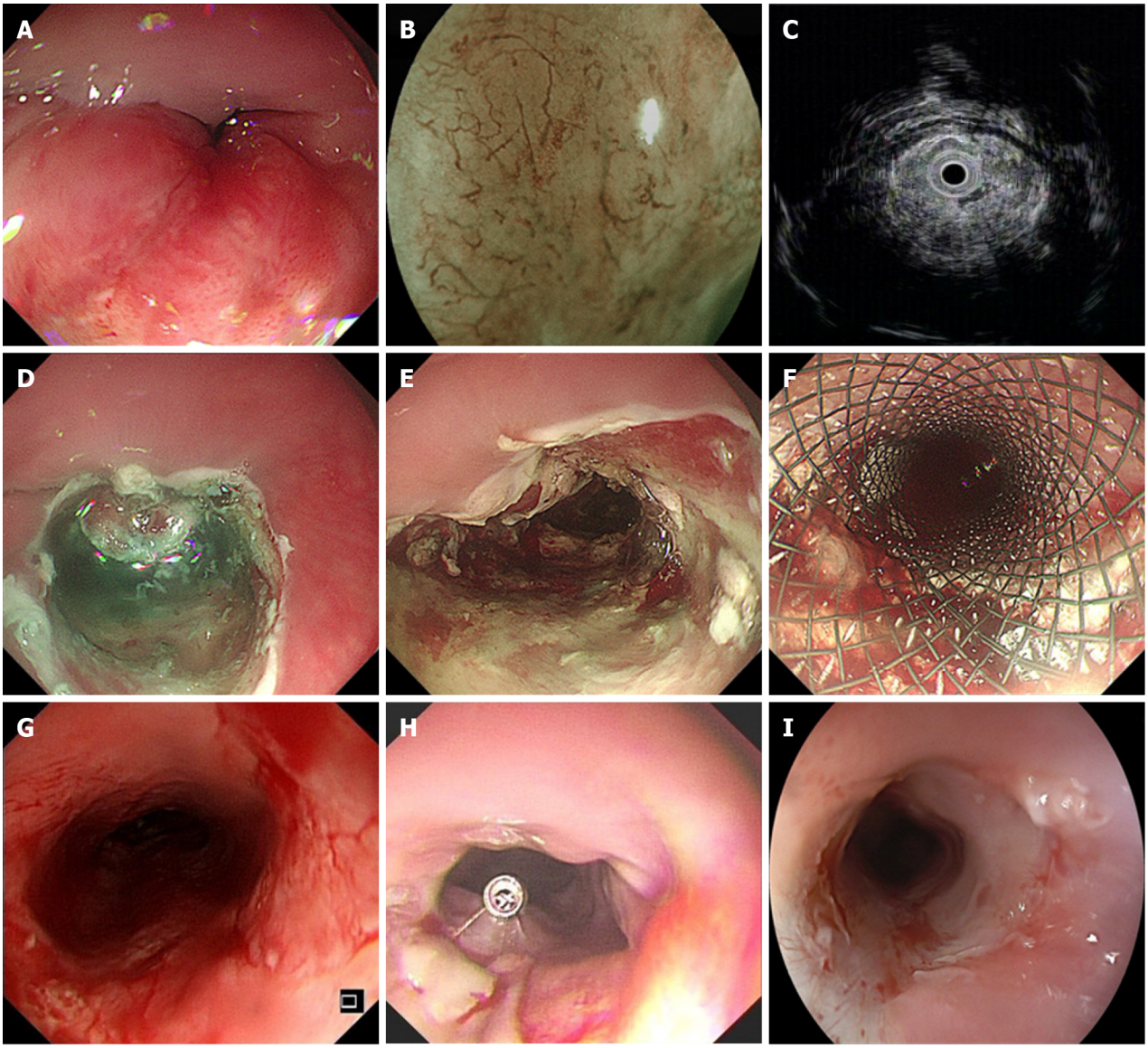
Debulking resection procedure

EdR was performed by experienced endoscopists in our center (Figure 1). All patients underwent operation under intubation anesthesia. Carbon dioxide insufflation and a GIF-H260 endoscope (Olympus, Tokyo, Japan) fitted with a transparent cap (Tokyo, Japan) were used during the therapy. We handled a VIO-300D electrosurgical generator (ERBE, Tübingen, Germany), set to Endocut I mode, with Effect 2 for incision and coagulation and Effect 3 (40 W) for dissection. The lesion border was marked by making spots around it with a Hybrid Knife (ERBE). A mixture of saline solution diluted with methylthionine chloride and epinephrine was injected into the fundus of the lesion. Sometimes, hyaluronic acid is used for its efficiency and persistency; however, as the lesions in our study were always deep in the submucosa, it was difficult to create submucosal fluid cushion and lift the lesion completely. In these cases, we did the separation along the stripping imaginary line and dissected lesions carefully step by step. The tumor was removed with a snare by fragment resection. Bleeding vessels were coagulated by hemostatic forceps (FD-410LR; Olympus, Japan). A fully covered esophageal stent (Micro-Tech Co., Ltd., Nanjing, China) was chosen depending on the postoperative wound, which was resected to the muscularis propria. After the operation, all patients fasted for at least 24 h and were treated with acid suppression, hemostasis, and anti-infection agents. The specimens were examined by experienced pathologists who referred to the Japanese Classification of Esophageal Cancer, 11th edition.

Chemoradiotherapy

Radiotherapy was administered 2 mo after EdR. A megavoltage photon beam (16-18 MV), a CT simulator, and a radiation treatment planning system were used at our institution. Tumor bed volume (GTVtb) was defined as the volume of the primary tumor. GTVtb was expanded to the planning target volume (PGTVtb) by extending 1 cm in all three dimensions. The clinical tumor volume (CTV) included the tumor bed and some optional areas of the regional lymph nodes (bilateral supraclavicular, perieosophageal, mediastinal, and perigastric). The planning target volume (PTV) included the CTV plus a margin of 0.5 cm. Three-dimensional radiotherapy treatment planning was performed to reduce the dose to the normal organs. A total dose of 40 Gy to 46 Gy in 20 fractions was delivered with intensity-modulated radiotherapy or anterior/posterior opposed portals according to the normal organs. A tumor boost of 4-6 Gy was delivered to the tumor bed after EdR. All patients were treated 5 d a week.

Based on the patient's physical state, different chemotherapy regimens were administered based on the pathological diagnosis and the patient's physical condition. The chemotherapy regimens in our study comprised (1) Cisplatin plus 5-fluorouracil (5-FU): Two cycles of cisplatin (70 mg/m^2/d) on day 1 and 5-FU (700 mg/m^2/d) on days 1-4 at an interval of 4 wk; (2) Nedaplatin plus 5-FU: the dosage and administration schedule were the same as those for cisplatin plus 5-FU; and (3) Docetaxel plus 5-FU: docetaxel (7.5 mg/m^2/d, days 1, 8, 22, and 29) and continuous infusion of 5-FU (250 mg/m^2) on days 1-5, 8-12, 15-19, 22-26, 29-33, 36-40, and 43-45 (Supplementary Table 1).



DOI: 10.4251/wjgo.v14.i9.1758

Figure 1 Technical notes of endoscopic debulking resection. This is a typical case from our study, A: An irregular, protruding neoplasm was seen in the esophagus 21 cm away from the incisor, occupying approximately 2/3 of the circumference of the lumen, and the endoscope could not pass through; B: Blue laser imaging magnification showed the type B2 intrapapillary capillary loop vessel in the lesion, with small aortic valve area observed; C: Endoscopic ultrasonography showed hypoechogenicity, an obvious thick mucosal layer and a submucosal layer. The submucosal layer of the lesion was discontinuous and involved the muscularis propria; D: A mixture of saline solution diluted with methylthionine chloride and epinephrine was injected into the lesion, and then incision of the lesion was made using the Dual-Knife; E: The final wound was hemostatically treated; F: A fully covered esophageal stent was finally implanted; G: One month later, the stent was removed endoscopically; H: After another week, the lumen of the endoscopic resection was slightly narrowed, but the ordinary gastroscope was still passable. A titanium clip was used to mark the lesion for later radiotherapy positioning; I: Six months of follow-up after chemoradiotherapy showed slight narrowing of the lumen of the esophagus.

Follow-up

Patients were monitored with weekly hematological examinations, including blood cell counts, liver and kidney function tests, tumor marker tests, electrocardiography, esophagogastroduodenoscopy (EGD), and neck-to-abdominal CT every 3 mo. Local recurrence and metastatic recurrence were defined as a positive biopsy at endoscopy, metastatic lesions to distant organs, and/or local lymph nodes enlarged inside of the irradiation area on ^{18}FDG-PET/CT or CT. The follow-up cutoff date was 31 December 2021.

Outcomes

The primary end point was overall survival (OS), defined as the time from the date of the initial treatment to the date of death from any cause or the date of the last contact. The key secondary end point was progression-free survival (PFS), which was measured as the time from treatment to either progression or death from any cause or the date of the last follow-up. Other secondary endpoints were

adverse events (AEs) of treatments, according to the National Cancer Institute Common Terminology Criteria (NCI-CTCAE ver. 4.0).

Statistical analysis

Categorical data were compared between groups by Fisher's exact test or the chi-square test. Quantitative data with a nonnormal distribution were compared with the nonparametric Mann-Whitney *U* test. Quantitative data are expressed as mean ± SD or median (range). Kaplan-Meier survival analysis was performed using SPSS 26.0 statistical software (IBM Corp., Armonk, NY, United States). Univariate and multivariate analyses using the Cox proportional hazards regression model were run to evaluate the influence of covariates on OS and PFS. $P < 0.05$ was considered significant.

RESULTS

Baseline characteristics

A total of 41 eligible patients were included retrospectively, the study flow diagram is shown in Figure 2. Among them, 12 patients had a high surgical risk of esophagectomy, 10 patients suffered proximal esophageal carcinoma, 13 patients were unwilling to receive esophagectomy, and the other 6 patients were unable to undergo esophagectomy due to a previous surgical history ($n = 1$, massive small intestine resection; $n = 1$, lung cancer resection; $n = 2$, gastric cancer resection; $n = 2$, cardia cancer resection). Twenty-eight patients underwent EdR plus CRT (EdR + CRT group), while 13 received EdR without CRT (EdR group). Among the 13 patients in the EdR group, 2 preferred not to undergo CRT due to poor physical condition, 9 due to older age (> 70 years), and 2 due to complications with fistulas.

The median age of the 41 enrolled patients was 69 years (range: 38-91). The median follow-up period was 36 mo (range: 1-83). Among the 41 cases, there were 22 (54%) primary tumors located less than 25 cm from the incisors, while 19 (46%) were located more than 25 cm from the incisors. There were 17 (42%), 23 (56%), and 1 (2%) patients diagnosed with clinical stage T1b-SM3, T2, and T3 disease, respectively. Seven (17%) patients had lymph node metastases, while 2 (5%) patients had M1 metastases. The clinical characteristics of the enrolled patients are listed in Table 1. There were no significant differences in the baseline clinical characteristics between the two groups.

Outcomes and AEs of EdR and CRT

The EdR was performed successfully in all 41 patients (Table 2). Ten (24%) received R0 resection, while 31 (76%) patients received R1 resection (deemed positive horizontal/vertical margins or unjudged margins). The procedure time, measured from the start of marking the lesions to the end of treatment, was 65 ± 29 min (range: 25-150 min). No intraoperative adverse events were observed except for one (1/41) case of mild subcutaneous emphysema, whose symptoms were relieved after 2 d of conservative treatment. Two patients (2/41) suffered delayed bleeding 7 d after the procedure but recovered with anti-acid therapy. Two patients (2/41) developed tracheoesophageal fistula within 2 mo after EdR, of which one died at 24 mo and the other was lost to follow-up at 25 mo. A total of 19 patients (19/41) developed degrees of esophageal stenosis: 2 patients were lost to follow-up at 25 mo and 30 mo, 16 had alleviated dysphagia after receiving retrievable stenting or bougie dilation, and 1 died due to an tracheoesophageal fistula at 24 mo.

After EdR, 28 patients received additive CRT. Complications such as myelosuppression were observed in 7 patients, including 5 cases of Grade I, 1 of Grade II, and 1 of Grade III. Three patients developed Grade I radiation pneumonia and 3 patients suffered Grade II mucous toxicity. No severe adverse events were observed during the CRT procedure.

Survival outcomes

The median follow-up period was 36 (1-83) mo, and 2 patients were lost to follow-up at 25 mo and 30 mo. The estimated 1-, 2-, and 3-year cumulative OS rates of the EdR + CRT group were 92.6%, 85.2%, and 79.5%, respectively, which were higher than those of the EdR group (1-year OS, 83.3%; 2-year OS, 58.3%; 3-year OS, 50%; $P = 0.05$) (Figure 3A). As shown in Figure 3B, the estimated 2-year PFS rate of the EdR + CRT group was 85.7%, higher than that of the EdR group (61.5%, $P = 0.043$). Univariate Cox regression analyses showed that clinical stage, additive CRT, lymphoid metastasis and distant metastasis were potential influencing factors of cumulative OS. These variables were included in a multivariate Cox regression analysis, which identified clinical stage as the only factor affecting OS. Similarly, early clinical stage and no lymphoid or distant metastasis were independent protective prognostic indicators for PFS in univariate Cox analyses, but multivariate analysis found only early clinical stage was a protective factor associated with PFS (Table 3).

Table 1 Clinical characteristics of enrolled patients

Characteristics	Total (41)	EdR + CRT (28)	EdR (13)	*P* value
Age, median (range), yr	69 (38-91)	67 (38-87)	74 (61-91)	0.519
Sex, *n* (%)				0.524
Male	25 (61)	18 (43.9)	7 (17.1)	
Female	16 (39)	10 (24.4)	6 (14.6)	
Location, *n* (%)				0.184
≤ 25 cm	22 (54)	17 (41.5)	5 (12.5)	
> 25 cm	19 (46)	11 (26.8)	8 (19.2)	
TNM stage, *n* (%)				0.400
IB	16 (39)	10 (24)	6 (15)	
IIA	18 (43.9)	14 (34.1)	4 (9.8)	
IIB	1 (2.4)	1 (2.4)	0 (0)	
IIIA	1 (2.4)	0 (0)	1 (2.4)	
IIIB	3 (7.3)	2 (4.9)	1 (2.4)	
IVA	0 (0)	0 (0)	0 (0)	
IVB	2 (4.9)	1 (2.4)	1 (2.4)	
T stage, *n* (%)				0.348
T1b	17 (41.5)	11 (26.8)	6 (14.7)	
T2	23 (56.1)	17 (41.5)	6 (14.6)	
T3	1 (2.4)	0 (0)	1 (2.4)	
T4	0 (0)	0 (0)	0 (0)	
N stage, *n* (%)				0.52
N0	34 (82.9)	24 (58.5)	10 (24.4)	
N1	2 (4.9)	1 (2.45)	1 (2.45)	
N2	5 (12.2)	3 (7.3)	2 (4.9)	
M stage, *n* (%)				0.539
M0	39 (95.1)	27 (65.9)	12 (29.2)	
M1	2 (4.9)	1 (2.45)	1 (2.45)	

CRT: Chemoradiotherapy; EdR: Endoscopic debulking resection.

DISCUSSION

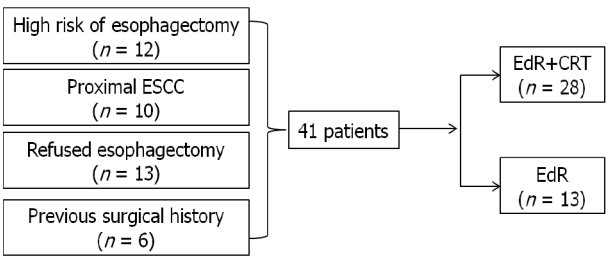
Patients who undergo noncurative R1 resection or have deeper than SM2 invasion always need additional esophagectomy. However, this concept faced challenges due to the possibility of no residual tumor present in the supplementary surgical specimen. Furthermore, esophagectomy is not a sensible choice for patients who are at high risk from surgery. Patients with advanced cervical or upper ESCC and have a history of lower esophageal or cardiac surgery usually cannot have an esophagectomy. Here, we tested a new treatment, EdR, on selected inoperable advanced ESCCs and extended it to patients with SM3 invasion who were unwilling to receive additional esophagectomy. The results revealed an encouraging short-term OS and low AE rate. Patients who received additive CRT after EdR had a better survival prognosis than those who received only EdR.

Although endoscopic resection (ER) is usually indicated for submucosal lesions, especially SM1-2 invasion, it is not recommended to perform ER for SM3 invasion because of the higher metastasis risk. The literature is unclear regarding additional esophagectomy following noncurative ER. ER can obtain accurate T staging while remove the primary lesion, and adjuvant CRT therapy can further reduce the potential of metastasis or recurrence. Therefore, ER plus CRT is considered an alternative strategy of esophagectomy for clinical stage I ESCC[19-21]. Follow-up studies also showed that ER followed by CRT displayed comparable outcomes of esophagectomy for T1b (SM1-2) cancer[22]. However, it is

Table 2 Outcomes and adverse events of endoscopic debulking resection and chemoradiotherapy

Procedure details and outcomes of EdR	
Technical success, *n* (%)	
Success	41 (100)
Failure	0 (0)
R0 resection, *n* (%)	
Yes	10 (24.4)
No	31 (75.6)
Procedure time [mean ± SD (range), min]	65 ± 29 (25-150)
Intraoperative complications, *n* (%)	
Mild subcutaneous emphysema	1 (2.4)
None	40 (97.6)
Post-operative complications, *n* (%)	
Delayed bleeding	2 (4.9)
Esophageal stenosis	19 (46.3)
Tracheoesophageal fistula	2 (4.9)
None	18 (43.9)
Complications of CRT after EdR, *n* (%)	
Myelosuppression	7 (25)
Radiation pneumonia	3 (10.7)
Mucous toxicity	3 (10.7)
None	15 (53.6)
Follow-up period [median (range), mo]	36 (1-83)

CRT: Chemoradiotherapy; EdR: Endoscopic debulking resection.



DOI: 10.4251/wjgo.v14.i9.1758

Figure 2 The patient flow diagram. CRT: Chemoradiotherapy; EdR: Endoscopic debulking resection; ESCC: Esophageal squamous cell carcinoma.

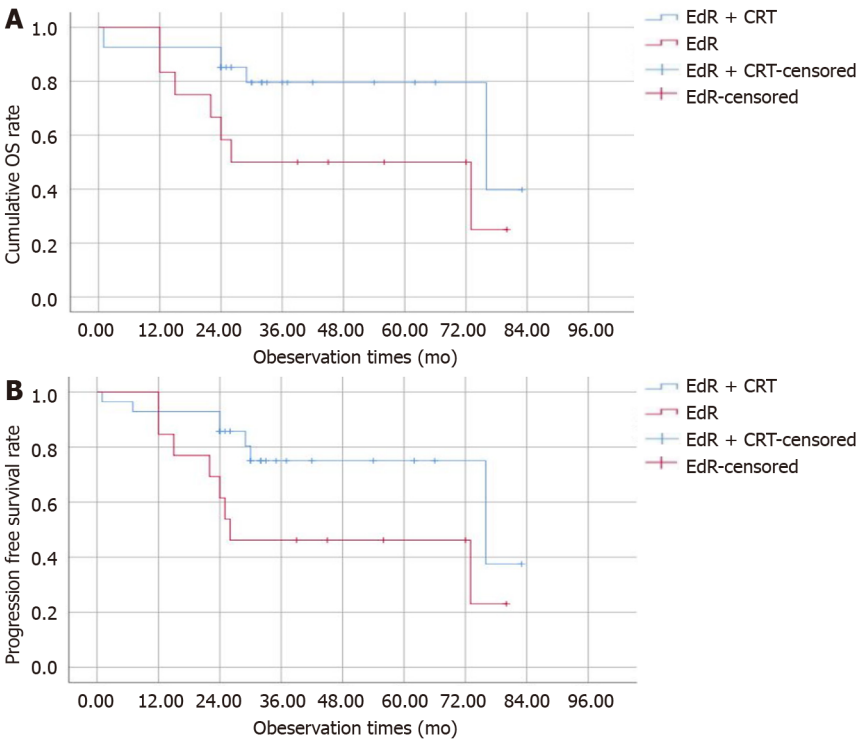
unclear whether ER followed by CRT is applicable to SM3 invasion. Here, we tentatively performed EdR plus CRT in patients with SM3 invasion. Among the 41 patients, 17 patients had T1b-SM3 cancer, and 5 out of 17 patients underwent R1 resection. Five patients with R1 resection received additional CRT, except 1 due to a history of severe emphysema. For these 17 patients, follow-up lasted 24 to 83 mo, and a favorable prognosis was found, except for 1 failed follow-up at 30 mo post-EdR. It is worth noting that 4 patients in our hospital with SM3 invasion who underwent R1 resection plus supplemental esophagectomy showed negative residual tumors and negative nodal metastases in the surgical specimens. Whether additional surgery or additive CRT be adopted for patients with lesions deeper than SM3 requires a large prospective study.

For advanced, inoperable ESCC, dCRT is the only choice. Previous studies reported the 5-year OS of ESCC patients who received dCRT was only 20%-27%, with a median survival of 14 mo[23,24]. Furthermore, the incidence of local failure of dCRT was up to nearly 50% with poor life quality[25]. Another randomized phase III trial enrolled 267 unresectable ESCC patients who received dCRT; these

Table 3 Univariate and multivariate Cox regression analyses on overall survival and progression-free survival

Characteristics	OS, *n* = 41				PFS, *n* = 41			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Clinical stage (> IIB *vs* ≤ IIB)	18.908 (4.629-77.235)	0.000	18.908 (4.629-77.235)	0.000	11.311 (3.397-37.622)	0.000	11.311 (3.397-37.622)	0.000
Intervention (EdR *vs* EdR + CRT)	4.861 (1.213-19.487)	0.026		0.198		0.063		0.411
T stage (≥ 2 *vs* < 2)	68.037 (0.304-15204.04)	0.126			66.824 (0.505-8840.4)	0.092		
N stage (≥ 1 *vs* 0)	13.329 (3.309-53.7)	0.000		0.737	4.937 (1.621-15.031)	0.005		0.318
M stage (≥ 1 *vs* 0)	9.13 (1.82-45.775)	0.007		0.876	7.035 (1.481-33.418)	0.014		0.906
Margin (positive/vague *vs* negative)	7.281 (0.84-63.144)	0.072				0.258		

CRT: Chemoradiotherapy; EdR: Endoscopic debulking resection; OS: Overall survival; PFS: Progression-free survival.



DOI: 10.4251/wjgo.v14.i9.1758

Figure 3 The overall survival and progression-free survival of two groups. A: The cumulative overall survival of the endoscopic debulking resection (EdR) + chemoradiotherapy (CRT) and EdR groups; B: The cumulative progression-free survival of the EdR + CRT and EdR groups. CRT: Chemoradiotherapy; EdR: Endoscopic debulking resection; OS: Overall survival.

data showed a median PFS was merely 9.7 mo[26]. There is an urgent need for a new strategy that is more effective than dCRT for unresectable ESCCs. Salvage ER, a complementary treatment after dCRT, has exhibited decent results in recent studies. Yano *et al*[13] showed that the 5-year survival rate of salvage EMR of stage I-III esophageal cancer patients after dCRT was 49.1%. Another retrospective study, reported by Nakajo *et al*[25], concluded that the 1-year local relapse-free survival (LRFS) rates of salvage ESD were 86%-100%, confirming the role of salvage ER in patients with dCRT failure[25,27,28]. Nevertheless, these patients all had localized and superficial lesions with no lymph node or distant metastasis. In addition, radiation-induced fibrosis and vessel vulnerability lead to a high risk of acute

AEs, such as bleeding or perforation.

In this study, we enrolled 41 patients, including 17 with T1b tumors, 23 with T2, and 1 with T3. Seven (17%) patients had lymph node metastases while 2 (5%) patients had M1 metastases. The primary tumor was partially or completely removed from all enrolled 41 patients, with a mean procedure time of 65 ± 29 min. Only two patients suffered delayed bleeding, and one suffered mild subcutaneous emphysema, with no severe intra- or postoperative AEs observed. All of the patients were cured by conservative therapy. It is recognized that lesions with a circumferential extension of > 3/4 of the esophageal lumen, depth of invasion above M2, and mucosal defects longer than 3 cm are independent risk factors for esophageal stricture[29,30]. Since the lesions in our study were mostly deeper than SM2 and had muscularis propria injuries, esophageal stents were implanted intraoperatively in 15 patients (15/41) to prevent postoperative stricture and delayed bleeding or perforation. Reassuringly, 7 of the 15 patients have no esophageal stenosis during follow-up, while the remaining 8 intake semi-fluid smoothly. It is well known that esophageal stenosis usually occurred late in the radiotherapy. Once the radiation esophagitis and stenosis occurred, the bleeding or perforation risks of endoscopic therapy were extremely high. Although the complication of stenosis in our study was 46% (19/41), it was manageable, as all of these patients intake semi-fluid smoothly. In spite of this, patients who have a high risk of stenosis and choose EdR should fully understand and accept this likely complication. Clinicians must also be cautious when choosing EdR for those with high stricture risk.

Patients who receive dCRT always suffer complications, such as hemorrhage, perforation, radiation esophagitis, pericarditis, pneumonia, and tracheal stenosis. The fatal complication of tracheoesophageal fistula occasionally occurred, especially under the conditions of a high RT dose[31,32]. One study reported that 6 of 49 patients (12%) with T1 or T2 esophageal cancer developed tracheoesophageal fistula, and 3 of them died. A list of studies reporting the rates of esophageal fistula in locally advanced ESCC patients who received dCRT varied from 3.7% to 24%. In our study, there were no fistulas in the EdR + CRT group, while 2 patients suffered fistulas in the single EdR group. We think that incomplete tumor resection and stent mechanical compression were the main reasons for the fistulas. Patients suffering from fistulas always have a poor prognosis due to the increased risk of severe infection and malnutrition. We usually plant a fully covered esophageal stent to plug the fistula, but as a residual necrotic tumor, the fistula cannot be completely cured. To our delight, patients who received EdR plus CRT had no fistulas up to our last follow-up. Our clinical experience tells us that the time point of additive CRT after EdR is extremely important. We implemented CRT at 2 mo after EdR, leaving sufficient time for esophageal mucosa repair. Furthermore, we reduced the ordinary radiation dose and reduced the scope of radiation treatment, relieving the toxicity of radiation. There were no severe adverse events in the EdR + CRT group. Complications, including mild myelosuppression, radiation pneumonia, and mucous toxicity, were observed in 25%, 11%, and 11% of patients, respectively.

One study reported that the 5-year relative survival rate of ESCC patients treated with surgery is only 19%-24%[33]. Zhang *et al*[34] showed that the 5-year OS of advanced ESCC patients who received adjuvant radiotherapy after surgery was 62.2%, which was much higher than the 5-year OS of patients who underwent surgery alone. The survival benefit of postoperative chemotherapy has also been confirmed[35]. A randomized phase II trial reported by Liu *et al*[36] reported that the 3-year OS rate of advanced ESCC patients in the CRT group was 38.1% while that in the induction chemotherapy group was 41.8%. Other cohort studies that included stage II-III ESCC patients reported a median DFS of 13 mo in the CRT group[37] and a median OS in the CRT group of 14.1 mo[38]. In our study, the estimated 1-, 2-, and 3-year cumulative OS rates after EdR + CRT were 92.6%, 85.2%, and 79.5%, respectively, and the estimated 2-year PFS rate after EdR + CRT was 85.7%, both satisfactory outcomes. The median survival time of the EdR + CRT group from Kaplan–Meier survival analysis was 76 mo. It is encouraging that 13 patients who received EdR alone also had fair outcomes, with a calculated median survival time of 26 mo. Although the number of EdR was small, the cumulative OS and PFS still were relatively good. According to univariate and multivariate Cox regression analyses, early clinical stage (stage ≤ IIB) and additive CRT after EdR were potential protective factors.

The initial aim of our study was to remove the primary lesion, reduce tumor burden, and enhance the effect of CRT. This strategy was only a daring attempt, and the conclusions in our study need to be treated with caution. As mentioned above, our study has several limitations. First, this was a small, retrospective, short-follow-up study. It is clinically preferable to evaluate the 5-year OS, but we deemed it important to obtain results as soon as possible, so we ultimately designated the primary endpoint as the 3-year OS. Due to the special and strict eligibility criteria of patients, the number of patients in our study was small. Second, the study was conducted at a single institution, which may limit its external generalizability. Large, multicenter, long-term follow-up studies are needed to validate the endoscopic advantages.

Given the limitations above, the results should be interpreted with caution. However, to the best of our knowledge, this is the first study to expand the ER indicator of lesions deeper than SM3, and it is the first study to provide evidence regarding the efficacy and safety of EdR followed by CRT for advanced inoperable ESCC, which might become an attractive therapeutic strategy for selected ESCC patients.

CONCLUSION

EdR is an alternative strategy for selected advanced inoperable ESCC patients. Additive CRT was not associated with more adverse events but showed better prognosis than EdR alone.

ARTICLE HIGHLIGHTS

Research background

Advanced esophageal squamous cell carcinoma (ESCC) patients who decline surgery or have high surgical risks have no treatment option but definitive chemoradiotherapy (dCRT). However, the complications from high doses of radiation and local recurrence result in a poor prognosis.

Research motivation

To explore a new therapy to treat patients diagnosed with advanced ESCC who were unable to undergo surgery and to extend this therapy to patients with deeper than T1b (≥ SM3) invasion who were unwilling or unable to receive additional esophagectomy.

Research objectives

To evaluate efficacy and safety of the strategy of endoscopic debulking resection (EdR) with additive chemoradiotherapy (CRT) for selected advanced ESCC patients.

Research methods

Patients who received (EdR) followed by CRT were deemed the EdR + CRT group and those without CRT were deemed the EdR group. Outcomes of overall survival (OS), progression-free survival (PFS), and adverse events were evaluated.

Research results

This study showed promising short-term overall and cancer-specific survival after EdR plus additive CRT, with estimated 1-, 2-, and 3-year cumulative OS rates of 92.6%, 85.2%, and 79.5%, respectively, and a 2-year cumulative PFS rate of 85.7%. Early clinical stage (stage ≤ IIB) and additive CRT were potential protective factors for cumulative OS.

Research conclusions

EdR plus CRT is relatively safe and feasible for selected advanced inoperable ESCC patients.

Research perspectives

The authors will continue to follow up the enrolled patients and increase the sample size to validate the endoscopic advantages and disadvantages.

ACKNOWLEDGEMENTS

We wish to thank Xie WH for help with statistics and Sachin Mulmi Shrestha for language editing.

FOOTNOTES

Author contributions: Ren LH contributed to data collection, data analysis, and manuscript writing; Zhu Y contributed to data collection and statistical methods; Chen R contributed to chemoradiotherapy; Sachin MS contributed to language editing; Lu Q contributed to technical guidance and the endoscopic debulking resection (EdR) procedure; Xie WH contributed to the data analysis; Lu T contributed to imaging interpretation; Wei XY contributed to pathology interpretation; Shi RH contributed to the conception and design of the study and performed the EdR procedure; All authors contributed to the revision and gave their final approval of the manuscript.

Supported by Fundamental Research Funds for the Central Universities, Postgraduate Research and Practice Innovation Program of Jiangsu Province, No. KYCX19_0118; Jiangsu Science and Technology Project, Innovative Team Project of Esophagus, No. 2017ZXK7QW08; and National Natural Science Foundation of China, No. 81570503.

Institutional review board statement: This study was approved by the institutional review board of Zhongda Hospital (No. 2019ZDSYLL023-Y01) and was conducted in accordance with the Declaration of Helsinki.

Conflict-of-interest statement: All authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets used and/or analyzed during the study are available from the corresponding author on reasonable request.

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S-Editor: Ma YJ
L-Editor: Filipodia
P-Editor: Ma YJ

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1771-1784

DOI: 10.4251/wjgo.v14.i9.1771

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery

Ren-Xiong Chen, Zhou-Qiao Wu, Zi-Yu Li, Hong-Zhi Wang, Jia-Fu Ji

Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Ahmed SK, Iraq; Saito R, Japan; Totoki T, Japan

Received: April 6, 2022
Peer-review started: April 6, 2022
First decision: June 12, 2022
Revised: June 19, 2022
Accepted: July 27, 2022
Article in press: July 27, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

There were few studies on the prognosis of tumor patients with sepsis after gastrointestinal surgery and there was no relevant nomogram for predicting the prognosis of these patients.

AIM

To establish a nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery in the intensive care unit (ICU).

METHODS

A total of 303 septic patients after gastrointestinal tumor surgery admitted to the ICU at Peking University Cancer Hospital from January 1, 2013 to December 31, 2020 were analysed retrospectively. The model for predicting the prognosis of septic patients was established by the R software package.

RESULTS

The most common infection site of sepsis after gastrointestinal surgery in the ICU was abdominal infection. The 90-d all-cause mortality rate was 10.2% in our study group. In multiple analyses, we found that there were statistically significant differences in tumor type, septic shock, the number of lymphocytes after ICU admission, serum creatinine and total operation times among tumor patients with sepsis after gastrointestinal surgery ($P < 0.05$). These five variables could be used to establish a nomogram for predicting the prognosis of these septic patients. The nomogram was verified, and the initial C-index was 0.861. After 1000 internal

validations of the model, the C-index was 0.876, and the discrimination was good. The correction curve indicated that the actual value was in good agreement with the predicted value.

CONCLUSION

The nomogram based on these five factors (tumor type, septic shock, number of lymphocytes, serum creatinine, and total operation times) could accurately predict the prognosis of tumor patients with sepsis after gastrointestinal surgery.

Key Words: Tumor; Surgery; Sepsis; Gastrointestinal; Nomogram

Core Tip: There were few studies on the prognosis of tumor patients with sepsis after gastrointestinal surgery and there was no relevant nomogram for predicting the prognosis of these patients. The aim of the study was to establish a nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery in the intensive care unit (ICU).The most common infection site of sepsis was abdominal infection and the 90-d all-cause mortality rate was 10.2% in our study group. In multiple analyses, we found that there were statistically significant differences in tumor type, septic shock, the number of lymphocytes after ICU admission, serum creatinine and total operation times among tumor patients with sepsis after gastrointestinal surgery ($P < 0.05$). These five variables could be used to establish a nomogram for predicting the prognosis of these septic patients. The nomogram was verified, and the initial C-index was 0.861. After 1000 internal validations of the model, the C-index was 0.876, and the discrimination was good. The correction curve indicated that the actual value was in good agreement with the predicted value.

Citation: Chen RX, Wu ZQ, Li ZY, Wang HZ, Ji JF. Nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery. *World J Gastrointest Oncol* 2022; 14(9): 1771-1784
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1771.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1771

INTRODUCTION

Since the definition of sepsis in version 1.0 (infection + systemic inflammatory response syndrome) was too sensitive and the specificity was poor and the definition of sepsis in version 2.0 was too cumbersome, the new definition of sepsis was life-threatening organ dysfunction caused by the imbalance of host response to infection, which was manifested in an increase in sequential organ failure score (SOFA) by more than one point[1]. Septic shock was identified as sepsis with severe circulatory, cellular and metabolic disorders, while its mortality was significantly higher than that of sepsis. Septic shock was mainly characterized by continuous hypotension with tissue hypoperfusion (lactic acid > 2 mmol/L).

The incidence rate of sepsis was notably high and mortality was especially high. It is estimated that tens of millions of septic patients die worldwide every year[2,3]. Sepsis not only increased the hospitalization expenses of patients but also prolonged the hospitalization time of patients. According to statistics, the total hospitalization cost of sepsis has jumped first in the United States, with an annual cost of approximately 38.2 billion United States dollars[4]. Therefore, we should pay more attention to the prevention and treatment of sepsis.

The predisposing factors of sepsis include community infection and nosocomial infection, and the mortality of septic patients induced by nosocomial infection is often higher[5,6]. Early identification of infection, infection source control, appropriate application of antibiotics and aggressive volume resuscitation of critically ill patients were the cornerstones of septic patient management[7-10]. These factors had a major influence on the prognosis of septic patients. It is well known that many factors could affect the prognosis of septic patients. However, there were few factors widely used to predict the prognosis of septic patients and there was no relevant nomogram for predicting the prognosis of these patients. In this study, we first retrospectively analysed 303 septic patients after gastrointestinal tumor surgery, collected some factors, analysed their relationship with prognosis, and then established a model for predicting the prognosis of these septic patients.

MATERIALS AND METHODS

Study population

The study which was registered at the Chinese Clinical Trial Registry (registration ID: ChiCTR2100051826) was conducted according to the Declaration of Helsinki (as revised in 2013). Ethical approval for the study was obtained from the Medical Ethical Committee of Peking University Cancer Hospital (ethics approval number 2020KT33) and informed consent from all the septic patients or their relatives. Inclusion criteria: From January 1, 2013 to December 31, 2020, a total of 4731 patients were admitted to the intensive care unit (ICU) at Peking University Cancer Hospital, of which 2448 patients were transferred to the ICU for various reasons (complicated with chronic medical diseases, sepsis, bleeding, acute myocardial infarction, acute heart failure, acute pulmonary embolism, acute cerebral infarction, pneumothorax, *etc*.) after gastrointestinal tumor surgery in the gastrointestinal tumor center. According to the new definition of sepsis, 303 septic patients were included in our study. Exclusion criteria: Those patients without surgery; postoperative patients with non-gastrointestinal tumors; patients without sepsis. The flow diagram is shown in Supplementary Figure 1.

The treatment of septic patients before 2016 mainly referred to the 2012 version of sepsis/septic shock guidelines[11], while the treatment of septic patients after 2016 mainly referred to the 2016 version of sepsis/septic shock guidelines[7]. For patients whose final culture results were negative, at least two experts would finally determine the most likely infection source of the patients after discussion.

For abdominal sepsis, we controlled the source of infection actively through minimally invasive drainage or surgical debridement by a multidisciplinary team.

Data collection and follow up

Clinical data and some laboratory tests of septic patients after gastrointestinal surgery were collected. Baseline information included age, body mass index (BMI), basic diseases, chronic diseases, Charlson score and tumor type. Clinical diagnosis and treatment data included whether the first operation was an emergency operation, laparoscopic or open in the first operation, the length of the first operation, drug sensitivity test results, antibiotics used, septic shock, number of blood leukocytes, number of lymphocytes, percentage of lymphocytes, percentage of neutrophils, activated partial thromboplastin time (APTT), albumin, serum creatinine, cardiac troponin I (cTnI), procalcitonin (PCT), blood lactic acid, oxygenation index (PaO2/FiO2), SOFA within 24 h after ICU admission, whether had gastrointestinal fistula or perforation and total operation times. Except for specially specified data, other data were the first data collected in the ICU.

The survival time of septic patients was calculated from entering the ICU and followed up to 90 d. If the patient's death occurred before 90 d, he was followed up to the day of death. Follow-up was carried out through an inpatient electronic case system or telephone and patient's survival status was recorded.

Statistical analyses

The data were processed by the R3.6.3 software package. Continuous variables were statistically described as the means ± SD and discontinuous variables were described by medians (Q1, Q3). The enumeration data were expressed as numerical values (percentages).

Univariate analysis was performed by the log rank test. Those factors with $P < 0.05$ in the univariate analysis were included in the multiple analysis and Cox regression analysis was used. Statistically significant factors in multiple survival analysis were used to establish a nomogram for predicting the prognosis of septic patients with the R3.6.3 software package. The performance of the model was evaluated by the C-index and calibration curve. The bootstrap method was used for the internal verification of the model. Two sided tests were used for all statistical analyses, and the statistical test $P < 0.05$ represented that the difference was statistically significant.

RESULTS

Patient characteristics

According to the new definition, 303 tumor patients were diagnosed with sepsis after gastrointestinal surgery, including 119 patients who needed vasopressors who were diagnosed with septic shock. The median age of these septic patients was 66 years. The most common complication was hypertension, followed by diabetes. According to the classification of tumor types, there were 138 patients with gastric cancer, 148 patients with colorectal cancer and 17 patients with other abdominal and pelvic tumors (5 cases of gastrointestinal stromal tumors, 4 cases of lymphoma, 2 cases of melanoma, 2 cases of implanted intestinal wall of ovarian cancer, 1 case of cervical cancer with postoperative intestinal perforation, 1 case of ileal metastasis of renal cancer, 1 case of abdominal fibromatosis and 1 case of colonic adenoma).

Among these septic patients, 35 underwent emergency surgery and 268 underwent limited surgery for the first operation. The median time of the first operation was 180 minutes. In the course of

treatment, 24 patients with sepsis were complicated with abdominal bleeding or gastrointestinal hemorrhage, 28 patients with venous thrombosis (including 9 cases of acute pulmonary embolism), 2 patients with acute cerebral infarction, 2 patients with acute myocardial infarction and 1 patient with cerebral hemorrhage. A total of 12 patients needed continuous renal replacement therapy due to renal failure, 149 patients received ventilator treatment and 1 patient received extracorporeal membrane oxygenation. The baseline information is shown in Table 1.

Pathogenic microorganisms in patients with sepsis

The most common infection site of sepsis after gastrointestinal surgery was abdominal infection, followed by pneumonia. Pathogenic microorganisms could be isolated in 255 cases (84.2%) of these septic patients, however 48 cases (15.8%) could not. Gram-negative bacilli (197 cases) were the most common pathogenic microorganisms, followed by gram-positive cocci (100 cases), fungi (28 cases) and gram-positive bacilli (2 cases). See Supplementary Table 1 for the microorganisms in each infection site.

The common isolated pathogens were as follows (≥ 5 cases): Ninety-seven cases of *Escherichia coli* (*E. coli*), 50 cases of Pseudomonas aeruginosa, 40 cases of Klebsiella pneumoniae, 30 cases of Enterococcus faecalis, 22 cases of Candida albicans, 20 cases of Enterococcus faecium, 12 cases of Staphylococcus aureus, 11 cases of Acinetobacter baumannii, 11 cases of Stenotrophomonas maltophilia, 10 cases of Streptococcus pharyngitis, 9 cases of Enterococcus avium and 8 cases of Staphylococcus epidermidis, 7 cases of hemolytic Staphylococcus, 7 cases of Klebsiella aerogenes and 6 cases of Enterobacter cloacae.

The distribution of common drug-resistant bacteria isolated was as follows: Seventy cases of *E. coli* producing extended spectrum β-lactamase (ESBL) and 7 cases of Klebsiella pneumoniae producing ESBL; 11 cases of carbapenem resistant Pseudomonas aeruginosa, 3 cases of carbapenem resistant Acinetobacter baumannii, and 2 cases of carbapenem resistant Enterobacteriaceae; 7 cases of methicillin resistant Staphylococcus epidermidis, 6 cases of methicillin resistant Staphylococcus aureus and 5 cases of methicillin resistant hemolytic Staphylococcus; 2 cases of vancomycin resistant enterococci.

Survival analysis of patients with sepsis

Three hundred and three septic patients were followed up for 90 d. A total of 31 patients died (27 patients died of multiple organ failure caused by septic shock, 2 patients died of hemorrhagic shock, 1 patient died of intracerebral hemorrhage and 1 patient died of respiratory failure). The 90 d all-cause mortality was 10.2%. Since there were slight differences in the sepsis/septic shock guidelines for the treatment of septic patients before and after 2016, we first performed a comparative analysis of the survival rate of septic patients before and after 2016. There was no significant difference in the 90-d survival rate among septic patients before and after January 1, 2016 ($P = 0.415$).

The univariate survival analysis showed that there were statistically significant differences in BMI, tumor type, empirical anti-infection evaluation, septic shock, number of lymphocytes after entering the ICU, the activated prothrombin time after entering the ICU, blood creatinine, PCT, blood lactic acid, oxygenation index, SOFA score within 24 h after entering the ICU and total operation times ($P < 0.05$). See Table 2 for the results of univariate analysis of these septic patients.

The twelve factors with $P < 0.05$ in univariate analysis were included in multiple analyses. The results showed that there were significant differences in tumor type, whether there was septic shock, number of lymphocytes after entering the ICU, serum creatinine and total operation times on the prognosis of these septic patients ($P < 0.05$). The areas under the receiver operating characteristic (ROC) curve of these five factors predicting the prognosis of postoperative sepsis of gastrointestinal tumors were 0.614, 0.766, 0.574, 0.629, and 0.513, respectively. The results of multiple analyses of tumor patients with sepsis after gastrointestinal surgery are shown in Table 3.

The 90-d survival rate of patients with postoperative sepsis of gastric cancer was worse than that of patients with postoperative sepsis of colorectal cancer ($P = 0.003$). However, there was no statistically significant difference in the survival rate between patients with postoperative sepsis of gastric cancer and patients with postoperative sepsis of other abdominal and pelvic tumors ($P = 0.739$). The 90-d survival rate of patients with postoperative sepsis of gastrointestinal tumors who underwent three operations was lower than that of patients who underwent only one operation ($P = 0.005$). However, there was no significant difference in the survival rate between patients with postoperative sepsis of gastrointestinal tumors who underwent two operations and patients who underwent only one operation ($P = 0.105$).

A nomogram for predicting the prognosis of patients with sepsis

The five factors affecting the 90-d survival rate of patients with postoperative sepsis of gastrointestinal tumors screened by Cox regression analysis were included in the prediction of the prognosis model. A nomogram for predicting the prognosis of patients with postoperative sepsis of gastrointestinal tumors was established and output by R statistical software (Figure 1). In clinical application, we found the corresponding value of each predictor in the nomogram and added the scores of each predictor to the total score. Finally, the total score was read on the axis of 90-d overall survival rate, which was the 90-d survival probability of the patient.

Table 1 Baseline characteristics of patients with sepsis

Baseline characteristics	Number (%)
Age, median (Q1, Q3)	66 (59,73)
Sex	
Male	235 (77.6)
Female	68 (22.4)
BMI, Mean (SD), kg/m^2	23.7 (4.0)
Tumor type	
Gastric cancer	138 (45.5)
Colorectal cancer	148 (48.8)
Other abdominal tumors	17 (5.6)
Coexisting disease[1]	
Hypertension	106 (35.0)
Diabetes	55 (18.2)
Coronary heart disease	32 (10.6)
Chronic obstructive pulmonary disease	15 (5.0)
Arrhythmia	22 (7.3)
Chronic renal insufficiency	4 (1.3)
Location of infection[2]	
Abdominal infection	229 (75.6)
Pneumonia	58 (19.1)
Intrathoracic infection	19 (6.3)
Enterogenous infection	16 (5.3)
Surgical wound infection	7 (2.3)
Skin and soft tissue infection	6 (2.0)
Central line-associated bloodstream infection	4 (1.3)
Urinary tract infection	4 (1.3)
Biliary infection	2 (0.7)
First surgery	
Laparoscopic	76 (25.1)
Open	227 (74.9)

[1]47 patients had more than one chronic disease.
[2]38 patients were infected with more than one site.
BMI: Body mass index.

Discrimination of the nomogram

We used the C-index to evaluate the differentiation of a nomogram for predicting the prognosis in septic patients after gastrointestinal surgery. The initial C-index of the nomogram was 0.861 and the 95%CI was 0.809-0.913, indicating that the nomogram for predicting the prognosis in septic patient after gastrointestinal tumor surgery had good discrimination.

Calibration

The calibration of the nomogram for predicting the prognosis in septic patients after gastrointestinal surgery was carried out through the correction curve. The correction curve revealed that the observed value was consistent with the predicted value (Figure 2). The above results showed that the nomogram for predicting the prognosis in septic patients after gastrointestinal surgery could accurately predict the 90-d survival rate.

Table 2 Univariate analysis of patients with sepsis

Parameters	Number (%)	Survival rate at 90-d	*P* value
Age, yr			0.405
≤ 65	149 (49.2)	0.913	
> 65	154 (50.8.0)	0.883	
Sex			0.190
Male	235 (77.6)	0.885	
Female	68 (22.4)	0.941	
BMI, kg/m^2			0.013
≤ 20	59 (19.5)	0.797	
20 < BMI ≤ 30	225 (74.3)	0.924	
> 30	19 (6.3)	0.895	
Charlson score			0.298
≤ 3	229 (75.6)	0.908	
> 3	74 (24.4)	0.865	
Tumor type			0.026
Gastric cancer	138 (45.5)	0.848	
Colorectal cancer	148 (48.8)	0.946	
Other abdominal tumors	17 (5.6)	0.882	
The first operation was emergency			0.725
No	268 (88.4)	0.896	
Yes	35 (11.6)	0.914	
First surgery			0.575
Laparoscopic	76 (25.1)	0.882	
Open	227 (74.9)	0.903	
Length of first operation, min			0.526
≤ 240	220 (72.6)	0.905	
> 240	83 (27.4)	0.880	
Empirical anti infection evaluation			0.001
Sensitive	229 (75.6)	0.917	
Resistance	26 (8.6)	0.692	
No pathogen detected	48(17.1)	0.917	
Septic shock			0.001
No	184 (60.7)	0.978	
Yes	119 (39.3)	0.773	
Leukocyte count, 10^9/L			0.143
≤ 4	49 (16.2)	0.837	
4 < WBC ≤ 12	142 (46.9)	0.930	
> 12	112 (37.0)	0.884	
Number of lymphocytes, 10^9/L			0.004
≤ 0.2	28 (9.2)	0.750	
> 0.2	275 (90.8)	0.913	
Neutrophil to lymphocyte ratio			0.883

≤ 20	218 (71.9)	0.899	
> 20	85 (28.1)	0.894	
APTT, S			0.003
≤ 50	244 (80.5)	0.922	
> 50	59 (19.5)	0.797	
Albumin, g/L			0.279
≤ 30	168 (55.4)	0.881	
> 30	135 (44.6)	0.919	
Serum creatinine, umol/L			0.001
≤ 120	256 (84.5)	0.926	
> 120	47 (15.5)	0.745	
Cardiac troponin I, ng/mL			0.130
≤ 0.05	253 (83.5)	0.909	
> 0.05	50 (16.5)	0.840	
Procalcitonin, ng/mL			0.034
≤ 10	214 (70.6)	0.921	
> 10	89 (29.4)	0.843	
Lactic acid, mmol/L			0.001
≤ 3	227 (74.9)	0.934	
> 3	76 (25.1)	0.789	
Oxygenation index, mmHg			0.001
≤ 200	146 (48.2)	0.836	
> 200	157 (51.8)	0.955	
SOFA score			0.001
≤ 6	175 (57.8)	0.983	
> 6	128 (42.2)	0.781	
Gastrointestinal fistula or perforation			0.364
No	183 (60.4)	0.885	
Yes	120 (39.6)	0.917	
Operation times			0.001
1	174 (57.4)	0.885	
2	123 (40.6)	0.943	
3	6 (2.0)	0.333	

BMI: Body mass index; SOFA: Sequential organ failure score; APTT: Activated partial thromboplastin time.

Internal validation of nomogram

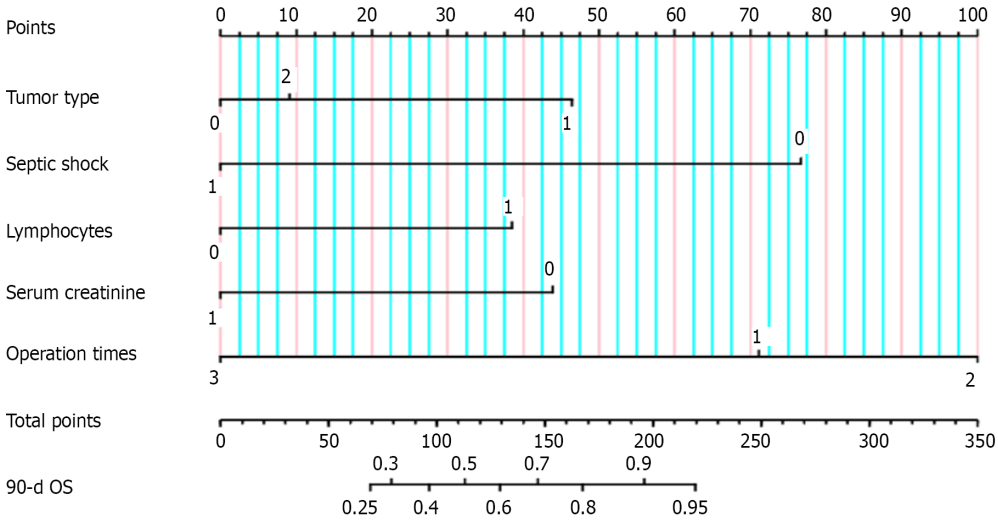
We used the bootstrap method to internally verify the nomogram for predicting the prognosis in septic patients after gastrointestinal surgery. After 1000 internal verifications using the R software package and a repeated bootstrap self sampling method, the C-index was 0.876 (Supplementary Figure 2). This result was consistent with the initial C-index of the nomogram, indicating that the nomogram had good discrimination.

Survival curve based on risk stratification

The total score was calculated according to the nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery, with a median of 233 points. According to the nomogram, 303 postoperative septic patients were divided into three subgroups: High-risk group (total score < 233), moderate-risk group (192 ≤ total score < 233), and low-risk group (total score ≥ 233).

Table 3 Multiple analysis of patients with sepsis

Factors	B	HR	95% interval		*P* value
			Lower	Upper	
Tumor type (Ref: Gastric cancer)					0.007
Colorectal cancer	-1.254	0.286	0.125	0.657	0.003
Other abdominal tumors	-0.249	0.780	0.180	3.370	0.739
Septic shock	2.204	7.569	2.539	22.557	0.001
Number of lymphocytes	-1.209	0.298	0.120	0.742	0.009
Serum creatinine	1.163	3.199	1.463	6.992	0.004
Operation times (Ref: 1)					0.006
2	-0.704	0.485	0.202	1.162	0.105
3	1.609	4998	1.613	15.490	0.005



DOI: 10.4251/wjgo.v14.i9.1771

Figure 1 Nomogram for predicting the 90-d overall survival. Tumor type: 0 represents gastric cancer, 1 represents colorectal cancer, and 2 represents other abdominal tumors; Septic shock: 0 represents no, 1 represents yes; Number of lymphocytes: 0 represents ≤ 0.2 × 10^9/L, 1 represents > 0.2 × 10^9/L; Serum creatinine: 0 represents ≤ 120 μmol/L, 1 represents > 120 μmol/L; Operation times: 1 represents 1 time, 2 represents 2 times, and 3 represents 3 times.

The survival curves of postoperative septic patients are shown in Figure 3. The 90-d overall survival rates of postoperative septic patients in the high-risk group, moderate-risk group and low-risk group were 0.645, 0.883, and 0.989, respectively. There was a statistically significant difference in the 90-d survival rate among the three groups ($P < 0.0001$).

DISCUSSION

The nomogram was a graphical representation of complex mathematical formulas. Medical nomograms mainly use biological and clinical data to describe statistical prediction models. As a graphical statistical prediction model, a nomogram could provide clinicians with a personalized prediction to quantitatively evaluate the prognosis of patients. This study established an effective nomogram for the first time, that could accurately predict the 90-d survival rate of septic patients after gastrointestinal tumor surgery. The calibration curve showed that the nomogram was highly reliable. At the same time, we used the bootstrap method for internal verification, which showed that the prediction ability of the model was very good. In addition, the nomogram could divide individuals into high-risk, moderate-risk and low-risk groups, which indicated that it might be a good tool for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery.

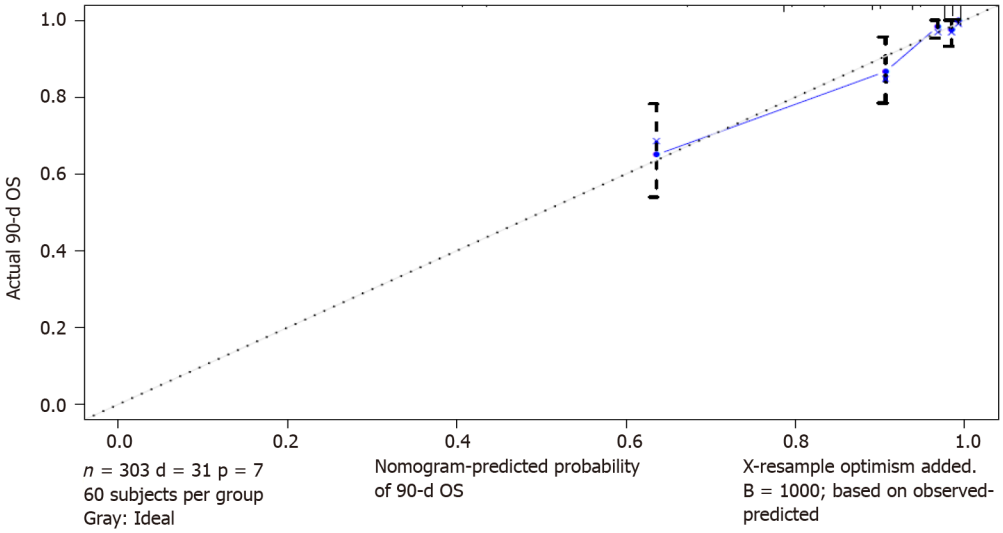


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Figure 2 Calibration plot.

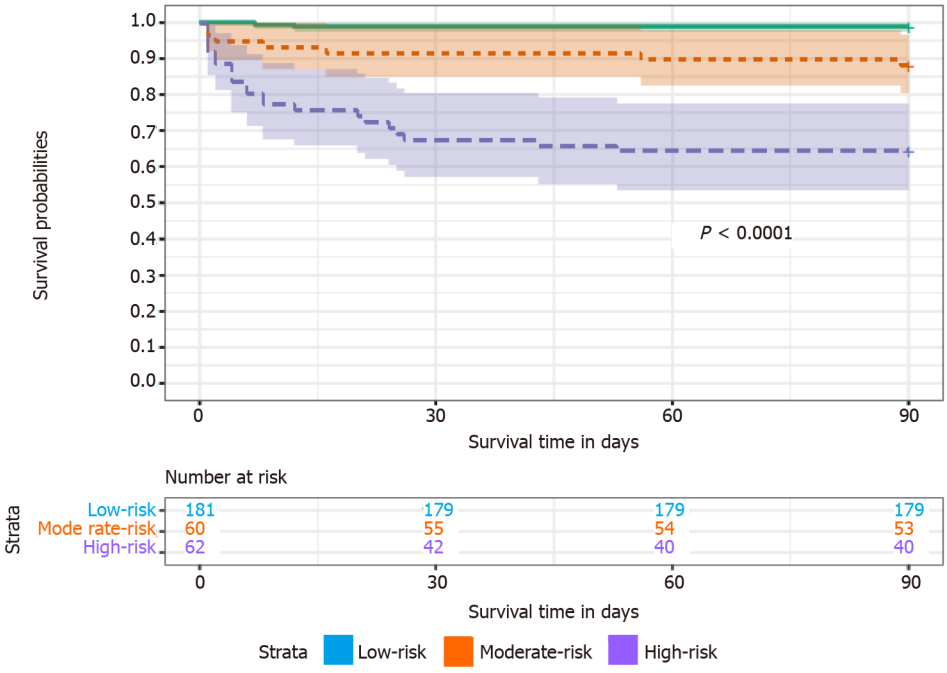

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Figure 3 Kaplan-Meier estimate of the 90-d overall survival and risk assessment using the nomogram.

The main purpose of analysing the prognostic factors of tumor patients with sepsis and establishing a prognostic prediction model was to identify high-risk patients with sepsis as soon as possible and improve the prognosis of these patients. At the same time, it also provided a reference for follow-up clinical research. How to quantify clinical features to achieve individualized prediction of prognosis in septic patients is still a great challenge. The nomogram listed each variable separately by graph and allocated a corresponding number of points for a given variable size. Then, the cumulative score of all variables was matched with the result scale to obtain the corresponding probability. Many studies have confirmed that the nomograms can predict the prognosis of clinical diseases[12-14]. Our study was also based on the nomogram established by the corresponding prognostic factors in septic patients, and we conducted internal validation.

Based on the nomogram for predicting the prognosis of the septic patients, the total score of patients could be calculated and patients could be divided into a high-risk group, moderate-risk group and low-risk group. According to the survival curve based on the nomogram to evaluate the prognosis of sepsis, we found a significant difference in the 90-d survival rate among the three groups, which might warn us

to take early intervention in patients with sepsis. For an individual, we could score the patients according to the nomogram, and the corresponding scores could be preliminarily divided into risk groups, which could provide a basis for clinicians to explain the condition to patients and their families and reduce some doctor-patient contradictions and disputes. Of course, whether the nomogram could be widely popularized remains to be verified.

Sepsis was one of the most common causes of mortality in the ICU. Due to its complex etiology and high heterogeneity, there were great differences in the mortality reported in various studies. At present, only a few studies on sepsis have been aimed at postoperative patients with gastrointestinal tumor[15-17]. The object of the study was septic patients after gastrointestinal tumor surgery, and the mortality was lower than that of septic patients reported in some literature[2,18], which might be closely related to the fact that most of the infection sources of septic patients in this study were abdominal infections that could be actively treated at an early stage by multidisciplinary cooperation in our hospital. In this study, 303 septic patients after gastrointestinal surgery were analysed retrospectively. Multiple survival analysis showed that there were statistically significant differences in tumor type, whether there was septic shock, number of lymphocytes after entering the ICU, serum creatinine and total operation times on the prognosis of these septic patients. Among these factors, except whether there was septic shock, which had a medium ability to predict the prognosis of these septic patients alone, the other factors had a low ability to predict the prognosis of these septic patients. The predictive ability of the nomogram established by combining the five factors was significantly higher than that of individual factors. In the following, we analysed some prognostic factors.

Data published in recent years by the National Cancer Center show that gastric cancer and colorectal cancer incidence rates were the second and third respectively[19]. It is well known that there are differences in the long-term survival rates between patients with gastric cancer and colorectal cancer. On the basis of the estimation of the World Health Organization's Global Cancer Observatory, the 1-year and 5-year survival rates of gastric cancer patients in the United Kingdom from 2010 to 2014 were 46.8% and 20.8% respectively; while in colorectal cancer they were 79.3% and 60% respectively[20]. However, there have been few studies on the prognostic difference between septic patients after gastric cancer surgery and septic patients after colorectal cancer surgery. A prospective, multicenter study in Finland showed that the inpatient mortality of sepsis after gastrointestinal surgery was 30.5%, but the study included fewer tumor patients and did not report the impact of tumor type on prognosis[16]. Our study was the first direct comparative analysis of the prognosis of septic patients after gastric cancer surgery and septic patients after colorectal cancer surgery. Because of the difference in prognosis between the two groups of septic patients, we considered that it was related to pathogenic microorganisms, the difficulty of infection source control and the stronger corrosiveness of digestive fluid in the upper gastrointestinal tract. In this study, the stratified analysis revealed that the pathogenic microorganisms isolated from septic patients after gastric cancer surgery included 83 cases of G-bacilli, 43 cases of G + cocci and 21 cases of fungi; pathogenic microorganisms isolated from patients with postoperative sepsis of colorectal cancer included 106 cases of G-bacilli, 53 cases of G + cocci and 6 cases of fungi. There was a significant difference in the pathogens isolated from these two groups.

Patients with sepsis often experience severe immunosuppression and have a poor prognosis[21]. The immunosuppression of sepsis included innate and acquired immunosuppression. Human leukocyte antigen HLA-DR on the surface of monocytes, dendritic cell count and NK cell count were mainly used to monitor congenital immunosuppression in patients with sepsis, while acquired immunosuppression could be monitored by the number of lymphocytes. Studies have shown that the decrease in lymphocytes in patients with sepsis was an independent prognostic risk factor[22]. Other studies have shown that the prognosis of septic patients is related to the ratio of neutrophils and lymphocytes[23], and its essence is consistent with the number of lymphocytes. In our study, we also found that the decrease in the number of lymphocytes in septic patients after gastrointestinal surgery was associated with poor prognosis, but our study showed that the ratio of neutrophils to lymphocytes had no significant effect on the prognosis of septic patients, which might be related to the cut-off value in our study.

The kidney is one of the vulnerable organs in septic patients, and acute kidney injury (AKI) can even be as high as 50% in septic patients[24]. With the aggravation of sepsis, the probability of acute kidney injury increases accordingly[25]. The pathogenesis of acute kidney injury in sepsis is complex. Current evidence suggests that acute kidney injury might be functional rather than structural for at least the first 48 h. For example, septic AKI lacked histopathological changes but had microvascular abnormalities and tubular stress changes. In this case, renal medullary hypoxia caused by the redistribution of intrarenal perfusion was becoming a key factor in acute kidney injury in sepsis. Risk factors for acute kidney injury in septic patients included advanced age, chronic renal insufficiency, diabetes, heart failure and cancer, *etc*. Septic patients complicated with acute kidney injury significantly worsen the prognosis[26,27]. At present, the diagnosis of sepsis related AKI followed the criteria of acute kidney injury issued by the global working group on improving the prognosis of kidney diseases (KDIGO) in 2012[28]. The treatment of AKI in sepsis mainly included volume resuscitation, antibiotics and renal replacement therapy. In our study, a serum creatinine level of 120 μmol/L was the cut-off value, and the patients were divided into two groups. There was a statistically significant difference in the 90-d survival rate between the two groups.

After gastrointestinal surgery, some patients with abdominal sepsis needed more than one operation to control the source of infection, which often suggested that the patient was in poor condition. The incidence of unplanned reoperation varies among hospitals due to the technical level of doctors[29]. As an important component of medical safety and quality management, unplanned reoperation is often used to assess the technical level of surgery. Therefore, we selected the number of operations to predict the prognosis of patients with sepsis. In our study, we found that there was a significant difference in the 90-d survival rate between septic patients after three operations and septic patients after one operation or two operations, although there was no statistically significant difference in the 90-d survival rate between septic patients after two operations and septic patients after only one operation. We considered that the mortality of patients with indirect operation-related infections (including pulmonary infection, urinary infection and central venous catheter-related infection) was higher than that of patients with direct operation-related infections (including thoracic and abdominal infection, intestinal infection, wound infection, skin and soft tissue infection and biliary tract infection). Among the patients who underwent only one operation, the proportion of indirect operation-related infections was higher. According to the stratified analysis of direct and indirect infections related to gastrointestinal surgery, the 90-d survival rate of patients in the group with two operations was slightly higher than that in the group with one operation, however the difference was not statistically significant. This suggested that we might need more active surgical intervention for the treatment of sepsis caused by infection directly related to gastrointestinal tumor surgery. Of course, it needs to be verified by subsequent randomized controlled trials.

Some limitations of this study should be stated. First, this study was a single-center study, and the sample size was limited, so the results of this study might have some bias. Second, although the nomogram was established to predict the prognosis of these septic patients, it was not externally verified due to the limited sample size. Since there might be differences in patients with sepsis in different research centers, multicenter studies and external validation should be considered in the follow-up. Third, the population in our study was septic patients after gastrointestinal surgery in the ICU. Whether the results could be extended to all septic populations remains to be confirmed. Fourth, new biomarkers were not included in the prognostic factors selected in this study. These factors will be considered for further research to elaborate on the value of these new biomarkers. Fifth, this study spanned a long time, but since there was no significant difference in the 90-d survival rate of septic patients after gastrointestinal surgery before and after January 1, 2016, we believed that this study was highly feasible. Finally, with the progress of technology and treatment, the survival rate of patients with sepsis may be improved. Therefore, the accuracy of predicting prognosis by nomogram may be affected, which needs our attention.

CONCLUSION

The nomogram based on these five factors (tumor type, septic shock, number of lymphocytes, serum creatinine, and total operation times) could accurately predict the prognosis of tumor patients with sepsis after gastrointestinal surgery.

ARTICLE HIGHLIGHTS

Research background

There were few studies on the prognosis of tumor patients with sepsis after gastrointestinal surgery and there was no relevant nomogram for predicting the prognosis of these patients.

Research motivation

To explore the prognostic predictors in patients with sepsis after gastrointestinal tumor surgery.

Research objectives

To establish a nomogram for predicting the prognosis of tumor patients with sepsis after gastrointestinal surgery in the intensive care unit (ICU).

Research methods

A total of 303 septic patients after gastrointestinal tumor surgery admitted to the ICU at Peking University Cancer Hospital from January 1, 2013 to December 31, 2020 were analysed retrospectively. The model for predicting the prognosis of these septic patients was established by the R software package.

Research results

The most common infection site of sepsis after gastrointestinal surgery in the ICU was abdominal infection. The 90-d all-cause mortality rate was 10.2% in our study group. In multiple analyses, we found that there were statistically significant differences in tumor type, septic shock, the number of lymphocytes after ICU admission, serum creatinine and total operation times among tumor patients with sepsis after gastrointestinal surgery ($P < 0.05$). These five variables could be used to establish a nomogram for predicting the prognosis of these septic patients. The nomogram was verified, and the initial C-index was 0.861. After 1000 internal validations of the model, the C-index was 0.876, and the discrimination was good. The correction curve indicated that the actual value was in good agreement with the predicted value.

Research conclusions

The nomogram based on these five factors (tumor type, septic shock, number of lymphocytes, serum creatinine and total operation times) could accurately predict the prognosis of tumor patients with sepsis after gastrointestinal surgery.

Research perspectives

Need external validation in the future to verify the results.

FOOTNOTES

Author contributions: Chen RX and Wu ZQ contributed equally to this work; Chen RX and Wu ZQ designed the research and wrote the first manuscript; Li ZY contributed conceiving the research and analyzing data; Wang HZ and Ji JF conducted the analysis and provided guidance of the research, and they were co-corresponding authors; all authors reviewed and approved the final manuscript.

Institutional review board statement: This study was reviewed and approved by Medical Ethical Committee of Peking University Cancer Hospital.

Informed consent statement: All ICU patients or their next of kin were given information that their data was stored in our registry for quality control and research purposes and the option to have their data deleted.

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: No additional data are available.

Country/Territory of origin: China

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1785-1797

DOI: 10.4251/wjgo.v14.i9.1785

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Retrospective Study

Efficacy and safety of laparoscopic radical resection following neoadjuvant therapy for pancreatic ductal adenocarcinoma: A retrospective study

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Trna J, Czech Republic; Zimmitti G, Italy

Received: May 23, 2022
Peer-review started: May 23, 2022
First decision: July 6, 2022
Revised: July 16, 2022
Accepted: August 15, 2022
Article in press: August 15, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Multiple studies have demonstrated that neoadjuvant chemotherapy (NACT) can prolong the overall survival of pancreatic ductal adenocarcinoma (PDAC) patients. However, most studies have focused on open surgery following NACT.

AIM

To investigate the efficacy and safety of laparoscopic radical resection following NACT for PDAC.

METHODS

We retrospectively analyzed the clinical data of 15 patients with pathologically confirmed PDAC who received NACT followed by laparoscopic radical surgery in our hospital from December 2019 to April 2022. All patients underwent abdominal contrast-enhanced computed tomography (CT) and positron emission tomography-CT before surgery to accurately assess tumor stage and exclude distant metastasis.

RESULTS

All 15 patients with pancreatic cancer were successfully converted to surgical resection after NACT, including 8 patients with pancreatic head cancer and 7 patients with pancreatic body and tail cancer. Among them, 13 patients received the nab-paclitaxel plus gemcitabine regimen (gemcitabine 1000 mg/m^2 plus nab-paclitaxel 125 mg/m^2 on days 1, 8, and 15 every 4 wk) and 2 patients received the modified FOLFIRINOX regimen (intravenous oxaliplatin 68 mg/m^2, irinotecan 135 mg/m^2, and leucovorin 400 mg/m^2 on day 1 and fluorouracil 400 mg/m^2 on day 1, followed by 46-h continuous infusion of fluorouracil 2400 mg/m^2). After

each treatment cycle, abdominal CT, tumor markers, and circulating tumor cell counts were reviewed to evaluate the treatment efficacy. All 15 patients achieved partial remission. The surgical procedures included laparoscopic pancreaticoduodenectomy (LPD, n = 8) and laparoscopic radical antegrade modular pancreatosplenectomy (L-RAMPS, n = 7). None of them were converted to a laparotomy. One patient with pancreatic head carcinoma was found to have portal vein involvement during the operation, and LPD combined with vascular resection and reconstruction was performed. The amount of blood loss and operation times of L-RAMPS *vs* LPD were 435.71 ± 32.37 mL *vs* 343.75 ± 145.01 mL and 272.52 ± 49.14 min *vs* 444.38 ± 68.63 min, respectively. The number of dissected lymph nodes was 16.87 ± 4.10, and 3 patients had positive lymph nodes. One patient developed grade B postoperative pancreatic fistula (POPF) after L-RAMPS, and one patient experienced jaundice after LPD. None of the patients died after surgery. As of April 2022, progressive disease was noted in 4 patients, 2 patients had liver metastasis, and one had both liver metastasis and lymph node metastasis and died during the follow-up period.

CONCLUSION

Laparoscopic radical resection of PDAC after NACT is safe and effective if it is performed by a surgeon with rich experience in LPD and in a large center of pancreatic surgery.

Key Words: Pancreatic ductal adenocarcinoma; Neoadjuvant chemotherapy; Laparoscopic pancreaticoduodenectomy; Laparoscopic radical antegrade modular pancreatosplenectomy; Complications

Core Tip: We retrospectively analyzed the clinical data of 15 patients with pathologically confirmed pancreatic cancer who received neoadjuvant therapy followed by laparoscopic radical surgery in our hospital from December 2019 to April 2022. All patients underwent abdominal contrast-enhanced computed tomography (CT) and positron emission tomography-CT before surgery to accurately assess tumor stages and exclude distant metastasis. This retrospective study demonstrated that laparoscopic radical resection of pancreatic cancer after neoadjuvant therapy is safe and effective if it is performed by a surgeon with rich experience in laparoscopic pancreaticoduodenectomy in a large center of pancreatic surgery.

Citation: He YG, Huang XB, Li YM, Li J, Peng XH, Huang W, Tang YC, Zheng L. Efficacy and safety of laparoscopic radical resection following neoadjuvant therapy for pancreatic ductal adenocarcinoma: A retrospective study. *World J Gastrointest Oncol* 2022; 14(9): 1785-1797
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1785.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1785

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant digestive system tumor with an extremely poor prognosis[1-4]. Surgical resection remains the only potentially curative therapy for pancreatic cancer, but only 10%-20% of PDAC patients are operable at diagnosis. Even among patients who have undergone surgery, the 5-year survival rate is below 20%[2-5]. Although surgery is still the main treatment to achieve long-term survival in PDAC patients, the cancer is often diagnosed in an advanced stage or a progressive stage, during which the large tumor size and increased number of nodules make surgical resection particularly risky and difficult to perform. Moreover, advances in surgical technology and increased surgical safety have not significantly improved the prognosis of PDAC patients, and surgical resection alone can no longer meet the comprehensive treatment needs of patients[6]. Therefore, the principle of the diagnosis and treatment of PDAC has gradually transitioned from "surgery first" to surgery-centered multidisciplinary modes to improve the overall outcomes of patients[7].

With the increased clinical application of neoadjuvant chemotherapy (NACT), many studies have indicated that by shrinking the primary tumor and reducing vascular invasion and micrometastatic lesions, NACT for PDAC can increase the resectability rate, lower the incidence of postoperative complications, and ultimately prolong survival and improve prognosis[8-10]. The 2021 National Comprehensive Cancer Network® (NCCN) guidelines recommend NACT for patients with high-risk resectable PDAC, borderline resectable PDAC, and locally advanced PDAC[11].

In recent years, with the development of surgical instruments and minimally invasive techniques, laparoscopic techniques have been increasingly applied in PDAC, and more studies have been performed in multicenter settings[12,13]. However, severe fibrosis of local tumor tissue may occur after NACT; in addition, most tumors are borderline resectable or advanced PDAC, with large tumor sizes and close relationships with blood vessels, making the surgical procedure more complicated and difficult[14]. Most reported patients with PDAC underwent open surgery after NACT[15-17]. On the basis of our experience in laparoscopic surgery and open surgery for PDAC[12,18], we performed laparoscopic radical resection of PDAC after NACT. This study aimed to investigate the efficacy and safety of laparoscopic radical resection following NACT for PDAC.

MATERIALS AND METHODS

Patients

We retrospectively analyzed the clinical data of patients with PDAC in our hospital from December 2019 to April 2022. The patients were diagnosed with borderline resectable PDAC or locally advanced PDAC, which was confirmed by pathology and further assessed by medical imaging, and received NACT followed by laparoscopic surgery. All patients underwent abdominal contrast-enhanced computed tomography (CT) and positron emission tomography (PET)-CT before surgery to accurately assess tumor stage and exclude distant metastasis. This retrospective observational study was approved by the Medical Ethics Committee of our hospital and was conducted in accordance with the Declaration of Helsinki and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (2022-r177-01). Written informed consent was obtained from all the patients.

Inclusion criteria were as follows: (1) PDAC was confirmed by pathology of endoscopic ultrasonography-guided fine-needle biopsy specimens; (2) NACT was recommended by a multidisciplinary team (MDT), as they believed that direct or immediate surgical resection was not feasible due to the inoperability of the tumor and/or other underlying diseases; and (3) Patients received at least 2 cycles of NACT. The exclusion criteria were as follows: (1) Distant metastasis found on preoperative PET-CT or other imaging examinations; (2) Received other antitumor treatments, such as radiotherapy or targeted therapy, before surgery; (3) Radical resection was not performed as scheduled during the operation; and (4) Presence of other malignant tumors.

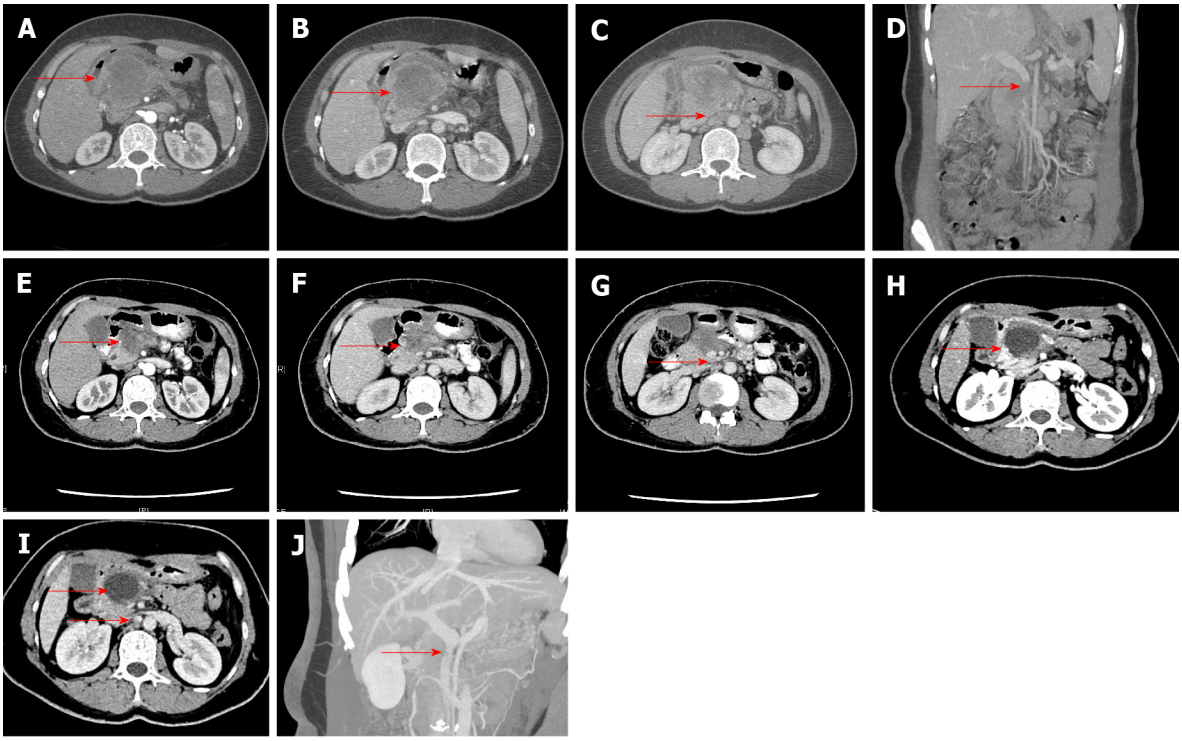
Methods

NACT: First-line treatment regimens were adopted. Nab-paclitaxel plus gemcitabine regimen (AG regimen) [11] (n = 13, 86.7%): Gemcitabine 1000 mg/m^2 plus nab-paclitaxel 125 mg/m^2 on days 1, 8, and 15 every 4 wk; modified FOLFIRINOX regimen[19,20] (n = 2, 13.3%): Intravenous oxaliplatin 68 mg/m^2, irinotecan 135 mg/m^2, and leucovorin 400 mg/m^2 on day 1 and fluorouracil 400 mg/m^2 on day 1, followed by 46-h continuous infusion of fluorouracil 2400 mg/m^2; 14 d as a cycle. Before each treatment, clinicians assessed the patient's physical status and individual differences to adjust the drug dose and treatment cycle.

After each treatment cycle, abdominal CT, tumor markers, and circulating tumor cell (CTC) counts were reviewed to evaluate the treatment efficacy. The course of treatment consisted of 2-4 treatment cycles. After 2 treatment cycles, the treatment efficacy was assessed using the 2021 NCCN Guidelines and the Response Evaluation Criteria in Solid Tumors (version 1.1)[21]. NACT was judged as effective by a MDT if: (1) The tumor diameter was reduced; (2) The carbohydrate antigen 19-9 (CA19-9) level markedly decreased; (3) The CTC count significantly decreased; (4) The patient's symptoms were obviously improved; and (5) There was no distant metastasis on PET-CT. After communicating with the patients and their families and obtaining written informed consent, we performed laparoscopic surgery. If the above criteria were not met, NACT might be continued. For borderline resectable or advanced PDAC, if the portal vein or superior mesenteric vein is partially involved or has a thrombus, resection should be considered only when appropriate vascular reconstruction at the distal and proximal ends is possible after vascular resection. The imaging findings before and after NACT are shown in Figure 1.

Surgical procedure: All patients laid in the prone position, with two legs apart. Under general anesthesia, the five-hole method was used to distribute the trocar position. The pneumoperitoneum pressure value was 12-14 mmHg, a 10-mm trocar was placed on the lower edge of the umbilicus to establish an observation port, two 12-mm trocars were placed on the left and right mid-clavicular lines parallel to the umbilicus, two 12-mm trocars were placed on the left and right anterior axillary lines, and one 5-12 mm trocar was placed on the costal edge to establish the main and auxiliary operating ports. The operator stood at the right side of the patient, the assistant stood at the left side of the patient, and the camera holder stood between the two legs of the patient.

The right-sided superior mesenteric artery (SMA) approach was used during laparoscopic pancreaticoduodenectomy (LPD), and the other surgical steps were the same as in the literature[18]. The lymph nodes on the right side of the SMA were dissected using a 180-degree arc incision. When the surgical maneuver was difficult, the "Easy First" approach was used instead, during which a vascular



DOI: 10.4251/wjgo.v14.i9.1785

Figure 1 Computed tomography changes in pancreatic cancer before and after neoadjuvant chemotherapy. A-C: Computed tomography (CT) before neoadjuvant chemotherapy revealed pancreatic cancer with multiple lymph node metastases (red arrow); D: Pancreatic cancer invaded the portal vein wall (red arrow); E-G: After 2 cycles of neoadjuvant chemotherapy, CT showed a decreased diameter of pancreatic cancer and a reduced number of lymph nodes (red arrow); H and I: After 4 cycles of neoadjuvant chemotherapy, CT showed an obviously decreased diameter of pancreatic cancer and a reduced number of retroperitoneal lymph nodes; J: The superior mesenteric vein had a regular shape.

occlusion belt was placed, which made the difficult LPD safe and feasible[22]. After the operation, one abdominal drainage tube was placed ahead the pancreatic duct-jejunal anastomosis and one behind the bile duct-jejunal anastomosis, respectively.

Laparoscopic radical antegrade modular pancreatosplenectomy (L-RAMPS) was performed for pancreatic body and tail tumors. A retropancreatic tunnel was established in front of the superior mesenteric vein in the neck of the pancreas. After the pancreas was severed, the splenic artery and vein were isolated and then severed at their roots. The lymph nodes on the left side of the celiac trunk and SMA were dissected. Then, the pancreatic body and tail containing the tumor, the spleen, the left prerenal fascia, the left adrenal gland, and the left prerenal fat sac were removed *en bloc* from the back of the left prerenal fascia to the left along the surface of the left renal vein. Finally, the lymph nodes on the right side of the SMA were dissected, and the Heidelberg triangle (*i.e.*, an anatomic triangle bordered by the SMA, celiac axis, and portal vein) was exposed (See Video).

Postoperative management: All patients received prophylactic antibiotics, proton-pump inhibitors, and parenteral nutrition during the perioperative period, and the nasogastric tube was removed on the 2nd postoperative day (POD 2). Patients started drinking water after feeling hungry, and a liquid diet was given after exhaustion. All patients were routinely tested for amylase levels in the drainage fluid on POD 3. When the amylase level in the drainage fluid was less than three times the normal upper limit of serum amylase and the risk of intra-abdominal hemorrhage was excluded, the abdominal drainage tube was removed (usually on day 5).

According to the International Study Group of Pancreatic Fistula[23], POPF was defined as a drainage amylase level of more than three times the normal serum amylase level on or after POD 3. The diagnosis of delayed gastric emptying (DGE) was based on the definition suggested by the International Study Group for Pancreatic Surgery in 2007[24]; *i.e.*, a diagnosis of DGE was made if one of the following conditions occurred after excluding mechanical factors such as anastomotic obstruction by upper gastrointestinal barium study or gastroscopy: (1) The gastric tube needed to be indwelled for more than three days after surgery; (2) The gastric tube needed to be reinserted due to vomiting and other reasons after extubation; or (3) Solid food was still not allowed seven days after surgery. The diagnosis of surgical site infection was based on the criteria developed by the United States Nosocomial Infections Surveillance System, United States Centers for Disease Control[25]. The short-term postoperative complications were graded using the 2004 Clavien-Dindo system[26].

Statistical analysis

Statistical analysis was performed using the SPSS 26.0 software package (SPSS Inc., IBM, Armonk, NY, United States). The measurement data were first tested for normality. Normally distributed data are presented as the means ± SD; otherwise, medians (interquartile ranges) are used. The count data are expressed as the number of cases. The survival curve was plotted by the Kaplan-Meier method.

RESULTS

General outcome

Fifteen patients with PDAC were included. After NACT, all patients were converted to resectable from borderline resectable or unresectable, including 8 patients (53.3%) with pancreatic head cancer and 7 patients (46.7%) with pancreatic body and tail cancer. Partial response to NACT was achieved in these 15 patients, and laparoscopic surgery was then performed. The demographic characteristics of all patients are shown in Table 1. There were 7 males (46.7%) and 8 females (53.3%) aged 55.53 ± 7.89 years. The average body mass index was 22.29 ± 2.94 kg/m^2. Compared with the measurement/count values before NACT, the tumor diameter, CA19-9 level, and CTC count decreased by 28.40% ± 9.71%, 57.07% ± 32.07%, and 65.33% ± 12.09%, respectively, after NACT. After the tumors were assessed as resectable, all patients underwent PET-CT to rule out the possibility of distant metastases.

Intraoperative conditions

All surgeries were completed under laparoscopy, and none of them were converted to laparotomy. The surgical procedures included LPD (n = 8, 53.3%) and L-RAMPS (n = 7, 46.7%). One patient (6.67%) with pancreatic head carcinoma was found to have portal vein involvement during the operation, and LPD combined with vascular resection and reconstruction was performed. The L-RAMPS time was 272.52 ± 49.14 min, and the average intraoperative blood loss was 435.71 ± 32.37 mL; LPD lasted 444.38 ± 68.63 min, and the average intraoperative blood loss was 343.75 ± 145.01 mL. Intraoperative blood transfusion was administered in 4 patients (26.66%). The number of dissected lymph nodes was 16.87 ± 4.10. In one patient (6.67%) with pancreatic body and tail cancer, grade B POPF occurred after L-RAMPS and was improved after drainage, pancreatic enzyme replacement therapy, and nutritional counseling. One patient (6.67%) with pancreatic head cancer developed jaundice after LPD, in whom percutaneous transhepatic biliary drainage (PTBD) was performed after surgery, and the drainage catheter was removed two weeks later; the condition was improved after the placement of a biliary metal stent. None of the patients died after surgery. The average hospital stay was 13 (12-14) d (Table 2).

Results of pathological examination

R0 resection was achieved in all 15 patients. The postoperative pathology showed that all the tumors were PDAC, and residual cancer was detected by multipoint sampling in one patient. The tumors were moderately differentiated in 11 patients (73.33%), moderately to poorly differentiated in 3 patients (20%), and poorly differentiated in 1 patient (6.67%). According to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system, 4 patients (26.66%) were in stage IA, 7 (46.66%) were in stage IB, 1 (6.67%) was in stage IIB, 1 (6.67%) was in stage IIIA, 1 (6.67%) was in stage IIIB, and 1 (6.67%) was in stage IIIC. The number of dissected lymph nodes was 16.87 ± 4.10, and 3 patients (20%) had positive lymph nodes (Table 3).

Postoperative results

One patient (6.67%) developed a grade B POPF after surgery, which improved after drainage, pancreatic enzyme replacement therapy, and nutritional counseling. One patient (6.67%) had jaundice after LPD, and abdominal ultrasonography and magnetic resonance cholangiopancreatography showed anastomotic stenosis and dilated intrahepatic bile duct above the anastomosis. Thus, jaundice was considered to be caused by biliary-enteric anastomotic stenosis after cholangiojejunostomy. PTBD was then performed, and the drainage catheter was removed two weeks later. The biliary obstruction was alleviated after the placement of a biliary metal stent. We assumed that the patient had a small bile duct diameter, and anastomotic stenosis was caused by continuous suturing.

Postoperative adjuvant therapy

All patients who underwent surgery after NACT were evaluated for their physical status and nutritional status, and postoperative adjuvant therapy was scheduled if they could tolerate it. The adjuvant treatment regimen was selected according to the efficacy of NACT. After multidisciplinary discussions, 15 patients received 6 cycles of treatment after surgery. Generally, adjuvant therapy was started 6 to 8 wk after surgery and repeated every 3 wk. Routine blood tests and biochemical tests were performed before each chemotherapy session. Gastrointestinal tumor marker detection, CTC counts, and contrast-enhanced CT or magnetic resonance imaging (MRI) were performed every 3 cycles to determine whether the tumor had recurred or metastasized.

Table 1 General data of the patients

Variables	
Sex, *n* (%)	
Male	7 (46.7)
Female	8 (53.3)
Age (yr)	55.53 ± 7.89
Body mass index, kg/m^2	22.29 ± 2.94
Resectability, *n* (%)	
Borderline resectable (*n*)	7 (46.7)
Advanced pancreatic cancer (*n*)	8 (53.3)
ASA grade, *n* (%)	
I	13 (86.7)
II	2 (13.3)
Chemotherapy regimen, *n* (%)	
AG	13 (86.7)
Modified FOLFIRINOX	2 (13.3)
ECOG score, *n* (%)	
0	11 (73.3)
1	2 (13.35)
2	2 (13.35)
Chemotherapy cycle	4 ± 1
Response to chemotherapy	
PR	15 (100%)
CR	0
Tumor diameter before chemotherapy (cm)	4.17 ± 1.40
Tumor diameter before surgery (cm)	3.03 ± 1.13
Tumor regression (%)	28.40 ± 9.71
CA19-9 level before chemotherapy (U/mL)	736.25 (8.44-1200.00)[1]
CA19-9 level before surgery (U/mL)	51.85 (4.81-341.3)[1]
Decrease in CA19-9 level (%)	57.07 ± 32.07
Total count of CTCs before chemotherapy (*n*)	16 (13-26)[1]
Total count of CTCs before surgery (*n*)	7.13 ± 2.88
Decrease in the total number of CTCs (%)	65.33 ± 12.09

[1]Data are presented as the mean ± SD or median (interquartile range).
ASA: American Society of Anesthesiologists; AG: Nab-paclitaxel + gemcitabine; modified FOLFIRINOX: Oxaliplatin + leucovorin + irinotecan + fluorouracil; CTC: Circulating tumor cells; CA19-9: Carbohydrate antigen 19-9; PR: Partial response; ECOG: Eastern Cooperative Oncology Group; CR: Complete response.

Postoperative follow-up

The patients were followed up every 3 mo after adjuvant chemotherapy was completed, during which routine blood tests, biochemical tests, gastrointestinal tumor marker detection, CTC counts, and contrast-enhanced CT or MRI examinations were performed. Follow-up was conducted by telephone, the WeChat app, and outpatient visits, and the date of the last follow-up visit was recorded. As of April 2022, all 15 patients had been followed up for 7 mo (range: 5-16 mo). Progressive disease was noted in 4 patients (26.66%), including liver metastases in 2 patients (13.3%), among whom one patient (6.67%) had both liver metastasis and lymph node metastasis. Adjuvant therapy was repeated when PD was detected during the follow-up period according to the opinions of the MDT. The patient with both liver

Table 2 Surgery-related data of 15 patients undergoing neoadjuvant therapy for pancreatic cancer

Variables	
Tumor location, *n* (%)	
Head of the pancreas	8 (53.3)
Pancreatic body and tail	7 (46.7%)
Surgical procedure, *n* (%)	
L-RAMPS	7 (46.7)
LPD	8 (53.3)
Vascular resection and reconstruction, *n* (%)	1 (6.67)
Operative time (min)	
L-RAMPS	326.43 ± 49.14
LPD	444.38 ± 68.63
Intraoperative blood loss (mL)	
L-RAMPS	435.71 ± 262.54
LPD	343.75 ± 145.01
Intraoperative blood transfusion, *n* (%)	
L-RAMPS	2 (13.35)
LPD	2 (13.35)
Conversion, *n* (%)	0 (100)
Complications, *n* (%)	
Jaundice	1 (6.67)
Grade B POPF	1 (6.67)
Postoperative hospital stay (d)	13 (12-14)[1]
Follow-up duration (mo)	7 (5-16)[1]
Recurrence/metastasis, *n* (%)	
Liver metastasis	3 (20)
Lymph node metastasis	1 (6.67)
Mortality within the follow-up period, *n* (%)	1 (6.67)

[1]Data are presented as the median (interquartile range).
L-RAMPS: Laparoscopic radical antegrade modular pancreatosplenectomy; LPD: Laparoscopic pancreatoduodenectomy; POPF: Postoperative pancreatic fistula.

metastasis and lymph node metastasis died due to tumor progression (Table 2). To date, the 1- and 2-year survival rates are both 50.00%, and the 1- and 2-year disease-free survival (DFS) rates are 60.00% and 40.00%, respectively (Table 2 and Figure 2).

DISCUSSION

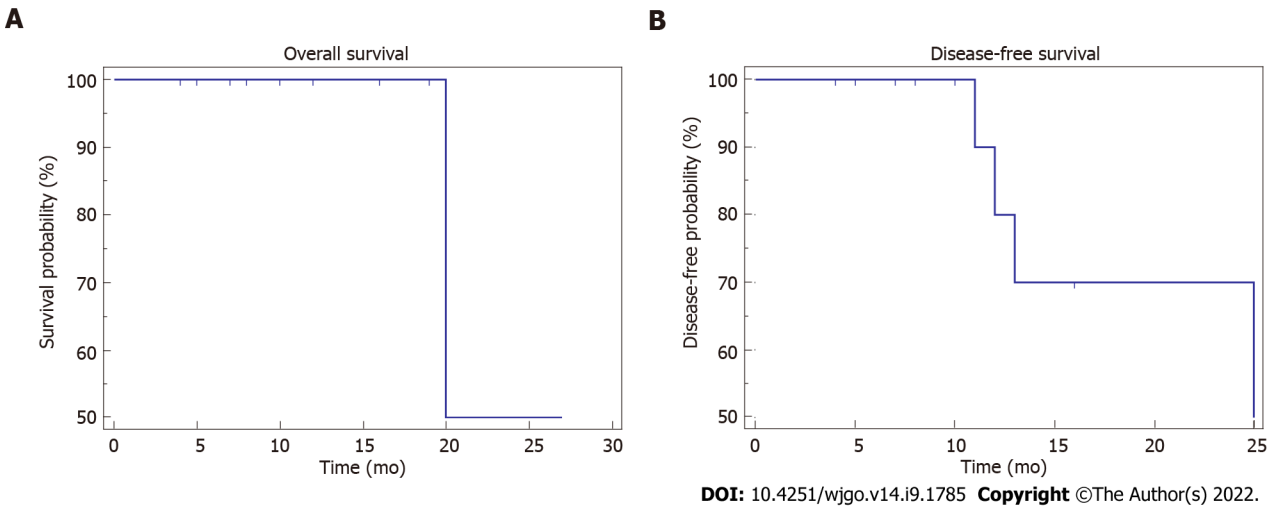
Many studies have demonstrated that NACT can increase the R0 resection rate, prolong DFS, and increase the long-term survival rate in patients with borderline resectable PDAC[8-10]. Therefore, guidelines on PDAC treatment have included NACT as a recommended treatment option for resectable PDAC, borderline PDAC, and locally advanced PDAC with high-risk factors (high serum CA19-9 level, large primary tumor, extensive lymph node metastasis, significant weight loss, and extreme pain)[11]. Treatments for PDAC include chemotherapy, radiotherapy, and immunotherapy. Among them, NACT is the core treatment, resulting in notable efficacy when combined with other therapies[11].

The currently available NACT regimens for PDAC include FOLFIRINOX, modified FOLFIRINOX, AG regimen, and gemcitabine + S-1[11,27]. It has been reported that FOLFIRINOX and the AG regimen showed no significant difference in terms of the R0 resection rate and overall survival[28]. However, the

Table 3 Pathological data

Variables	
Degree of differentiation, *n* (%)	
Moderately differentiated	11 (73.33)
Moderately to poorly differentiated	3 (20)
Poorly differentiated	1 (6.67)
AJCC pathological stage, *n* (%)	
IA	4 (26.66)
IB	7 (46.66)
IIB	1 (6.67)
IIIA	1 (6.67)
IIIB	1 (6.67)
IIIC	1 (6.67)
R0 resection, *n* (%)	15 (100)
Total number of lymph nodes dissected (*n*)	16.87 ± 4.10
Number of patients with positive lymph nodes (*n*)	3

AJCC: American Joint Committee on Cancer.



DOI: 10.4251/wjgo.v14.i9.1785

Figure 2 Kaplan-Meier curves of overall survival and disease-free survival. A: The 1- and 2-year survival rates were both 50%; B: The 1- and 2-year disease-free survival rates were 60.00% and 40.00%, respectively.

AG regimen has many advantages: Acceptable toxicities; good tolerance, which leads to good compliance and a high rate of treatment completion; and good feasibility for the Chinese population [29]. Therefore, the AG regimen is used in most of the patients in our center, and modified FOLFIRINOX is also used in some patients. For patients with poor physical performance, gemcitabine + S-1 may be used to improve the quality of life and prolong the survival time. In the present study, the 15 patients whose diseases were successfully converted after NACT were all treated with the AG regimen, and these patients were in good general condition during the treatment and had no chemotherapy-induced myelosuppression.

Based on the Chinese and foreign guidelines[27,30], we used imaging findings (tumor diameter, relationship between tumor and adjacent blood vessels, and surrounding lymph nodes) before and after NACT, tumor markers [mainly CA19-9 but also carcinoembryonic antigen (CEA)], and improvement in clinical symptoms to evaluate the treatment cycle. For patients receiving NACT for PDAC, an abdominal contrast-enhanced CT examination was performed upon the completion of each cycle, CTA was performed every 2 cycles, and the levels of CA19-9, CEA, and other tumor markers were measured during each follow-up visit. Changes in clinical symptoms were monitored and recorded. CA19-9 can be

easily influenced by various factors, such as inflammation and infection, and its potential as a biomarker for monitoring PDAC progression and recurrence has been compromised by false negative results before surgery. The use of microRNAs has been reported in the literature[31,32], but with limited clinical value. CTCs have been used as a predictor of long-term survival in patients with PDAC, and the overall survival and progression-free survival rates significantly decreased in patients with total CTCs ≥ 6[33,34]. In our study, we used the Canpatrol™ CTC assay (SurExam, Guangzhou, China) to detect CTCs before NACT and re-examined CTCs after each cycle of treatment. It was observed that the CTC count markedly increased in patients with elevated CA19-9 levels; in CA19-9-negative patients, the CTC count was also significantly higher than the normal value but gradually dropped with the application of NACT. Therefore, we speculate that CTCs may be one of the predictors of the resectability of PDAC. In the absence of standard evaluation criteria, monitoring changes in imaging features and tumor markers is currently an important method to evaluate the efficacy of NACT for PDAC[27].

Our criteria for the resectability of PDAC after NACT are as follows: (1) The diameter of the pancreatic tumor decreased, and the relationship between the tumor and the surrounding blood vessels improved; (2) The CA19-9 level and CTC count notably dropped (ideally, decreased by 50% or returned to the normal ranges); and (3) The pain was relieved or other symptoms were improved. If one or more of the above criteria are met, NACT is considered effective following multidisciplinary consultations, and surgery may be performed after communicating with patients and their families and obtaining written informed consent. If none of the above criteria is met, NACT will be continued.

R0 resection and lymph node negativity are key factors to ensure survival after PDAC surgery[35-37]. In addition to negative surgical margins, it is important to ensure a sufficient number of negative lymph nodes and negative vascular margins. After NACT for PDAC, the diameter of the primary lesion is decreased, along with a lowered rate of positive lymph nodes, which can reduce vascular invasion and micrometastases. According to the 8th edition of the AJCC guidelines, the number of lymph nodes to be dissected should be no less than 12. In the present study, the total number of lymph nodes dissected during surgery in the 15 patients was 16.87 ± 4.10, and the rate of positive lymph nodes was 2.1%, reaching the AJCC guideline-recommended requirement for lymph node dissection in pancreatic cancer. Another key factor in achieving R0 resection is a negative vascular margin. In a recent study, compared with laparotomy, LPD had similar short- and long-term prognoses, and LPD combined with venous resection and reconstruction was safe; notably, the laparoscopic technique was easier to perform.

Mokdad *et al*[15] reported on patients with resectable PDAC who received NACT followed by radical resection, and the study results showed that NACT significantly improved the overall survival of the patients (26 mo *vs* 21 mo, $P < 0.01$) and could reduce the positive rate of surgical margins (17% *vs* 24%, $P < 0.01$). The study by Reni *et al*[38] came to similar conclusions, with an overall survival time of 38.2 mo for patients with resectable PDAC who received NACT followed by surgery, compared with overall survival times of 20.4 mo and 26.2 mo for patients who underwent surgery followed by NACT. We have performed radical resection of PDAC following NACT since December 2019. Thus far, a total of 15 patients have achieved R0 resection, and the lymphadenectomy rate in these patients is high with a low positive rate, but during the follow-up process, 3 patients had liver metastasis, 1 patient had lymph node metastasis, and the rest were tumor-free. The 1-year survival rate and 2-year survival rate were 50.00%, the 1-year tumor-free survival rate was 60.00%, and the 2-year tumor-free survival rate was 40.00%. Most of our patients had been followed up for no more than 2 years (less than 1 year in most cases), which may explain the low 1- and 2-year survival rates.

We believe that resection is the challenging part of LPD after NACT for PDCA, and the difficulty of resection is the management of anatomical structure and vessels. Our experience is as follows: (1) Although more challenging, LPD after NACT can be performed by a surgeon with rich experience in LPD surgery; (2) It is very difficult to find a single approach that suitable for all cases. During the operation, we preferentially adopt the "early first" principle, and gradually separate and resect to complete. However, in some cases, we chose different arterial approaches according to the direction of tumor invasion; (3) Due to portal vein adhesion and tumor invasion after NACT in some pancreatic cases, procedures of the superior mesenteric vein behind the neck of the pancreas may cause bleeding, and the establishment of a retropanctreatic tunnel is more challenging. In these cases, the pancreas can be separated and resected from 2-3 cm to the left side of the superior mesenteric vein and the neck of the pancreas. The advantage of choosing here is that it is far away from the tumor, the tissue separation is easier than performing behind the neck of the pancreas, and the space between the splenic vein and the pancreas can be easily separated. It is safer to search the superior mesenteric vein after the resection of the pancreas and dissection of surrounding tissues from left to right; and (4) The digestive tract reconstruction was performed according to a routine procedure after lesion resection in pancreaticoduodenectomy and was barely affected by NACT.

CONCLUSION

Laparoscopic radical resection of PDAC after NACT is safe and feasible if it is performed by an operator

with experience of at least 100 cases of relevant surgeries in a specialist pancreas center. However, since our study was a retrospective analysis with a small sample from a single center, the safety and feasibility of this technique need to be verified by prospective large-sample randomized controlled trials in multiple pancreatic centers.

ARTICLE HIGHLIGHTS

Research background

Multiple studies have demonstrated that neoadjuvant chemotherapy (NACT) can prolong the overall survival of pancreatic ductal adenocarcinoma (PDAC) patients. However, most studies have focused on open surgery following NACT.

Research motivation

Despite the development of surgical instruments and minimally invasive techniques, laparoscopic techniques have been increasingly applied in pancreatic surgery. However, most reported cases of PDAC patients underwent open surgery after NACT. At present, we performed laparoscopic radical resection of PDAC after NACT.

Research objectives

Our aims were to investigate the efficacy and safety of laparoscopic radical resection following NACT for PDAC.

Research methods

We retrospectively analyzed the clinical data of 15 patients with pathologically confirmed PDAC who received NACT followed by laparoscopic radical surgery in our hospital from December 2019 to April 2022. All patients underwent abdominal contrast-enhanced computed tomography (CT) and positron emission tomography-CT before surgery to accurately assess tumor stage and exclude distant metastasis.

Research results

All 15 patients with PDAC were successfully converted to surgical resection after NACT, including 8 patients with pancreatic head cancer and 7 patients with pancreatic body and tail cancer. Among them, 13 patients received the nab-paclitaxel plus gemcitabine regimen (gemcitabine 1000 mg/m^2 plus nab-paclitaxel 125 mg/m^2 on days 1, 8, and 15 every 4 wk), and 2 patients received the modified FOLFIRINOX regimen (intravenous oxaliplatin 68 mg/m^2, irinotecan 135 mg/m^2, and leucovorin 400 mg/m^2 on day 1 and fluorouracil 400 mg/m^2 on day 1, followed by a 46-h continuous infusion of fluorouracil 2400 mg/m^2). After each treatment cycle, abdominal CT, tumor markers, and circulating tumor cell (CTC) counts were reviewed to evaluate the treatment efficacy. All 15 patients achieved partial remission. The surgical procedures included laparoscopic pancreaticoduodenectomy (LPD, n = 8) and laparoscopic radical antegrade modular pancreatosplenectomy (L-RAMPS, n = 7). One patient with pancreatic head carcinoma was found to have portal vein involvement during the operation, and LPD combined with vascular resection and reconstruction was performed. One patient developed grade B postoperative pancreatic fistula after L-RAMPS, and one patient experienced jaundice after LPD. None of the patients died after surgery.

Research conclusions

Laparoscopic radical resection of PDAC after neoadjuvant therapy is safe and effective if it is performed by a surgeon with rich experience in LPD and L-RAMPS in a large center of pancreatic surgery.

Research perspectives

With the increased clinical application of NACT, many studies have indicated that by shrinking the primary tumor and reducing vascular invasion and micrometastatic lesions, NACT for PDAC can increase the resectability rate, lower the incidence of postoperative complications, and ultimately prolong survival and improve prognosis. Most reported cases of pancreatic cancer patients underwent open surgery after NACT. LPD has certain advantages, such as less trauma, quick recovery, less bleeding, and a good postoperative quality of life. Therefore, laparoscopic surgery after NACT for PDAC has certain advantages.

FOOTNOTES

Author contributions: He YG and Huang XB, Zheng L contributed to the conception and design of the study and

drafted the manuscript; Zheng L, Li YM, Peng XH, Li J, Huang W and Tang YC contributed to the analysis and interpretation of the data and revised the manuscript; Zheng L, Li YM, and He YG participated in the clinical treatment operation and literature research; and all authors read and approved the final manuscript.

Supported by Chongqing Municipal Science and Technology Talent Project, No. csct2017jcyj-yszxX0002; Chongqing Municipal Science and Technology Innovation Project for Social Undertakings and People's Livelihood Guarantee, No. cstc2018jscx-mszdX0012; and the Natural Science Foundation of Chongqing, No. cstc2021jcyj-msxmX0991.

Institutional review board statement: This retrospective observational study was approved by the Medical Ethics Committee of our hospital and was conducted in accordance with the Declaration of Helsinki and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (2022-r177-01).

Informed consent statement: Written informed consent was obtained from all the patients.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The authors declare that all the data have been provided in the article and that there are no supplemental data.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1798-1807

DOI: 10.4251/wjgo.v14.i9.1798

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

To scope or not - the challenges of managing patients with positive fecal occult blood test after recent colonoscopy

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Batyrbekov K, Kazakhstan; Tadros M, United States

Received: January 21, 2022
Peer-review started: January 21, 2022
First decision: February 24, 2022
Revised: March 12, 2022
Accepted: July 27, 2022
Article in press: July 27, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Colorectal cancer (CRC) is a major health problem. There is minimal consensus of the appropriate approach to manage patients with positive immunochemical fecal occult blood test (iFOBT), following a recent colonoscopy.

AIM

To determine the prevalence of advanced neoplasia in patients with a positive iFOBT after a recent colonoscopy, and clinical and endoscopic predictors for advanced neoplasia.

METHODS

The study recruited iFOBT positive patients who underwent colonoscopy between July 2015 to March 2020. Data collected included demographics, clinical characteristics, previous and current colonoscopy findings. Primary outcome was the prevalence of CRC and advanced neoplasia in a patient with positive iFOBT and previous colonoscopy. Secondary outcomes included identifying any clinical and endoscopic predictors for advanced neoplasia.

RESULTS

The study included 1051 patients (male 53.6%; median age 63). Forty-two (4.0%) patients were diagnosed with CRC, 513 (48.8%) with adenoma/sessile serrated lesion (A-SSL) and 257 (24.5%) with advanced A-SSL (AA-SSL). A previous colonoscopy had been performed in 319 (30.3%). In this cohort, four (1.3%) were diagnosed with CRC, 146 (45.8%) with A-SSL and 56 (17.6%) with AA-SSL. Among those who had a colonoscopy within 4 years, none had CRC and 7 had

AA-SSL. Of the 732 patients with no prior colonoscopy, there were 38 CRCs (5.2%). Independent predictors for advanced neoplasia were male [odds ratio (OR) = 1.80; 95% confidence interval (CI): 1.35-2.40; $P < 0.001$), age (OR = 1.04; 95%CI: 1.02-1.06; $P < 0.001$) and no previous colonoscopy (OR = 2.07; 95%CI: 1.49-2.87; $P < 0.001$).

CONCLUSION

A previous colonoscopy, irrespective of its result, was associated with low prevalence of advanced neoplasia, and if performed within four years of a positive iFOBT result, was protective against CRC.

Key Words: Colorectal cancer; Adenoma; Screening; Fecal occult blood test; Colonoscopy

Core Tip: Currently, there is minimal consensus to inform clinicians of the appropriate approach to manage patients presenting with positive immunochemical fecal occult blood test (iFOBT) following a recent colonoscopy. This may lead to additional unnecessary, invasive procedure which confers procedure-related risks, as well as avoidable patient anxiety and a higher cost-burden on the healthcare system. Our study revealed that a previous colonoscopy, irrespective of its result, was associated with low prevalence of advanced neoplasia, and if performed within 4 years of a positive iFOBT result, was protective against colorectal cancer.

Citation: Rattan N, Willmann L, Aston D, George S, Bassan M, Abi-Hanna D, Anandabaskaran S, Ermerak G, Ng W, Koo JH. To scope or not - the challenges of managing patients with positive fecal occult blood test after recent colonoscopy. *World J Gastrointest Oncol* 2022; 14(9): 1798-1807
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1798.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1798

INTRODUCTION

Colorectal cancer (CRC) is the fourth most-commonly diagnosed malignancy and second-highest cause of cancer mortality in Australia[1]. Screening for CRC with a fecal occult blood (FOBT) test is essential in early detection and management, leading to reduction in CRC-related mortality[2,3]. When diagnosed early, CRC has excellent prognosis, with a 5-year survival rate of up to 93%[4,5]. In Australia, the National Bowel Cancer Screening Program (NBCSP) invites those 50 to 74 years of age to participate in biennial immunochemical FOBT (iFOBT) screening. Of those undergoing colonoscopy, 1 in 41 had a CRC diagnosis, resulting in a 15% reduction in mortality in the screened population when compared with non-screened population[1,4]. The NBCSP automatically invites subjects to participate in screening at the designated ages, irrespective of having had a previous colonoscopy. In individuals who have had a recent colonoscopy, this may lead to an unnecessary, invasive procedure which confers procedure-related risks, as well as avoidable patient anxiety and a higher cost-burden on the healthcare system[6,7]. Despite aiming to shift resources from surveillance to screening, this may paradoxically place greater burden on the need for repeat procedures, and potentially drain resources. Hence, there is a need to optimize the utilization of available resources, specifically to determine the widest acceptable surveillance interval in those with a prior colonoscopy that still confers a reduction in CRC mortality. Currently, there is limited data and minimal consensus to inform clinicians of the appropriate approach to manage patients presenting with positive iFOBT following a recent colonoscopy. The primary aim of this study was to determine the prevalence of advanced neoplasia, defined as CRC and advanced adenoma or sessile serrated lesions, in a patient presenting with positive iFOBT, after having had a previous colonoscopy. The secondary aim was to determine any clinical, biochemical, and endoscopic predictors of advanced neoplasia in these patients.

MATERIALS AND METHODS

Study design

This cohort study included iFOBT-positive patients between the ages of 50 and 75 years who were referred for a colonoscopy at a high-volume Australian tertiary referral center between July 2015 to March 2020. A positive iFOBT result was determined during population-based or opportunistic

screening.

Data collection and statistical analysis

Data was prospectively collected from patients including demographics such as age, gender, family history of CRC, aspirin use, diabetes and gastrointestinal symptoms (rectal bleeding, altered bowel habits, abdominal pain, unexplained weight loss and anemia). Prior and current colonoscopy timing and findings were retrieved from the centre's electronic medical records and treating proceduralists' records. Data obtained included quality of bowel preparation, completion to cecum or terminal ileum, pathology identified and histopathology. Only completed colonoscopies were included for patients who required a repeat procedure if the initial colonoscopy was unable to be completed due to poor quality of bowel preparation. All colonoscopies were performed by 12 experienced gastroenterologists. Statistical analysis was performed using IBM SPSS statistics (version 22; IBM Corp., Armonk, NY, United States) including χ^2 test for categorical variables, the Mann-Whitney *U* test to assess differences between non-parametric continuous variables and binary logistic regression to assess for predictors of advanced neoplasia and CRC.

Definitions

Polyps were classified as adenomas/sessile serrated lesions (A-SSL), or non-adenomas based on histopathology. An advanced A-SSL (AA-SSL) was defined as an adenoma measuring ≥ 10mm in diameter, having high-grade dysplasia or villous or tubulovillous architecture or a sessile serrated lesion measuring ≥ 10mm in diameter with or without dysplasia. Advanced neoplasia was defined as an AA-SSL, carcinoma *in situ* or invasive CRC. A colonoscopy was deemed complete if the endoscope was advanced to the cecum or terminal ileum.

Ethics

The local institution's Human Research and Ethics Committee approved the study (HREC/LNR/15/LPOOL/186).

RESULTS

Patient demographics

The study involved data collected from 1051 iFOBT-positive patients (male 563, 53.6%; median age 63, range 50 to 75 years) from July 2015 to March 2020. Within this group, 108 patients (10.3%) had a family history of CRC with this being a first degree relative in 78 (father 31, mother 22, sibling 25). A total of 407 patients (38.7%) were symptomatic at the time of presentation, with symptoms including rectal bleeding (n = 178; 16.9%), altered bowel habits (n = 181, 17.2%), abdominal pain (n = 81, 7.7%), unintentional weight loss (n = 53, 5.0%) and anemia (n = 59, 5.6%). Just over thirty percent of patients had a previous colonoscopy (n = 319), and 47 patients (4.5%) could not recall having undergone a colonoscopy.

Current colonoscopy findings

The bowel preparation was reported as excellent or good in 736 (70%), fair/adequate/satisfactory in 246 (23.4%) and poor in 69 (6.6%) patients. Complete colonoscopy was achieved in 1026 (97.6%) patients. Overall, 42 (4.0%) patients were diagnosed with CRC. The A-SSL detection rate was 48.8% (n = 513) while 54 (5.1%) patients had non-adenomatous polyps and 466 (44.3%) patients had no polyps. There were 257 (24.5%) patients with AA-SSL and cumulatively 281 (26.7%) with advanced neoplasia detected. The number of polyps detected ranged from 1 to 13 (mean 2.26 ± 1.69, median 2.0). The size of the polyps ranged from 1 to 65 mm (mean 9.24 ± 6.50 mm, median 8.0 mm). Other pathology identified at colonoscopy included diverticulosis (n = 240, 22.8%), hemorrhoids (n = 215, 20.4%), colonic angioectasia (n = 14, 1.3%) and inflammatory bowel disease (n = 2), while 121 (11.5%) patients had a normal colonoscopy. Demographics and colonoscopy outcomes in patients with and without a previous colonoscopy are described in Table 1.

Previous colonoscopy findings

For most patients who had a previous colonoscopy, it was performed more than 5 years earlier (63.9%). The time of previous colonoscopy in relation to current procedure is depicted in Table 2. With respect to previous colonoscopies, the quality of bowel preparation was reported as excellent or good in 66 patients, fair/satisfactory/adequate in 28, poor in 21 and unknown in 204 (63.9%) patients. The colonoscopy was complete in 106 (33.2%) cases, incomplete in eight patients and the extent of insertion was unknown for 205 (64.2%) patients. In 84 (26.3%) patients, the previous colonoscopy findings were unable to be obtained. Where results were available, colonoscopy findings included one CRC and 95 patients had at least one polyp detected (25 patients had adenomas, and the remaining were non-adenomatous polyps). Other findings included diverticulosis (n = 19) and hemorrhoids (n = 20). There

Table 1 Patient demographics and current colonoscopy findings

n = 1051	Previous colonoscopy = Yes, *n* = 319 (30.4%)	Previous colonoscopy = No/unknown, *n* = 732 (69.6%)	*P* value
Sex			
Male	174 (54.5%)	389 (53.1%)	0.68
Age			
50-64	151 (47.3%)	438 (59.8%)	< 0.001
65 +	168 (52.7%)	294 (40.2%)	
Median age	65 (range 50-75)	62 (range 50-75)	< 0.001
Family history of CRC			
Yes	52 (16.3%)	56 (7.7%)	< 0.001
Symptomatic			
Any symptoms	149 (46.7%)	258 (35.2%)	< 0.001
Rectal bleeding	66 (20.7%)	112 (15.3%)	0.03
Change in bowel pattern	65 (20.4%)	116 (15.8%)	0.09
Abdominal pain	35 (11%)	46 (6.3%)	0.014
Weight loss	11 (3.4%)	34 (4.6%)	0.28
Anemia	19 (6.0%)	40 (5.5%)	0.75
Current colonoscopy bowel preparation			
Good/excellent	204 (63.9%)	532 (72.7%)	0.014
Poor	23 (7.2%)	46 (6.3%)	
Complete colonoscopy	311 (97.5%)	715 (97.7%)	0.42
CRC detected	4 (1.3%)	38 (5.2%)	0.003
A-SSL detected	146 (45.8%)	367 (50.1%)	0.19
AA-SSL detected	57 (17.8%)	200 (27.3%)	0.002
Advanced neoplasia	60 (18.8%)	221 (30.2%)	< 0.001

CRC: Colorectal cancer; A-SSL: Adenoma/sessile serrated lesion; AA-SSL: Advanced adenoma/sessile serrated lesion.

Table 2 Time of previous colonoscopy in relation to current procedure

Time since previous colonoscopy; *n* = 319	Frequency (%)
< 1 yr	2 (0.6)
1-2 yr	11 (3.4)
2-3 yr	18 (5.6)
3-4 yr	37 (11.6)
4-5 yr	37 (11.6)
> 5 yr	204 (63.9)
Timing unknown	10 (3.1)

were 100 patients who had a previous normal colonoscopy.

Current colonoscopy findings in the context of previous colonoscopy

Of the 319 patients who had a previous colonoscopy, four (1.3%) were diagnosed with CRC and 56 (17.6%) had AA-SSL on their current colonoscopies. Of the four CRC cases, one patient was diagnosed 4 years and 7 mo after a normal index colonoscopy, where the bowel preparation was reported as good.

Another patient had a prior colonoscopy 7 years earlier and was symptomatic with abdominal pain prior to the current procedure. The remaining two patients diagnosed with CRC had a prior colonoscopy greater than 10 years ago, and their prior colonoscopy findings including bowel preparation were unavailable. Details of these four patients' previous and current colonoscopy findings and American Joint Committee on Cancer (AJCC) staging of CRC at diagnosis are summarized[8] in Table 3.

Among the 732 patients who had no prior colonoscopy or were uncertain about a previous procedure, 38 (5.2%) and 200 (27.3%) patients were diagnosed with CRC and AA-SSL respectively, and these were significantly higher than those who had an index colonoscopy. Also, these patients were younger, had fewer family members with CRC and were more likely to be asymptomatic at the time of their current colonoscopy (Table 1). The prevalence of AA-SSL, advanced neoplasia, and CRC on the current colonoscopy according to the time since the previous colonoscopy, are presented in Table 4. Among patients who had their index colonoscopy within 4 years (n = 68), there was no CRC detected on their current colonoscopy, while 7 patients had an AA-SSL detected. Details of these seven patients' previous and current colonoscopy findings are summarized in Table 5.

Predictors of advanced neoplasia

In multi-variate analysis using binary logistic regression of the entire cohort, male gender, age, and no previous colonoscopy were independent predictors of advanced neoplasia. The univariate and multivariate predictors of advanced neoplasia of the entire cohort are reported in Table 6. In the cohort with a previous colonoscopy, univariate analysis using binary logistic regression identified age over 65 years [odds ratio (OR) = 1.94; 95% confidence interval (CI): 1.08-3.46; P = 0.03) as the only predictor of advanced neoplasia. Male gender, family history of CRC, symptoms, quality of bowel preparation and completion of the index colonoscopy were not statistically significant. Due to the small number of CRC diagnosis in this cohort, we were unable to analyze the clinical predictors of CRC detection.

DISCUSSION

In Australia, nationwide biennial iFOBT invitations have resulted in a significant influx in patients presenting for colonoscopy, thus anticipating a sustained increase over time. Strategies to avoid unnecessary procedures would help distribute resources more effectively, leading to improved management of waitlists, reducing patient anxiety and the cost-burden on the healthcare system[6,7]. While a colonoscopy is recommended in a patient with a positive iFOBT, the decision to proceed in those with a previous colonoscopy is often unclear and guidelines are lacking. The concern exists for interval pathology, especially CRC, likely influenced by the timing between procedures and quality of the preceding colonoscopy. Colonoscopy is not a perfect procedure and rates of missed lesions are well documented, with the quality of colonoscopy dependent on multiple factors including the proceduralist's adenoma detection rate, withdrawal times and quality of bowel preparation[9,10]. However, avoiding an unnecessary colonoscopy would be ideal if one can be confident that the preceding colonoscopy did not miss advanced colorectal pathology.

Our study aimed to determine the widest acceptable interval between consecutive colonoscopies that maintains patient safety through a reduction in CRC incidence whilst optimizing healthcare resource utilization. We found that despite presenting with a positive iFOBT, there was no CRC detected among the 68 patients with an index colonoscopy within 4 years of their current procedure, irrespective of the results of their index procedures. Of these patients, 7 had an AA-SSL detected, although four were classified based on size greater than 10 mm alone, without having other high-risk features such as villous architecture or high-grade dysplasia. Excluding these patients, the rate of AA-SSL detection was 4.4%. In three patients with AA-SSL, the bowel preparation of the index procedure was suboptimal, thereby increasing the possibility of missed lesions. Two patients were symptomatic at the time of their current examination, and none had a family history of CRC. Our study found that having a previous colonoscopy for any clinical indication was associated with a lower risk of advanced neoplasia in subsequent testing. A similar protective effect of a prior colonoscopy has been reported by another study, with a risk reduction of CRC of 67%-85% for up to 10 years[11].

Several studies have supported deferring a colonoscopy after a positive FOBT in patients who have had a previous procedure. A prospective study of asymptomatic, average-risk, predominantly male Veteran Affairs healthcare population reported an advanced adenoma detection rate of 1.1% and no CRC cases in positive guaiac-FOBT patients following a normal colonoscopy within 5 years[12]. The study recommended a cut-off interval of 5 years for an asymptomatic average-risk screening population after a recent normal colonoscopy. Compared with our study, the prevalence of advanced adenoma was considerably lower in this cohort, as it only included an asymptomatic, average-risk patient population who had a previously normal colonoscopy. Our study also utilized iFOBT, which has greater sensitivity for detecting occult colonic bleeding, as compared with guaiac-FOBT.

Similarly, another study compared the prevalence of CRC and advanced neoplasia following positive iFOBT in average-risk, asymptomatic patients with or without an index colonoscopy, categorized

Table 3 Patients with colorectal cancer - previous and current colonoscopy findings

Patient	Gender	Age at current colonoscopy	Family history	Symptoms	Year of previous colonoscopy	Year of current colonoscopy	Quality of bowel preparation of previous colonoscopy	Quality of bowel preparation of current colonoscopy	Result of previous colonoscopy	Site of CRC	AJCC stage of CRC
1	Male	71	Nil	Nil	Oct 2012	May 2017	Good	Fair	Normal	Sigmoid colon	1
2	Male	59	Nil	Abdominal pain	2010	2017	Good	Good	Normal	Hepatic flexure	3B
3	Female	72	Nil	Nil	> 10 yr	2016	Unknown	Good	Unknown	Rectum	1
4	Female	72	Nil	Nil	> 10 yr	2019	Unknown	Good	Unknown	Cecum	1

CRC: Colorectal cancer; AJCC: American Joint Committee on Cancer.

Table 4 Diagnosis of advanced adenoma/sessile serrated lesion, advanced neoplasia and colorectal cancer as per time since previous colonoscopy

Total = 1051	Had a previous colonoscopy, *n* = 319				Never had or uncertain of previous colonoscopy, *n* = 732	
	0-4 yr, (*n* = 68)	4-5 yr (*n* = 37)	> 5 yr (*n* = 204)	Unsure when (*n* = 10)	Never (*n* = 685)	Unsure (*n* = 47)
AA-SSL	7 (10.3%)	7 (18.9%)	41 (20.1%)	1 (10%)	181 (26.4%)	19 (40.4%)
Advanced neoplasia	7 (10.3%)	8 (21.6%)	44 (21.6%)	1 (10%)	202 (29.5%)	19 (40.4%)
CRC	0	1 (2.7%)	3 (1.5%)	0	37 (5.4%)	1 (2.1%)

AA-SSL: Advanced adenoma/sessile serrated lesion; CRC: Colorectal cancer.

according to specific time frames following their previous procedure[13]. The prevalence of CRC in those without a previous colonoscopy, with a colonoscopy within 5 years and greater than 5 years were comparable with our study (5.7%, 0.3% and 1.2% respectively, compared with our study of 5.4%, 0.9% and 1.4%). After stratifying their results according to the severity of adenomas in the previous colonoscopy, the prevalence of advanced neoplasia was only 2.9% among patients who had low-risk adenomas detected within 5 years. They concluded that a colonoscopy should not be recommended within 5 years of a prior colonoscopy in average-risk patients with previous low-risk adenomas.

However, several studies have reported conflicting outcomes. Kim *et al*[14] reported 16 (2.1%) iFOBT positive patients were diagnosed with CRC after having an index colonoscopy within 3 years. Carrera *et al*[15] reported 3.8% of 157 guaiac-FOBT positive patients were diagnosed with CRC in second-round biennial screening after a negative colonoscopy. Similarly, a study revealed CRC was diagnosed in 0.4% (3 of 740) patients with positive guaiac-FOBT within 28 mo after their index negative colonoscopy[16]. A recent study by Peng *et al*[17] reported that the incidence of CRC following a negative colonoscopy was significantly lower in patients who recommenced iFOBT as compared to those who did not (incidence: 1.34 *vs* 2.69 per 1000 person years; adjusted OR = 0.47). Notably, of those who undertook iFOBT screening, the incidence of CRC was highest in those who had their subsequent iFOBT between 1.5 to 3 years, as compared to those performed 5 years or more (1.46 *vs* 1.08 per 1000 person years). While these studies demonstrated a benefit from undergoing colonoscopy within 3 years of the index procedure when presenting with a positive FOBT, the results are difficult to interpret as quality indicators of the index colonoscopy were not reported and these are key predictors of missed lesions[14-17]. The colonoscopies done at such short intervals were principally to detect missed or rapidly evolving lesions to compensate for the compromised effectiveness of a potentially inadequate quality index colonoscopy.

The latest consensus by the US Multi-Society Task Force on Colorectal Cancer is to offer colonoscopy following positive FOBT even if colonoscopy was performed recently; however, the recommendation was considered weak and the available quality of evidence low[6]. It recommended that the clinician considers the clinical context, such as presence or absence of symptoms of CRC, CRC risk factors such as family history, the quality and results of the index colonoscopy including the adequacy of bowel preparation, completion of procedure to the cecum and the proceduralist's adenoma detection and cecal intubation rates, and then balances this with the procedural risks of having another colonoscopy within

Table 5 Patients with advanced adenoma/sessile serrated lesion - previous and current colonoscopy findings

Patient	Gender	Age at current colonoscopy	Family history	Symptoms	Year of previous colonoscopy	Year of current colonoscopy	Quality of bowel preparation of previous colonoscopy	Quality of bowel preparation of current colonoscopy	Result of previous colonoscopy	Most advanced histology on current colonoscopy	Size of largest polyp (mm)
1	Male	74	Nil	Altered bowel pattern, abdominal pain	2012	2015	Unknown	Fair	Melanosis coli	Serrated adenoma	13
2	Female	70	Nil	Abdominal pain	2015	2017	Good	Good	Angioectasia	Tubular adenoma with LGD	10
3	Male	74	Nil	Nil	2016	2019	Fair	Excellent	Tubular adenomas × 4	Tubulovillous adenoma with LGD	20
4	Male	75	Nil	Nil	2016	2017	Poor	Fair	Tubular adenoma × 1	Tubulovillous adenoma with LGD	15
5	Male	71	Nil	Nil	2015	2018	Poor	Good	Normal	Tubular adenoma with LGD	10
6	Male	70	Nil	Nil	2012	2015	Unknown	Good	Unknown	Tubular adenoma with LGD	10
7	Male	58	Nil	Nil	2015	2018	Unknown	Fair	Unknown	Tubular adenoma with LGD	10

LGD: Low-grade dysplasia.

a short time frame.

Strengths and limitations

A high-quality colonoscopy is paramount in reducing the likelihood of missed lesions and interval CRC. A limitation of our study is that quality indicators of the previous colonoscopy such as the proceduralists' adenoma detection rate and assessment of bowel preparation were not available, thus may have impacted upon our findings and the likelihood of detecting advanced neoplasia on their current procedures. We were unable to retrieve a proportion of patients' index colonoscopy reports and hence could not make any conclusions on the important association of advanced lesions at the index colonoscopy with the current colonoscopy. Furthermore, due to the small number of CRC cases in patients with a prior colonoscopy, we were unable to report on the clinical predictors of CRC detection in this cohort. Additional studies assessing quality indicators and presence of advanced lesions of the index colonoscopy should be performed to determine predictors of interval lesions in patients with positive iFOBT following previous colonoscopy. Our study did not include patients who had a normal index colonoscopy but were subsequently diagnosed with interval CRC without iFOBT being performed. Further studies evaluating all CRCs diagnosed and reviewing colonoscopy findings and FOBT screening history may be worthwhile. Data on previous colonoscopy was obtained retrospec-

Table 6 Univariate and multivariate analyses of predictors of advanced neoplasia in the entire cohort

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Gender: Male	1.78	1.34-2.36	< 0.001	1.80	1.35-2.40	< 0.001
Increasing age (continuous variable)	1.04	1.02-1.06	< 0.001	1.04	1.02-1.06	< 0.001
Family history of CRC	1.07	0.68-1.68	0.77	2.07	1.49-2.87	< 0.001
No previous colonoscopy	1.83	1.39-2.52	< 0.001			
Aspirin use	0.96	0.58-1.60	0.89			
Diabetes	0.81	0.52-1.26	0.36			
Symptoms of CRC	0.90	0.68-1.19	0.65			

CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval.

tively, and patient recall was relied upon where procedure or histopathology reports were inaccessible, which may be subject to recall bias. In our study, two of the four patients with CRC detected on current colonoscopy recalled their prior procedures as more than 10 years earlier but the specific time interval was unable to be confirmed with procedure reports. Nevertheless, despite these limitations, this study represents a large cohort of patients in a "real-world" scenario, where healthcare provision is often fragmented, screening programs are centrally driven, and primary care physicians are not always involved with delivering or coordinating screening programs for their patients. Therefore, our study results are applicable within similar clinical settings, as our population of patients are of varying demographics and heterogenous risk profiles, therefore reflecting real-life clinical practice and improving the overall reproducibility of the study. Furthermore, the overall A-SSL detection rates, cecal intubation rates and bowel preparation quality exceeded the recommended level, further supporting the validity of this cohort as representative of a real-life population[8].

CONCLUSION

The decision to perform a colonoscopy following a positive iFOBT in a patient with a recent colonoscopy remains a challenging one. In our study, a previous colonoscopy, irrespective of its indication or findings, was associated with low prevalence of advanced neoplasia, and was protective against the detection of CRC if performed within 4 years of the positive iFOBT result. Our study suggests that a colonoscopy could be deferred following a positive iFOBT result for patients with a high-quality colonoscopy performed within 4 years. However, a colonoscopy should be repeated if there are concerns about the quality of the prior colonoscopy or presence of high-risk clinical features.

ARTICLE HIGHLIGHTS

Research background

There is currently minimal consensus to inform clinicians of the best approach to manage patients presenting with positive immunochemical fecal occult blood test (iFOBT) after having a recent colonoscopy. Repeating the colonoscopy within a short time frame may expose to the patient to unnecessary procedure-related risks, avoidable patient anxiety and a higher cost-burden on the healthcare system.

Research motivation

The primary motivation for this study was to determine the widest acceptable interval between consecutive colonoscopies that maintains patient safety through a reduction in colorectal cancer (CRC) incidence whilst optimizing healthcare resource utilization.

Research objectives

To determine the prevalence of CRC and advanced neoplasia in patients with a positive iFOBT after a recent colonoscopy, and clinical and endoscopic predictors for advanced neoplasia.

Research methods

This study included iFOBT-positive patients who were referred for a colonoscopy at a high-volume Australian tertiary referral center. Data was prospectively collected including demographics, quality indicators and results of current and previous colonoscopy. The main outcome was to determine the prevalence of CRC and advanced neoplasia in a patient with positive iFOBT who had a previous colonoscopy.

Research results

Of the 1051 patients included in the study, 319 (30.3%) had a previous colonoscopy. In this group, four patients were diagnosed with CRC. Among those who had a colonoscopy within four years, none were diagnosed with CRC and 7 had advanced adenomas/sessile serrated lesions. Of the 732 patients with no prior colonoscopy, there were 38 CRC (5.2%).

Research conclusions

Our study revealed that a previous colonoscopy, irrespective of its result, was associated with low prevalence of advanced neoplasia, and if performed within 4 years of a positive iFOBT result, was protective against CRC.

Research perspectives

Our study suggests that a colonoscopy could be deferred following a positive iFOBT result for patients who had a high-quality colonoscopy performed within 4 years. However, a colonoscopy should be repeated if there are concerns about the quality of the prior colonoscopy or presence of high-risk clinical features.

FOOTNOTES

Author contributions: Koo JH was the guarantor of the study; Koo JH, Bassan M, Abi-Hanna D, and Ng W designed the study; Rattan N, Willmann L, Aston D, George S, Anandabaskaran S, Ermerak G participated in the acquisition of the data; Koo JH, Rattan N, Willmann L and Ng W participated in the analysis and interpretation of the data; Rattan N drafted the initial manuscript; Koo JH, Bassan M, Abi-Hanna D and Ng W revised the article critically for important intellectual content; and all authors have read and approved the final manuscript.

Institutional review board statement: The local institution's Human Research and Ethics Committee approved the study (HREC/LNR/15/LPOOL/186).

Informed consent statement: Informed consent was waived.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

STROBE statement: The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of item.

Country/Territory of origin: Australia

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1808-1822

DOI: 10.4251/wjgo.v14.i9.1808

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Clinical implications of interleukins-31, 32, and 33 in gastric cancer

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

P-Reviewer: Kawabata H, Japan; Yakar M, Turkey

Received: February 28, 2022
Peer-review started: February 28, 2022
First decision: April 17, 2022
Revised: April 21, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality.

AIM

To determine whether interleukin (IL)-31, IL-32, and IL-33 can be used as biomarkers for the detection of GC, *via* evaluating the correlations between their expression and clinicopathological parameters of GC patients.

METHODS

Tissue array (n = 180) gastric specimens were utilised. IL-31, IL-32, and IL-33 expression in GC and non-GC tissues was detected immunohistochemically. The correlations between IL-31, IL-32, and IL-33 expression in GC and severity of clinicopathological parameters were evaluated. Survival curves were plotted using the Kaplan-Meier method/Cox regression. Circulating IL-31, IL-32, and IL-33 were detected by ELISA.

RESULTS

We found that the expression levels of IL-31, IL-32, and IL-33 were all lower in GC than in adjacent non-GC gastric tissues (P < 0.05). IL-33 in peripheral blood of GC patients was significantly lower than that of healthy individuals (1.50 ± 1.11 *vs* 9.61 ± 8.00 ng/mL, P <0.05). Decreased IL-31, IL-32, and IL-33 in GC were observed in younger patients (< 60 years), and IL-32 and IL-33 were lower in female patients (P < 0.05). Higher IL-32 correlated with a longer survival in two

GC subgroups: T4 invasion depth and TNM I-II stage. Univariate/multivariate analysis revealed that IL-32 was an independent prognostic factor for GC in the T4 stage subgroup. Circulating IL-33 was significantly lower in GC patients at TNM stage IV than in healthy people ($P < 0.05$).

CONCLUSION

Our findings may provide new insights into the roles of IL-31, IL-32, and IL-33 in the carcinogenesis of GC and demonstrate their relative usefulness as prognostic markers for GC. The underlying mechanism of IL-31, IL-32, and IL-33 actions in GC should be further explored.

Key Words: Diagnosis and therapy; Gastric cancer; Immune cell interactions; Interleukin-31; Interleukin32; Interleukin-33

Core Tip: Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality. This study aimed to determine whether interleukin (IL)-31, IL-32, and IL-33 can be used as biomarkers for the detection of GC, *via* evaluating the correlations between their expression and clinicopathological parameters of GC patients. IL-31, IL-32, and IL-33 expression in GC was correlated with the severity of clinicopathological parameters. Circulating IL-33 was significantly low in GC patients. Our findings may provide new insights into the roles of IL-31, IL-32, and IL-33 in the carcinogenesis of GC.

Citation: Liu QH, Zhang JW, Xia L, Wise SG, Hambly BD, Tao K, Bao SS. Clinical implications of interleukins-31, 32, and 33 in gastric cancer. *World J Gastrointest Oncol* 2022; 14(9): 1808-1822
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1808.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1808

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity (approximately 24%) and mortality (approximately 17%)[1] and is ranked third amongst malignant tumours[2]. Despite the more widespread use of recently developed diagnostic techniques, including endoscopic examination, many GC patients are diagnosed at advanced stages, resulting in a poor 5-year survival rate (< 20%). This emphasises the critical need for development of a reliable biomarker(s)[3] with high specificity and sensitivity, to improve the prediction of prognosis for more successful outcomes for GC patients. Endoscopic examination provides a useful approach in the early detection of GC and in reducing cancer-related mortality.

Immunity is critically important in inhibiting the development of malignancy[4], but the precise underlying mechanism concerning how host defence is involved in the oncogenesis of GC remains to be explored[5]. The role of pro-inflammatory and anti-inflammatory responses during the development of malignancy has been well established to be able to either stimulate or inhibit the growth of a cancer[4, 6]. The actions of the immune checkpoint molecules PD-1 and CTLA-4 have been elegantly demonstrated[7] to inhibit anti-cancer immunity during oncogenesis[8]. In addition, the molecular basis of carcinogenesis has also been studied within the gastrointestinal system[9]. Furthermore, a new classification of GC has recently been proposed based on subtype pathway clustering[10].

Helicobacter pylori (*H. pylori*), a spiral Gram-negative rod that infects and colonizes the human stomach in 50% of the human population, is a definite human oncogenic agent[11]. In addition, it has been suggested that *H. pylori* contributes to > 60% of all GCs, although the precise underlying mechanisms are complex[12]. It has been well illustrated by the Nobel laureate Barry Marshal that chronic gastric ulceration is caused by *H. pylori* infection, which can be eliminated by a cocktail of antibiotics[13]. It has been reported that the constitutive levels of interleukin 32 (IL-32) in both the gastric mucosa and GC tissue is upregulated in *H. pylori* infection[14]. Thus, it is reasonable to speculate that host immunity plays a critical role during the development of GC.

The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity is known to contribute to the establishment, proliferation, and metastasis of malignant tumours[15]. Although high host inflammatory status has been reported in the tumour microenvironment, an incompetent inflammatory/immune response will lead to tumour progression[16].

IL-31, an immunoregulatory cytokine secreted mainly by activated Th2 cells, plays a major role in the process of chronic inflammation[17]. However, the involvement of IL-31 in the pathogenesis of cancer is unclear. Recent studies have shown that malignant Tcells produce IL-31, with an associated increase in serum levels of IL-31[18]. Additionally, in the advanced stages of cutaneous T cell lymphoma, improved pruritus in patients correlates with lower levels of IL-31[19].

IL-32, a pro-inflammatory cytokine, is highly produced in several autoimmune diseases, *e.g.*, rheumatoid arthritis, inflammatory bowel disease, and atopic dermatitis[20,21]. However, by contrast with autoimmune and inflammatory diseases, the role of IL-32 appears to differ amongst different forms of cancer, *e.g.*, IL-32 exhibits anti-tumour effects in human colon cancer and leukaemia[22,23], however, it promotes tumorigenesis in human pancreatic cancers[24]. The role of IL-32 in GC is controversial, *i.e.*, one study found that IL-32 expression is elevated in GC compared with normal stomach tissue[14], while another study reported that there is no significant difference between GC and normal stomach tissue[25]. The precise role of IL-32 in tumorigenesis of GC and other malignancies remains to be fully explored. An additional controversial finding, however, has also reported that there is substantially reduced IL32 expression in the GC tissue of patients with the diffuse type of GC[26]. These divergent observations concerning IL-32 expression in GC may be due to different races and/or different tumour microenvironments.

IL-33, a member of the IL-1 family, regulates innate and adaptive immunity as a potent inducer of pro-inflammatory cytokines. The involvement of IL-33 in non-small cell lung cancer is controversial, *i.e.*, high IL-33 has been found to be of diagnostic and prognostic value[27], but another group has reported no significant associations[28]. The possible role of IL-33 in GC remains to be explored. IL-33 promotes GC invasion and migration *via* stimulating production of MMP-3 and IL-6 *in vitro*, using the ST2ERK1/2 pathway[29], which has been confirmed in a GC animal model by ablation of the cognate IL-33 receptor ST2[30]. *IL-33* mRNA expression is significantly higher in GC tissue compared to that of non-cancer tissue[31], suggesting that IL-33 promotes the development of GC. However, another controversial report failed to demonstrate an association between IL-33 and the overall 5-year survival rate[32].

In this study, we specifically assessed the relationships among IL-31, IL-32, and IL-33 in GC utilising the same cohort of patients. We aimed to identify the expression of IL-31, IL-32, and IL-33 in GC and assess their inter-correlations and clinical significance. Our data may provide useful information for both basic understanding of tumour immunology and/or therapeutic targets for GC patients.

MATERIALS AND METHODS

Patients and samples

GC tissues and adjacent histologically normal gastric tissues (control) were obtained from 180 GC patients undergoing subtotal gastrectomy at the Affiliated Hospital, Xuzhou Medical University, China between 2015 and 2020. None of these patients had a total gastrectomy. These GC patients were comprised of 140 males and 40 females, aged from 23 to 85 years. No chemotherapy was administered to these patients prior to subtotal gastrectomy. There were no cases of local recurrences within the stomach after subtotal gastrectomy among the 180 GC patients included in the study. Non-cancer tissues were also collected (n = 159), but did not include cases without a mucosal layer present under microscopic examination (n = 21). This study was approved by the Human Ethical Committee, the Institutional Review Boards of Affiliated Hospitals of Xuzhou Medical University.

Immunohistochemistry

Sections (5 μm) from tissue microarray blocks were labelled with three antibodies, as described previously[33]. The antibodies used are: Rabbit anti-IL-31 polyclonal antibody (22859-1-AP, Proteintech, China), rabbit anti-IL-32 polyclonal antibody (11079-1-AP, Proteintech), and rabbit anti-IL-33 polyclonal antibody (12372-1-AP, Proteintech, China). The dilution for all three antibodies was 1:100. A horseradish peroxidase-conjugated secondary antibody (12127A07, Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) was used. The specific target(s) were visualized with a DAB detection kit (Beijing Sequoia Jinqiao Biological Technology Co., Ltd.) and counterstained with hematoxylin.

Photomicrographs from each of the tissue arrays were taken with a fixed exposure time and colour balance to ensure consistency. IL-31, IL-32, and IL-33 production was quantified using ImagePro Plus9.1 (Media Cybernetic, Silver Spring, MD, United States), as described previously[34].

ELISA for IL-31, IL-32, and IL-33

To determine if there was a correlation between GC and circulating IL-31, IL-32, and IL-33, we enrolled prospectively ten GC patients prior to preoperative chemotherapy in the Affiliated Hospital, Xuzhou Medical University, China. Blood from ten healthy age and sex matched persons presenting for a routine health check-up were collected as controls. Consent was obtained from both GC patients and healthy controls. The circulating cytokine study was also approved by the Human Ethical Committee, the Institutional Review Boards of the Affiliated Hospitals of Xuzhou Medical University. Plasma samples were collected from subjects and stored at -80 °C until analysis. The concentrations of IL-31, IL-

32, and IL-33 were determined using an ELISA instrument (Bio-Rad 550, United States) at 450 nm, following the manufacturers' instructions for human IL-31 (KGEHC141, KeyGEN BioTECH, Nanjing, Jiangsu Province, China), IL-32 (SEB802Hu, Cloud-Clone Corp, Wuhan, Hubei Province, China) and IL-33 (KGEHC151, KeyGEN BioTECH). All samples were tested in duplicate.

Statistical analysis

GraphPad Prism 6.0 and SPSS 16.0 statistical software packages were used for the statistical analysis of the results of immunohistochemistry and ELISA. Comparison between two groups was performed *via* the Mann-Whitney *U*-test. Comparisons among multi-groups were performed *via* the Kruskal-Wallis test. Low and high cut-off values for cytokine expression were defined by receiver operating characteristic (ROC) curve analysis. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards model was used to identify the prognostic factors that influenced survival. $P < 0.05$ was considered statistically significant[35].

RESULTS

Baseline characteristics of patients

The detailed patients' information is presented in Table 1. Notably, there were four early GC patients, specifically stage T1 patients, among the 180 GC patients involved (Table 1). The management of patients after gastric resection uniformly followed the 2018 Chinese guidelines for diagnosis and treatment of GC, the National Health Commission of The People's Republic of China[36]. All patients had complete clinical information. Among them, 77 had follow-up until their death or until their most recent contact. The other patients were lost to follow-up (Figure 1). There were 42 cancerrelated deaths among the 77 patients (54.5%). Thus, amongst the 77 cases, 6 were stage I, and 32 were stage II.

Local expression of IL-31, IL-32, and IL-33 in GC tissue and in peripheral blood of GC patients

The expression levels of IL-31 (Figure 2A and B), IL-32 (Figure 2E and F), and IL-33 (Figure 1I and J) in GC tissue were investigated using immunohistochemistry. The densities of IL31 (Figure 1C), IL-32 (Figure 2G), and IL-33 (Figure 2K) are presented as box plots, including medians and 25th and 75th percentiles. IL-31, IL-32, and IL-33 were decreased by 9.4%, 28.2% and 27.5%, respectively, in GC compared to histologically normal adjacent gastric tissues ($P < 0.05$).

There was no significant difference in IL-31 (Figure 2D) or IL-32 (Figure 2H) concentration in the peripheral blood between GC patients and heathy controls. However, the mean value for IL-33 levels in peripheral blood of GC patients was 1.50 ± 1.11 ng/mL, which was significantly lower than that of healthy individuals (9.61 ± 8.00 ng/mL; $P < 0.05$) (Figure 2L).

Correlation between IL-31, IL-32, and IL-33 expression in GC and clinicopathological parameters

Associations between clinicopathological parameters and IL-31, IL-32, and IL-33 expression are listed in Table 1, Figures 3 and 4, and Supplementary Figures 1 and 2. All three ILs were associated with the age of GC patients (Figure 3A-D, IL-31; Figure 3E-H, IL-32; Figure 3I-L, IL-33). There was significantly lower expression of IL-31, IL-32, and IL-33 in the group of GC patients aged ≤ 60 years compared to the patients aged > 60 ($P < 0.05$). Significantly lower IL-32 (Figure 4A-D) and IL-33 (Figure 4E-H) expression was also observed in female GC patients compared to male GC patients ($P < 0.05$). However, no significant difference was observed in IL-31 expression when GC patients were stratified by sex (Supplementary Figure 2). Additionally, there were no correlations observed among IL31, IL-32, and IL-33 and other parameters, such as tumour size, lymph node metastasis, tumour differentiation, tumour invasion depth (Supplementary Figure 1), and TNM stage (Supplementary Figure 2) of GC.

Prognostic cytokines for overall survival of GC patients

To evaluate whether decreased IL-31, IL-32, and IL-33 correlate with survival of GC patients, low and high cut-off points for IL-31 (Figure 5A), IL-32 (Figure 5B), and IL-33 (Figure 5C) were defined by ROC curve analysis (Figure 5). The cut-off values for the three ILs were determined to be: IL-31, 1486000 AU; IL-32, 64893 AU; IL-33, 166291 AU. Kaplan-Meier survival curves were constructed to compare the survival of GC patient with high and low expression of IL-31 (Figure 5D), IL-32 (Figure 5E), and IL33 (Figure 5F). The data revealed that there were no correlations between IL-31, IL-32, and IL-33 expression and the prognosis of GC patients (Figure 4). However, Kaplan-Meier analysis was applied to further compare overall survival according to IL-31 (Figure 5G), IL-32 (Figure 5H), and IL33 (Figure 5I) expression in different subgroups of GC (Figure 5). Figure 4 shows that decreased IL-32 staining correlated with a significantly worse survival of patients in the TNM I-II stage subgroup ($P = 0.006$) (Figure 5K) and in the tumour invasion depth T4 subgroup ($P = 0.004$). There were no significant differences in the other clinicopathological subgroups of GC for IL-31, IL-32, and IL-33 (Supplementary Figures 3-5). Furthermore, there was no significant differences in the combination of IL-31, IL-32, and IL-33 expression for the prognosis of GC patients (Supplementary Figure 6).

Table 1 Correlations between interleukin-31, interleukin-32, and interleukin-33 expression and clinical/pathological features in patients with gastric cancer (*n* = 180)

Characteristic	Patient number	IL-31 median	*P* value	IL-32 median	*P* value	IL-33 median	*P* value
All cancer	180	1.333×10^6		99245		125998	
Noncancer (non)	159	1.472×10^6	0.043	138164	0.001	173818	< 0.0001
Gender							
Male	140	1.344×10^6		106075		143830	
Female	40	1.208×10^6	0.329	81009	0.040	89697	0.029
Age							
≤ 60	79	1.082×10^6		74098		106857	
> 60	101	1.404×10^6	0.007	122682	0.001	148615	0.026
Tumour size (diameter)							
< 5 cm	87	1.325×10^6		98583		122572	
≥ 5 cm	93	1.335×10^6	> 0.999	101583	> 0.999	126415	> 0.999
Lymph node metastasis							
No	75	1.404×10^6		106075		143359	
Yes	105	1.267×10^6	0.284	93196	0.671	113657	0.3
Differentiation							
High	14	1.609×10^6	H/M > 1	114379	H/M > 1	171038	H/M: > 0.999
Moderate	78	1.393×10^6	H/L: 0.6	113024	H/L> 1	142850	H/L: 0.2
Low	88	1.146×10^6	M/L: 0.3	91551	M/L: 0.4	104570	M/L: 0.1
Invasion depth							
T1	4	2.072×10^6		218529	T1/T3: 0.5, T1/T4: 0.6	156096	
T2	27	1.600×10^6		110353		143582	
T3	75	1.208×10^6		98367		116081	
T4	74	1.318×10^6	All > 1	96542	T1/T2, T2/T3, T2/T4, T3/T4, all > 1	125941	All > 1
TNM							
I	12	1.355×10^6		98583		142117	I/IV: 0.8
II	70	1.414×10^6		113560	II/ IV: 0.6	147031	II/III: 0.3, II/IV: 0.1
III	92	1.288×10^6		87667		107919	III/IV: 0.7
IV	6	0.950×10^6	All > 1	54851	I/II, I/III, I/IV, II/ III, III/IV, all > 1	52195	I/II, I/III>1

IL: Interleukin.

Correlation of IL-32 with overall survival in subgroups of GC patients

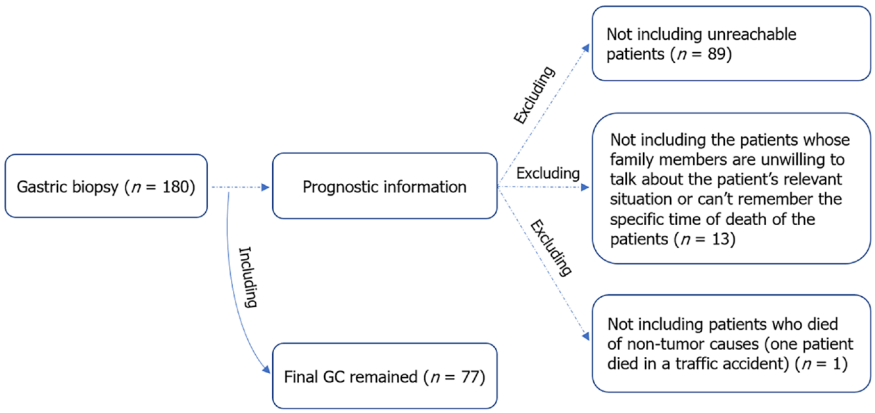
Univariate and multivariate Cox regression analyses were used to examine whether IL-32 is an independent prognostic marker for subgroups of GC patients, including IL32 expression level, age, sex, tumour differentiation, lymph node invasion, tumour size, depth of tumour invasion, and TNM stage.

Data from patients within the T4 stage subgroup, analysed by univariate analysis, exhibited a correlation between the survival of GC patients and IL-32 expression and TNM stage. In multivariate analysis, IL-32 expression and TNM stage remained as significant independent prognostic factors for survival of GC patients (Table 2).

Furthermore, decreased survival of GC patients in the TNM I-II stage subgroup was found to correlate with lymph node metastasis and tumour size on univariate analysis, but not on multivariate analysis. However, both univariate and multivariate analyses revealed no significant correlations

Table 2 Univariate and multivariate analyses of clinicopathological factors affecting survival of patients with gastric cancer at T4 stage

Variables analysis	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
IL-32 (low/high)	4.338 (1.450-12.980)	0.009	3.287 (1.024-10.555)	0.046
Tumour differentiation (low/moderate)	0.710 (0.225-2.237)	0.559		
TNM		0.008		0.037
IV (reference)	1		1	
II	0.034 (0.003-0.423)	0.008	0.069 (0.005-0.946)	0.045
III	0.203 (0.018-0.464)	0.004	0.127 (0.025-0.646)	0.013
Lymph node metastasis (no/yes)	0.441 (0.098-1.982)	0.285		
Diameter (< 5/≥ 5, cm)	0.475 (0.161-1.404)	0.178		
Female/male	0.912 (0.323-2.573)	0.862		
Age (≤ 60/> 60)	1.950 (0.688-5.529)	0.209		



DOI: 10.4251/wjgo.v14.i9.1808

Figure 1 Flow chart for recruitment of gastric cancer patients. GC: Gastric cancer.

between decreased IL-32 expression and survival of GC patients in the TNM I-II stage subgroup of GC patients (Table 3).

DISCUSSION

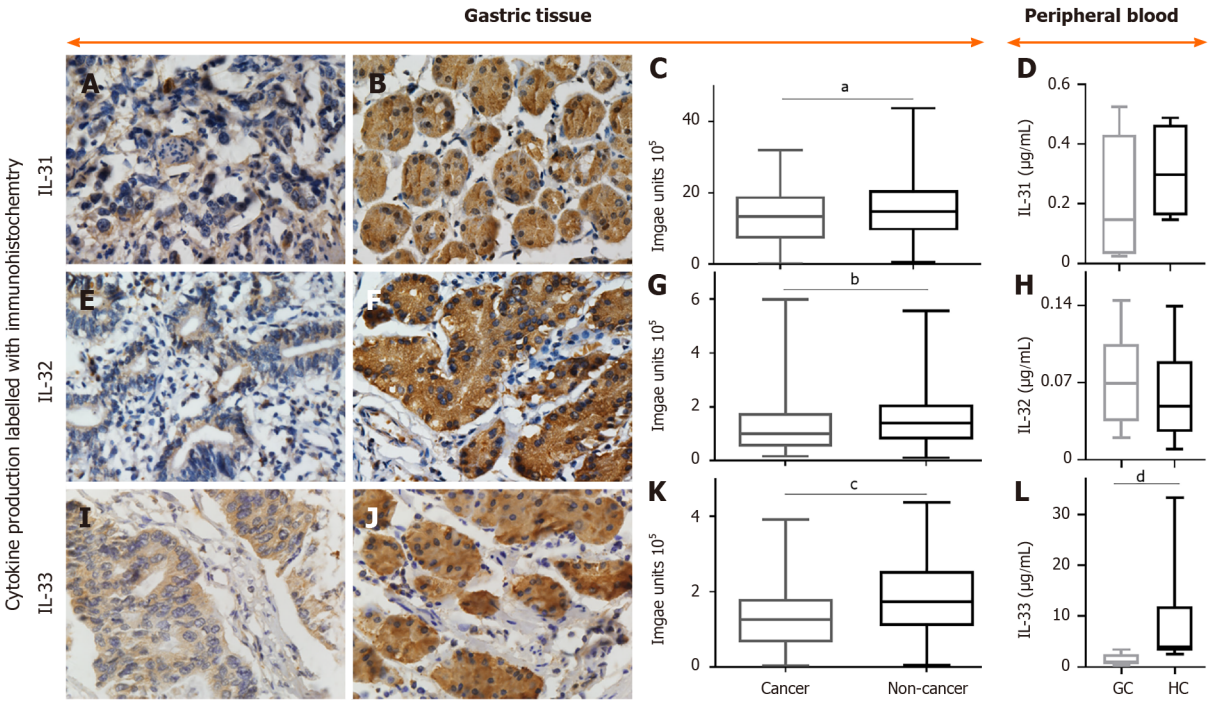
The current study demonstrated that the levels of expression of IL-31, IL-32, and IL-33 were all decreased in GC tissue compared to adjacent non-cancer gastric tissue and that the extent of these reductions in expression was higher in younger patients below the age of 60 years. Additionally, in the case of IL-32 and IL-33, their expression was found to be lower in females compared to males. However, the levels of expression of all three ILs amongst all the GC patients as a group did not correlate with a survival benefit, although subgroup analysis did reveal a survival benefit associated with higher levels of expression of IL-32 in the T4 stage and the TNM I-II stage subgroups.

H. pylori, a spiral Gram-negative rod that infects the human stomach in 50% of humans, is a definite human oncogenic agent[11], consistent with the previous finding that *H. pylori* contributed to > 60% of all GCs[12]. It has been clearly demonstrated by the Nobel laureate Barry Marshal that chronic gastric ulceration is caused by *H. pylori* infection[13]. The constitutive level of IL-32 is upregulated in both the gastric mucosa and GC tissue infected with *H. pylori*[14]. The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity contributes to the establishment, proliferation, and metastasis of malignant tumours[15], a concept that is further supported by others who have shown that incompetent inflammation/immunity leads to tumour progression[16].

IL-31, an immunoregulatory cytokine secreted mainly by activated Th2 cells, plays a major role in the process of chronic inflammation[17]. However, the involvement of IL31 in the pathogenesis of cancer is unclear. Malignant Tcells produce IL-31, consistent with increased circulating IL-31[18]. Additionally, in the advanced stages of cutaneous T cell lymphoma, improved pruritus in patients correlates with lower

Table 3 Univariate and multivariate analyses of clinicopathological factors affecting survival of patients with gastric cancer in TNM I-II stage

Variable	Univariate HR (95%CI)	*P* value	Multivariate HR (95%CI)	*P* value
IL-32 (low/high)	0.180 (0.024-1.370)	0.098		
Tumour differentiation		0.947		
Low (reference)	1			
High	1.259 (0.258-6.133)	0.776		
Moderate	0.964 (0.324-2.871)	0.947		
Tumour invasion depth		0.546		
T4 (reference)	1			
T2	0.567 (0.059-5.491)	0.624		
T3	1.460 (0.187-11.379)	0.718		
Lymph node metastasis (no/yes)	0.307 (0.108-0.868)	0.026	0.490 (0.152-1.578)	0.232
Diameter (< 5/≥ 5, cm)	0.259 (0.092-731)	0.011	0.368 (0.112-1.165)	0.088
Female/male	0.522 (0.116-2.340)	0.396		
Age (≤ 60/> 60)	0.562 (0.192-1.646)	0.293		

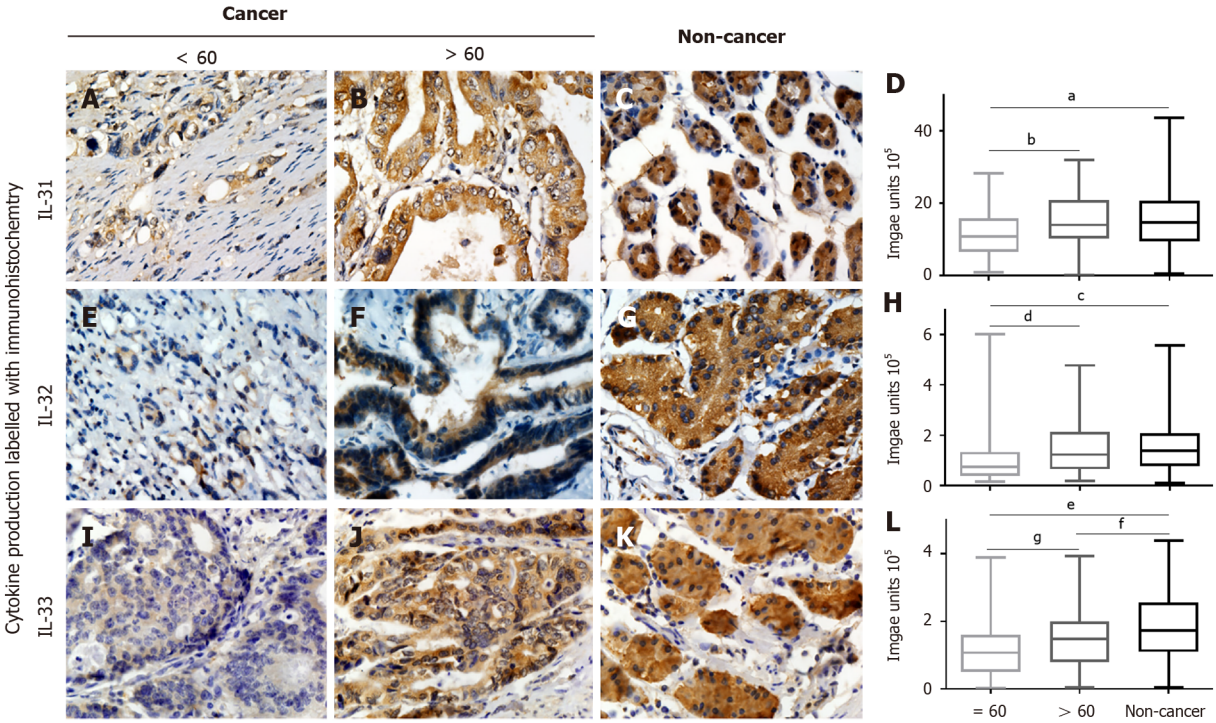


DOI: 10.4251/wjgo.v14.i9.1808

Figure 2 Representative images of immunohistochemical staining for interleukin-31, interleukin-32, and interleukin-33 and their densities in non-cancerous and gastric cancer tissues, as well as their levels in peripheral blood of gastric cancer patients and healthy individuals. A-C: Positive (brown) interleukin (IL)-31 expression in gastric cancer (A) and noncancerous tissues (B) and quantified data (C); D, H, and L: IL-31 (D), IL-32 (H), and IL-33 (L) levels in peripheral blood from gastric cancer (GC) patients and healthy controls (HC); E-G: Positive IL-32 expression in gastric cancer (E) and noncancerous tissues (F) and quantified data (G); I-K: Positive IL-33 expression in gastric cancer (I) and noncancerous tissues (J) and quantified data (K). The densities of IL-31 and IL-33 were all decreased in GC compared to tumour-adjacent normal gastric tissues. Magnification, 600 ×. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.0001$; $^{d}P < 0.05$. GC: Gastric cancer; IL: Interleukin.

levels of IL-31[19].

We found decreased IL-31 in GC patients, particularly in younger patients. Our data are consistent with other studies that have shown that younger patients are more likely to have more poorly differentiated tumours compared to older patients with GC, suggesting that younger GC patients have more malignant types of GC[37]. The activity of IL-31 is mediated through the IL31 receptor A (IL-31RA) and



DOI: 10.4251/wjgo.v14.i9.1808

Figure 3 Correlation of interleukin-31, interleukin-32, and interleukin-33 expression with age. A-D: Positive (brown) interleukin (IL)-31 expression in gastric cancer tissues from patients aged < 60 (A) *vs* > 60 (B) years and noncancerous tissues (C) plus quantified data (D); E-H: Positive (brown) IL-32 expression in gastric cancer tissues from patients aged < 60 (E) *vs* > 60 (F) years and noncancerous tissues (G), and quantified data (H); I-L: Positive (brown) IL33 expression in gastric cancer tissues from patients aged < 60 (I) *vs* > 60 (J) years and noncancerous tissues (K) plus quantified data (L). IL-32 and IL-33 were all decreased in the group of gastric cancer patients aged less than or equal to 60 years. $^{a}P < 0.01$; $^{b}P < 0.01$; $^{c}P < 0.0001$; $^{d}P < 0.01$; $^{e}P < 0.0001$; $^{f}P < 0.05$; $^{g}P < 0.05$. IL: Interleukin.

the oncostatin M receptor[38,39]. The two different isoforms of the IL-31RA consist of either long (745 residues) or short (560 residues) isoforms which may induce contrary functions[40]. Proliferation of follicular lymphoma is enhanced *via* the long IL-31RA isoform, whereas germinal centre-derived B-cell malignancy is inhibited *via* the short IL-31RA isoform[41]. There is no direct evidence available that identifies which isoform/s of IL-31RA are activated in GC *via* the IL-31 signalling pathway. However, our data are consistent with the hypothesis that IL-31 mediates an anti-cancer role in GC through the short IL-31RA isoform.

The involvement of IL-33 in non-small cell lung cancer is controversial, *i.e.*, high IL33 has been found to be of diagnostic and prognostic value[27], but another report shows no significant associations[28] between IL-33 and the overall 5-year survival rate[32]. IL-33 promotes GC invasion/migration *via* stimulating MMP-3 and IL6 *in vitro*[29], which has been confirmed in a GC animal model by ablation of the cognate IL-33 receptor ST2[30]. *IL-33* mRNA is significantly higher in GC tissue compared to that of non-cancer tissue[31], suggesting that IL-33 promotes the development of GC.

We observed similar levels of expression of IL-31 and IL-33 in GC, with decreased IL33 in both younger GC patients and in female GC patients, which is consistent with data from others, who have shown that female sex is a significant factor for predicting a higher likelihood of lymph node metastasis in mucosa-confined, poorly differentiated GC[42]. IL-33 is a multifunctional cytokine that can bind to the IL-33 receptor (ST2), to regulate immunity *via* activating Th1 cells, Th2 cells, $CD8^+$ T cells, and NK cells[43,44]. There are two forms of ST2: The transmembrane form ST2L that when bound to IL-33, is able to activate target cells[45], and the soluble, secreted form of ST2 (sST2) that acts as a decoy receptor and negatively regulates IL-33 signalling[46]. The possible role of IL33 in carcinogenesis has been demonstrated in an IL-33 transgenic mouse metastasis model, demonstrating inhibition of the growth and metastasis of B16 melanoma and Lewis lung carcinoma cells, *via* activating $CD8^+$ T cells and NK cells[47]. Thus, these data may be useful for future therapeutic design, utilising the anti-cancer role of IL-33 in GC.

IL-32, a proinflammatory cytokine, is highly expressed in several autoimmune diseases, *e.g.*, rheumatoid arthritis, inflammatory bowel disease, and atopic dermatitis[20,21]. However, the role of IL-32 appears to vary amongst different forms of cancer, *e.g.*, IL32 has been reported to inhibit colon cancer and leukaemia[22,23], but promotes pancreatic cancer[24]. The role of IL-32 in GC is also controversial, *i.e.*, IL-32 is elevated in GC compared with normal stomach tissue[14], but other groups have found either substantially reduced IL32 expression in GC for the diffuse type of GC[26], or no significant difference has been observed between GC and normal stomach tissue[25]. These divergent observations

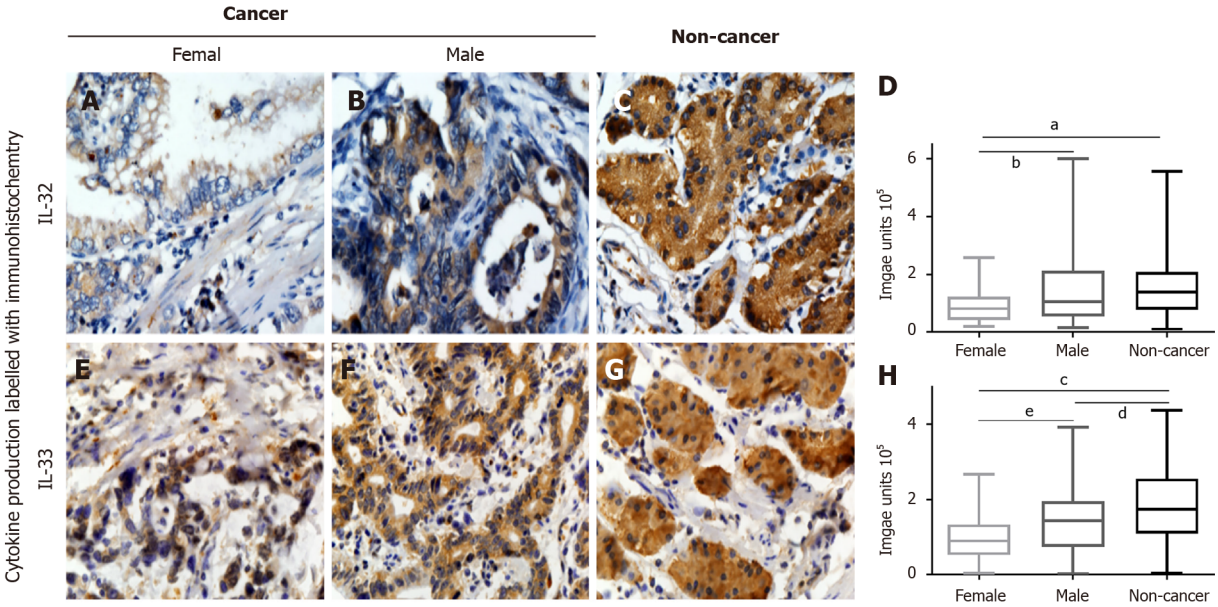


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Figure 4 Correlation of interleukin-32 and interleukin-33 expression with sex. A-D: Positive (brown) interleukin (IL)-32 expression in gastric cancer tissues from female (A) *vs* male (B) patients and noncancerous tissues (C) plus quantified data (D); E-H: Positive (brown) IL-33 expression in gastric cancer tissues from female (E) *vs* male (F) patients and noncancerous tissues (G) plus quantified data (H). IL-32 and IL-33 both decreased in female patients with gastric cancer. [a]*P* < 0.001; [b]*P* < 0.05; [c]*P* < 0.0001; [d]*P* < 0.01; [e]*P* < 0.05. IL: Interleukin.

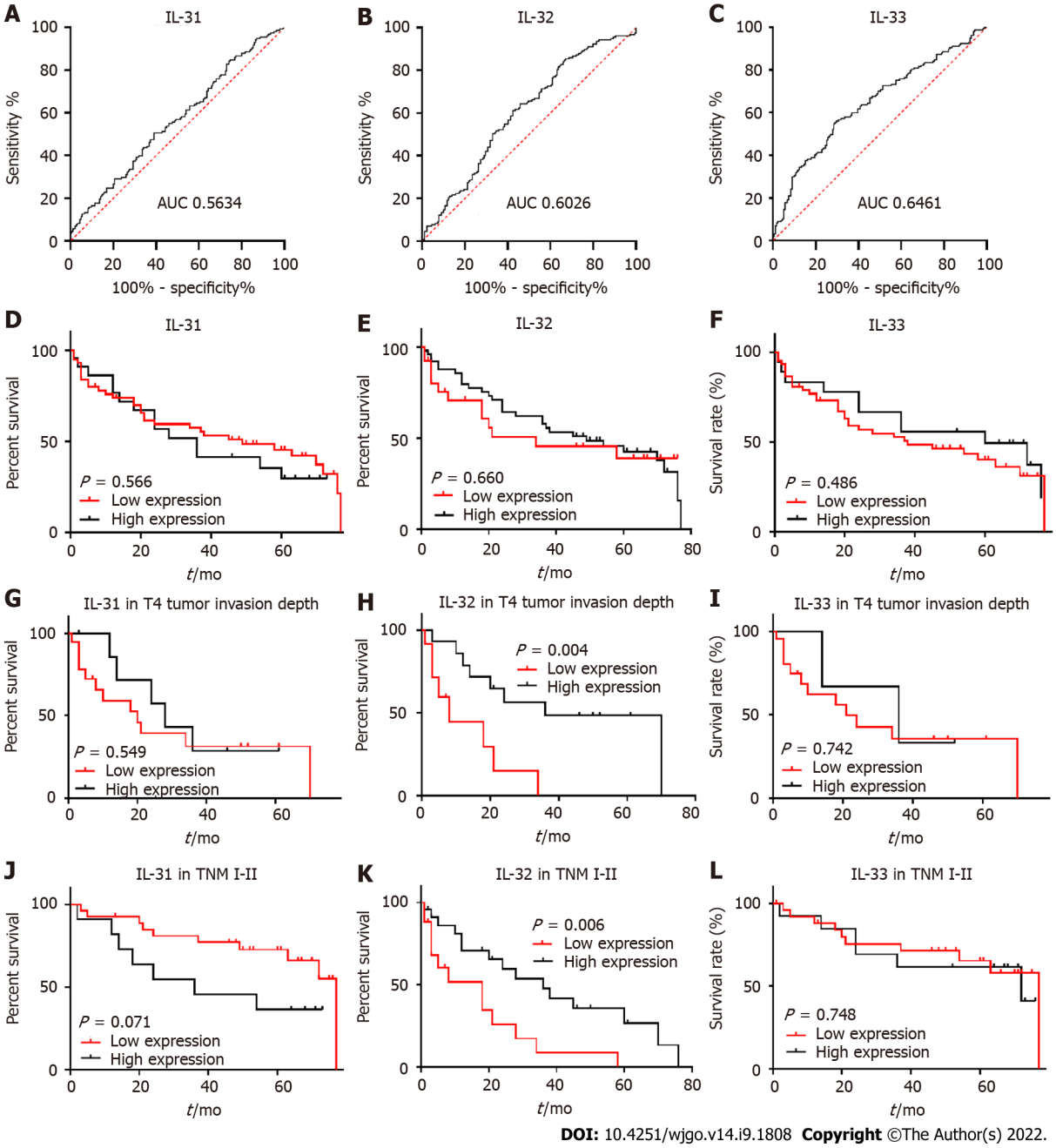
concerning IL32 expression in GC may be due to different races and/or different tumour microenvironments.

We found that the expression of IL-32 was decreased in both younger patients and in female patients with GC, consistent with more severe forms of GC in younger and female patients, suggesting that IL-32 may mediate host defence against the development of GC. Furthermore, we found that high IL-32 expression correlated with a longer survival of GC patients, in the T4 stage and TNM I-II stage subgroups and that IL32 was an independent prognostic factor for survival in the T4 stage subgroup. Interestingly, the IL-32 positive rate in GC (12%) has been reported to be much lower than the rate in oesophageal squamous cell carcinoma (60%), but no comparison to non-cancerous tissues has been made[48,49]. Thus, we propose a hypothesis for the possible mechanism of IL-32 involvement in carcinogenesis as follows: Because IL-32 contributes to the host defence *via* enhancing differentiation of monocytes into macrophages[50], decreased IL-32 in GC tissue, seen particularly amongst the younger or female patients, may compromise host innate immunity, and subsequently contribute to poorly controlled development of cancer. Notably, macrophages are classified as either classical M1 macrophages that promote the inflammatory response against microorganism invasion and are thought to inhibit carcinogenesis, or as M2 macrophages that regulate host immunity and are thought to promote carcinogenesis[51]. It remains to be clarified whether tumourassociated macrophages in GC are derived from one subset or the other, which either promote the development of cancer (M2) or suppress cancer growth (M1), which is perhaps dependent on the tumour microenvironment[52]. For example, IL-32 can induce cell death in thyroid cancer cells through the induction of IL-8 and caspase-8[53], subsequently up-regulating the proinflammatory response.

IL-32 may also be able to inhibit tumour growth indirectly, hence it may be efficacious as a clinical anti-cancer therapy[54]. For example, the application of siRNA to inhibit IL-32 enhances angiogenesis in HUVECs[55] *via* up-regulation of VEGF and PDGF. Our current findings showed an inverse correlation between IL-32 and the development of GC, suggesting that IL-32 inhibits the development of cancer directly and/or indirectly, which will be further investigated in future experiments.

Finally, the levels of circulating IL-31, IL-32, and IL-33 were found to be consistent with their respective expression levels in GC tissue, further supporting the relevance of the potential role for these cytokines in mediating tumour-related immunity. However, we hypothesise that the host systemic and/or local inflammatory/immune response may be insufficient to inhibit the development of GC, among the GC cohorts studied, leading to tumour progression[16].

Unfortunately, no correlation with survival of GC patients was observed among any combination of IL-31, IL-32, and IL-33 expression, a similar result that we have reported previously for the relationship with IL-34 in GC[35]. The current observations are consistent with others, showing that there is no significant correlation between IL-33 expression and overall survival[32]. However, the advantage of our current data is the analysis for the combined IL-31, IL-32, and IL-33 data, to determine the correlation with GC patients from the same cohort. It remains to be explored why there is a discrepancy



DOI: 10.4251/wjgo.v14.i9.1808

Figure 5 Receiver operating characteristic curves, correlation of interleukin-31, interleukin-32, and interleukin-33 with prognosis of gastric cancer, and subgroup analysis for patients at T4 stage and TNM stage I-II. A-C: The specificity (X-axis) *vs* sensitivity (Y-axis) of interleukin (IL)-31 (A), IL-32 (B), and IL-33 (C); D-F: Comparison of 5-year survival rate between patients with high and low IL-31 (D), IL-32 (E), and IL-33 (F) expression; G-I: Comparison of 5-year survival rate between patients with high and low IL-31 (G), IL-32 (H), and IL-33 (I) expression in the T4 subgroup; J-L: Comparison of 5-year survival rate between patients with high and low IL-31 (J), IL-32 (K), abd IL-33 (L) expression in the TNM stage 2 subgroup. ROC curves analysis displayed the poor diagnostic potential of IL-31, IL-32, and IL-33 expression for GC. The cut-off point and area under the curve were: IL-31: 1.486 × 10^6, area under the curve (AUC) = 0.563; IL-32: 64893, AUC = 0.603; IL-33: 166291, AUC = 0.646. Kaplan-Meier survival analysis of GC patients showed that decreased IL-32 expression correlated with a poor survival of GC patients in the T4 and TNM I-II subgroups. IL: Interleukin; AUC: Area under the curve.

among IL-31, IL-32, IL-33, and IL-34 during the development of GC, which may be due to different receptors and/or signalling pathways, which will be clarified in the conditioning knockout mice in future studies.

There are some limitations for the current study. First, the number of GC patients and normal individuals who were sampled was rather small for the evaluation of circulating cytokines, using ELISA. However, this pilot study was undertaken to simply provide proof of concept that a systemic response is involved compared to only local cytokine expression in the affected gastric tissues, as well as to support our immunohistochemistry findings. A study with a larger sample size and a range of different backgrounds will be performed in the future.

Second, the stomach tissue of normal healthy people would be the ideal control for GC for comparison, and would offer more convincing evidence. However, we were unable to collect any normal healthy stomach tissue due to ethical issues. We are applying for human ethics approval for the collection of normal healthy stomach tissue from organ donors in the future.

The GC patient cohort recruited for this study was initially set at a reasonable size, *i.e.*, 180 in total, to establish sufficient power to detect clinically relevant differences in the expression levels of the ILs that we examined. Regrettably, more than half of the patients were lost to follow-up during the course of the study, and only 77 GC patients had complete followup data (Figure 5). The data in relation to expression levels were based on all 180 patient samples that were initially recruited to ensure that the study was sufficiently powered to detect the potential role of IL31, IL32, and IL33 during the development of GC. If we had only selected the 77 GC patients with complete follow-up data for all aspects of this study, we would be highly likely to lose some important information and/or statistical power in exploring the correlation of these cytokines with clinical presentations. However, the survival analysis could only be performed on the adequately followed sub-cohort of 77 patients. We are currently collecting more samples with a full history and complete follow-up data in collaboration with other institutes, *i.e.*, a larger number of samples for more convincing information for our future studies.

Because there was no local recurrence of GC within the current cohort, we cannot explore the potential role of these cytokines in the prediction of local recurrence of GC. We are currently searching for both primary and recurrent GC cases for future study.

CONCLUSION

In summary, our data demonstrate that IL-31, IL-32, and IL-33 expression in GC is all decreased, which correlates with younger age of the GC patients. IL-32 and IL-33 also correlate with the sex of the GC patients. Decreased IL-32 correlates with a poorer survival of GC patients in the T4 stage and TNM I-II stage subgroups. Downregulation of IL-32 is an independent prognostic factor for survival of T4 GC patients. Finally, low IL-33 in peripheral blood may be considered as an objective predictive marker for the development of GC. However, further studies are required to investigate the mechanism of action of these ILs in GC.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is one of the most common malignancies in China with a high morbidity and mortality. Despite the more widespread use of recent diagnostic techniques, including endoscopic examination, many GC patients are diagnosed at advanced stages, resulting in a poor 5-year survival rate, emphasizing the critical need for development of a reliable biomarker(s) with high specificity and sensitivity to improve the prediction of prognosis for more successful outcomes for GC patients. Endoscopic examination provides a useful approach in the early detection of GC, and in reducing cancer-related mortality.

Research motivation

The cell-mediated immune response is extremely important in defence against tumour development, since compromised host immunity is known to contribute to the establishment, proliferation, and metastasis of malignant tumours. Although high host inflammatory status has been reported in the tumour microenvironment, an incompetent inflammatory/immune response will lead to tumour progression.

Research objectives

We aimed to identify the expression of interleukin (IL)-31, IL-32, and IL-33 in GC and assess their inter-correlation and clinical significance.

Research methods

GC tissues were obtained from patients without local recurrences for immunohistochemistry to determine the expression of IL-31, IL-32, and IL-33. Additionally, circulating levels of IL-31, 32, 33 were determined using ELISA. The Mann-Whitney *U* test or the Kruskal-Wallis test was used for statistical analysis.

Research results

IL-31, IL-32, and IL-33 expression was all lower in GC than in adjacent non-cancer gastric tissues ($P < 0.05$). IL-33 level in peripheral blood of GC patients was significantly lower than that of healthy

individuals (1.50 ± 1.11 *vs* 9.61 ± 8.00 ng/mL, ($P < 0.05$). Decreased IL-31, IL-32, and IL-33 expression in GC was observed in younger patients (< 60 years), and IL-32 and IL-33 expression was lower in female patients ($P < 0.05$). Higher IL-32 expression correlated with a longer survival in two GC subgroups: T4 invasion depth and TNM stage I-II. Univariate/multivariate analysis revealed that IL-32 was an independent prognostic factor for GC in the T4 stage subgroup. Circulating IL-33 was significantly lower in GC patients at TNM stage IV than in healthy people ($P < 0.05$).

Research conclusions

IL-31, IL-32, and IL-33 expression in GC is all decreased, which correlates with younger age of the GC patients. IL-32 and IL-33 expression also correlates with the sex of the GC patients. Decreased IL-32 correlates with a poorer survival of GC patients in the T4 stage and TNM stage I-II subgroups. Down-regulation of IL-32 is an independent prognostic factor for survival of T4 GC patients. Finally, low IL-33 in peripheral blood may be considered as an objective predictive marker for the development of GC.

Research perspectives

Further studies are required to investigate the mechanism of action of these ILs in GC.

ACKNOWLEDGEMENTS

We acknowledge the staff from the Department of Pathology, Xuzhou Medical University for their support.

FOOTNOTES

Author contributions: Liu QH collected the samples, performed the histopathological and immunohistochemical examinations, analysed the data, and wrote the paper; Zhang JW collected the samples, analysed the data, and wrote the paper; Liu QH and Zhang JW contributed equally to the study; Xia L performed ELISA and data analysis; Wise SG provided intellectual input; Hambly BD and Tao K revised the manuscript and provided intellectual input; Bao SS designed the experiment and revised the manuscript.

Supported by the National Natural Science Foundation of China, No. 81502030.

Institutional review board statement: This study was approved by the Human Ethics Committee, the Institutional Review Boards of Affiliated Hospital of Xuzhou Medical University and conducted in accordance with the Declaration of Helsinki.

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

Conflict-of-interest statement: The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

Data sharing statement: The data can be available upon request.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

Country/Territory of origin: China

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S-Editor: Yan JP
L-Editor: Wang TQ
P-Editor: Li X

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1823-1832

DOI: 10.4251/wjgo.v14.i9.1823

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Construction and analysis of an ulcer risk prediction model after endoscopic submucosal dissection for early gastric cancer

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Specialty type: Gastroenterology and hepatology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Albillos A, Spain; McDowell HR, United Kingdom; Shroff RT, United States

Received: April 19, 2022
Peer-review started: April 19, 2022
First decision: May 11, 2022
Revised: May 14, 2022
Accepted: August 14, 2022
Article in press: August 14, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Endoscopic submucosal dissection (ESD) has been widely used in the treatment of early gastric cancer (EGC). A personalized and effective prediction method for ESD with EGC is urgently needed.

AIM

To construct a risk prediction model for ulcers after ESD for EGC based on LASSO regression.

METHODS

A total of 196 patients with EGC who received ESD treatment were prospectively selected as the research subjects and followed up for one month. They were divided into an ulcer group and a non-ulcer group according to whether ulcers occurred. The general data, pathology, and endoscopic characteristics of the groups were compared, and the best risk predictor subsets were screened by LASSO regression and tenfold cross-validation. Multivariate logistic regression was applied to analyze the risk factors for ulcers after ESD in patients with EGC. A receiver operating characteristic (ROC) curve was used to estimate the predictive model performance.

RESULTS

One month after the operation, no patient was lost to follow-up. The incidence of ulcers was 20.41% (40/196) (ulcer group), and the incidence of no ulcers was

79.59% (156/196) (non-ulcer group). There were statistically significant differences in the course of disease, *Helicobacter pylori* infection history, smoking history, tumor number, clopidogrel medication history, lesion diameter, infiltration depth, convergent folds, and mucosal discoloration between the groups. Gray's medication history, lesion diameter, convergent folds, and mucosal discoloration, which were the 4 nonzero regression coefficients, were screened by LASSO regression analysis. Further multivariate logistic analysis showed that lesion diameter [Odds ratios (OR) = 30.490, 95%CI: 8.584-108.294], convergent folds (OR = 3.860, 95%CI: 1.060-14.055), mucosal discoloration (OR = 3.191, 95%CI: 1.016-10.021), and history of clopidogrel (OR = 3.554, 95%CI: 1.009-12.515) were independent risk factors for ulcers after ESD in patients with EGC ($P < 0.05$). The ROC curve showed that the area under the curve of the risk prediction model for ulcers after ESD in patients with EGC was 0.944 (95%CI: 0.902-0.972).

CONCLUSION

Clopidogrel medication history, lesion diameter, convergent folds, and mucosal discoloration can predict the occurrence of ulcers after ESD in patients with EGC.

Key Words: Endoscopic submucosal dissection; Early gastric cancer; Endoscopic features; Ulcer; Model

Core Tip: In recent years, with the development of endoscopic techniques, endoscopic submucosal dissection (ESD) has been widely used in the treatment of early gastric cancer (EGC). Nevertheless, it is difficult to determine the presence of histological ulcers before ESD, and the presence of ulcers in EGCs is closely related to their depth of invasion and lymphatic invasion. In this study, we aimed to build a personalized prediction model for EGC patients after ESD.

Citation: Gong SD, Li H, Xie YB, Wang XH. Construction and analysis of an ulcer risk prediction model after endoscopic submucosal dissection for early gastric cancer. *World J Gastrointest Oncol* 2022; 14(9): 1823-1832
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1823.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1823

INTRODUCTION

Gastric cancer is a common, widespread cancer. According to the "2020 Latest Global Cancer Burden" released by the World Health Organization, there were 1.089 million new gastric cancer cases and 768000 deaths worldwide, of which 478000 new cases and 373000 deaths were in China, accounting for nearly half of the cases, equivalent to 1022 Chinese people dying every day due to gastric cancer[1]. The prognosis of early gastric cancer is significantly better than that of advanced gastric cancer due to the low rate of lymphatic metastasis and distant metastasis[2].

In recent years, with the development of endoscopic techniques, endoscopic submucosal dissection (ESD) has been widely used in the treatment of early gastric cancer (EGC)[3,4]. Compared to previous treatments, the scope of ESD treatment is expanded, the resection rate is improved, the residual lesion is reduced, the recurrence rate is reduced, and the cure rate of digestive tract lesions is improved[5]. Therefore, ESD is currently the main endoscopic resection treatment for early gastric cancer; however, due to the wide range of ESD peeling, deep lesion peeling, difficult operations, and relatively high risk of complications such as bleeding and perforation[6-8], personalized and effective methods to predict the outcome are urgently needed in clinical practice.

The Japanese Gastric Cancer Association proposed that the absolute indications for ESD for EGC radical resection initially included non-ulcerative, well-differentiated mucosal lesions ≤ 2 cm in diameter. However, the absolute indications are so strict that unnecessary surgery may be performed. Subsequently, after a rigorous investigation of surgical specimens, the indications for ESD were expanded to include a larger diameter, undifferentiated mucosal lesions, and differentiated lesions with mild submucosal infiltration[9,10].

A recent meta-analysis showed that the postoperative ulcer risk was relatively low in patients who met the absolute indications, suggesting that if radical endoscopic dissection is accurately predicted based on histopathology, it may be possible to avoid intraoperative specimen excision[11]. Nevertheless, it is difficult to determine the presence of histological ulcers before ESD, and the presence of ulcers in EGCs is closely related to their depth of invasion and lymphatic invasion. Ruptures are considered ulcers, which undoubtedly overestimate the disease and lead to unnecessary surgery[12,13]. In addition, an endoscopy study reported that EGC ulcers might heal spontaneously without mucosal rupture. The

presence of an ulcer is critical in deciding on the treatment modality[14].

In our study, LASSO regression was performed to screen the factors influencing the risk of ulcers in EGC patients after ESD. Based on the differential indicators, we aimed to build a personalized prediction model that may provide a theoretical basis for the prevention of ulcers in EGC patients after ESD.

MATERIALS AND METHODS

Study subjects

This study was approved by the ethics committee of the hospital. After signed informed consent was obtained, 196 EGC patients who received ESD treatment in our hospital from March 2019 to March 2021 were enrolled in our study. The inclusion criteria were as follows: (1) Met the diagnostic criteria for early gastric cancer confirmed by pathological examination; (2) the depth of invasion was limited to the mucosa and submucosa without lymph node metastasis; and (3) all patients provided informed consent and signed the consent form. The exclusion criteria were as follows: (1) gastric cancer combined with tumors in other parts; (2) epithelial tumor, adenocarcinoma or gastric adenoma; and (3) received radiotherapy, chemotherapy and/or surgery before ESD[15]. The occurrence of postoperative ulcers was evaluated 1 mo after ESD. At the same time, according to previous literature reports and clinical references, the baseline data and endoscopic characteristics of patients before ESD treatment were collected, and the factors influencing postoperative ulcers were discussed. A risk prediction model for ulcers after ESD in patients with early gastric cancer was constructed, and receiver operating characteristic (ROC) curves were drawn to verify the effectiveness of the prediction model.

Scheme of ESD treatment

General intravenous anesthesia was performed on all patients during ESD in our study. The size and scope of the lesions were determined by endoscopy before surgery, and the depth of invasion of the lesions was determined to exclude the possibility of lymph node metastasis. The detailed scheme of EDS treatment was as follows: (1) Marking: the periphery of the lesion was marked by electrocoagulation at a distance of 5.0 mm from the outer edge of the lesion by subion coagulation; (2) submucosal injection: indigo rouge injection (Southwest Pharmaceuticals; batch no. H50021944; 10 mL: 40 mg) for multipoint submucosal injection to ensure that the lesion mucosa was uplifted; (3) circular incision: a needle knife was used to cut the outer edge of the lesion along the marked point of the lesion edge; (4) mucosal peeling: repeated submucosal injection and separation to strip and excise the lesion from the submucosa; (5) wound treatment: thermal biopsy forceps and titanium clips were used to treat the postoperative bleeding points and lesion edges; and (6) postoperative treatment: the size of the lesion was measured, it was fixed with 4% formaldehyde solution and sent for histopathology to clarify the nature of the lesion.

Data collection and data quality control

The data collection included the general information of the patients, their pathological features and the endoscopic features. The general information of the patients included age, sex (male/female), course of disease, body mass index [weight (kg)/height (m^2)], history of *Helicobacter pylori* (*H. pylori*) infection, family history of gastric cancer, lesion site, comorbid diseases (hypertension, diabetes, coronary heart disease), residence (rural, urban), smoking history, drinking history, and drug history (aspirin, clopidogrel). The pathological features included lesion diameter, pathological type (differentiated carcinoma, undifferentiated carcinoma), number of tumors (single, multiple), depth of invasion (submucosal, muscularis mucosa), and vascular invasion. The endoscopic features included the lesion site (upper 1/3 of the stomach, middle 1/3 of the stomach, lower 1/3 of the stomach), lesion surface (convex, flat, depressed), mucosal rupture (regardless of the depth of invasion, any mucosal defect represents the presence of mucosal ruptures), mucosal discoloration (discoloration of any part of the lesion or the entire lesion contrasts with that of the surrounding mucosa, indicating a color change), and converging folds (the presence of any centripetal folds in the lesion indicates converging folds).

Data quality control was performed according to the inclusion and exclusion criteria, which were strictly implemented to ensure the authenticity of the patient data. Specialized personnel collected and checked the general data of the patients, and the data were double-entered in parallel into EpiData software to ensure accuracy.

Follow-up and ulcer occurrence criteria

Follow-up and observation were performed for one month. Endoscopic review within 1 mo after the operation, local anesthesia and gastroscopic observation of the patient's lesions were performed. Then, 500 mL of 400% degassed distilled water was injected into the stomach, and endoscopic examination was performed under immersion. The occurrence of ulcers after ESD in the patients was recorded. The criteria for ulceration were mucosal defects involving the submucosa, muscularis propria malformation,

or fibrosis in the submucosa or deeper layers under endoscopy[16,17].

Statistical analysis

SPSS 22.0 statistical software was used in our study. The measurement data were first tested for normality; the normally distributed data are expressed as the mean ± SD, and two independent samples *t*-tests were used for comparisons between groups. Count data are given as *n* (%), and differences between groups were compared using the χ^2 test. Based on *R* software (glmnet package), LASSO regression was performed, and the tenfold cross-validation method was used to screen the best risk predictor subset. Multivariate logistic regression analysis was performed to evaluate the odds ratio. ROC analysis was performed to evaluate the effectiveness of the prediction model. A *Z* score test was performed to compare the ROC curves of the different indicators. A *P* value less than 0.05 represents a significant difference.

RESULTS

General information of the patients

One month after the operation, no cases were lost to follow-up, the incidence of ulcers was 20.41% (40/196) (ulcer group), and the incidence of no ulcers was 79.59% (156/196) (non-ulcer group). There was no significant difference in age, sex, body mass index, drinking history, family history of gastric cancer, number of tumors, comorbidities, residence, or aspirin medication history between the two groups ($P > 0.05$). There were significant differences in the course of disease ($P = 0.032$), history of *H. pylori* infection ($P = 0.041$), smoking history ($P = 0.045$), and proportion of clopidogrel medication history ($P < 0.001$) between the two groups ($P < 0.05$) (Table 1).

Comparison of pathological features between the ulcer group and the non-ulcer group

The pathological features in the ulcer group ($n = 40$) and non-ulcer group ($n = 156$) were compared. There was no significant difference in pathological type or vascular invasion between the two groups ($P > 0.05$), but there were statistically significant differences in lesion diameter ($P < 0.001$), the number of tumors ($P = 0.041$), and infiltration depth ($P = 0.046$) between the two groups (Table 2).

Comparison of endoscopic features between the ulcer group and the non-ulcer group

The endoscopic features in the ulcer group ($n = 40$) and non-ulcer group ($n = 156$) were compared. There was no significant difference in lesion site or lesion surface between the two groups ($P > 0.05$), but there were statistically significant differences in mucosal discoloration ($P < 0.001$) and convergent folds ($P < 0.001$) between the two groups, as shown in Table 3.

LASSO regression analysis

After the differential information of the patients, pathological features and endoscopic features was obtained, LASSO regression analysis was performed on the above independent variables (the course of disease, history of *H. pylori* infection, smoking history, clopidogrel medication history, lesion diameter, number of tumors, infiltration depth, mucosal discoloration, and convergent folds) (Figure 1). With the change in the penalty coefficient λ, the coefficients of the independent variables initially included in the model were gradually compressed, and finally, the coefficients of some independent variables were compressed to 0. Then, the 10-fold cross-validation method was used to validate the independent variables. After validation, clopidogrel medication history, lesion diameter, convergent folds, and mucosal discoloration were the 4 independent variables that predicted postoperative ulceration (Figure 2).

Multivariate logistic regression analysis of the risk of ulcers after ESD in EGC patients

Taking the occurrence of ulcers as the dependent variable (ulcer occurrence = 1, no ulcer occurrence = 0), the above variables with statistically significant differences were used as independent variables for logistic regression analysis, and variable selection was performed by the stepwise method (α in = 0.05, α out = 0.1). Multivariate logistic analysis showed that lesion diameter [Odds ratios (OR)= 30.490, 95%CI: 8.584-108.294], convergent folds (OR = 3.860, 95%CI: 1.060-14.055), mucosal discoloration (OR = 3.191, 95%CI: 1.016-10.021) and clopidogrel medication history (OR = 3.554, 95%CI: 1.009-12.515) were independent risk factors for ulcers after ESD in EGC patients ($P < 0.05$) (Table 4).

Evaluation of the ROC risk prediction model for ulcer occurrence after ESD in EGC patients

ROC curve analysis showed that the area under the curve (AUC) of the risk prediction model for ulcers after ESD in patients with EGC was 0.916 (95%CI: 0.865-0.967). In addition, ROC curves of the lesion diameter, convergent folds, mucosal discoloration and clopidogrel medication history for ulcer occurrence after ESD in EGC patients were also evaluated. Among the four indicators alone, the AUC of the lesion diameter was the best, 0.885 (95%CI: 0.814-0.955), and the AUCs of convergent folds, mucosal

Table 1 Comparison of general information of ulcer group and non-ulcer group, *n* (%)

General Information	Ulcer group (*n* = 40)	Non-ulcer group (*n* = 156)	t/χ^2 value	*P* value
Sex			0.83	0.362
Male	26 (65.00)	89 (57.05)		
Female	14 (35.00)	67 (42.95)		
Age	48.98 ± 8.23	47.11 ± 9.02	1.257	0.213
BMI	22.25 ± 2.01	21.83 ± 1.98	1.183	0.242
Course of disease (yr)	2.85 ± 0.48	2.66 ± 0.52	2.195	0.032
History of *H. pylori* infection	7 (17.50)	11 (7.05)	4.168	0.041
Family history of GC	8 (20.00)	22 (14.10)	0.854	0.355
Drinking history	9 (22.50)	26 (16.67)	0.739	0.39
Smoking history	24 (60.00)	66 (42.31)	4.013	0.045
Comorbidities				
Hypertension	9 (22.50)	26 (16.67)	0.739	0.39
Diabetes	6 (15.00)	22 (14.10)	0.021	0.885
Coronary heart disease	7 (17.50)	23 (14.75)	0.187	0.666
Residence			0.116	0.733
Rural	25 (62.50)	102 (65.38)		
Town	15 (37.50)	54 (34.62)		
Medication history				
Aspirin	12 (30.00)	28 (17.95)	2.847	0.092
Clopidogrel	19 (47.50)	27 (17.31)	16.158	< 0.001

BMI: Body mass index; *H. pylori*: *Helicobacter pylori*; GC: Gastric cancer.

Table 2 Comparison of pathological features of ulcer group and non-ulcer group, *n* (%)

Pathological features	Ulcer group (*n* = 40)	Non-ulcer group (*n* = 156)	t/χ^2 value	*P* value
Lesion diameter (cm)	4.40 ± 0.97	2.97 ± 0.62	8.871	< 0.001
Number of tumors			4.185	0.041
Single shot	18 (45.00)	98 (62.83)		
Multiple	22 (55.00)	58 (37.18)		
Pathological type			0.268	0.605
Differentiated carcinoma	19 (47.50)	67 (42.95)		
Undifferentiated carcinoma	21 (52.50)	89 (57.05)		
Infiltration depth			3.988	0.046
Submucosa	21 (52.50)	55 (35.26)		
Mucosal layer	19 (47.50)	101 (64.74)		
Vascular invasion	2 (5.00)	6 (3.85)	0.108	0.742

discoloration and clopidogrel medication history were 0.651 (95%CI: 0.549-0.753), 0.648 (95%CI: 0.554-0.742) and 0.693 (95%CI: 0.601-0.785), respectively. Compared to the four indicators alone, the combined prediction model should significantly increase the accuracy of the prediction of ulcer occurrence after ESD in EGC patients (Table 5).

Table 3 Comparison of endoscopic features of ulcer group and non-ulcer group, *n* (%)

Endoscopic features	Ulcer group (*n* = 40)	Non-ulcer group (*n* = 156)	χ^2 value	*P* value
Lesion site			2.132	0.344
Upper 1/3 of stomach	12 (30.00)	61 (39.10)		
1/3 of stomach	20 (50.00)	76 (48.72)		
Lower 1/3 of stomach	8 (20.00)	19 (12.18)		
Mucosal discoloration	28 (70.00)	63 (40.38)	11.227	< 0.001
Convergence folds	28 (60.00)	49 (31.41)	19.877	< 0.001
Lesion surface			1.105	0.576
Bulge	11 (27.50)	52 (33.33)		
Flat	15 (37.50)	62 (39.74)		
Sag	14 (35.00)	42 (26.93)		

Table 4 Multivariate logistic regression analysis on the risk of ulcers after endoscopic submucosal dissection in early gastric cancer patients

Related indicator	β	SE	Wald	*P* value	OR	95%CI	
						Lower	Upper
Lesion diameter	3.417	0.647	27.927	< 0.001	30.490	8.584	108.294
Clopidogrel medication history	1.268	0.642	3.899	0.048	3.554	1.009	12.515
Convergent folds	1.351	0.659	4.195	0.041	3.860	1.060	14.055
Mucosal discoloration	1.160	0.584	3.950	0.047	3.191	1.016	10.021

OR: Odds ratios.

Table 5 Evaluation of prediction model for ulcer occurrence after endoscopic submucosal dissection in early gastric cancer patients

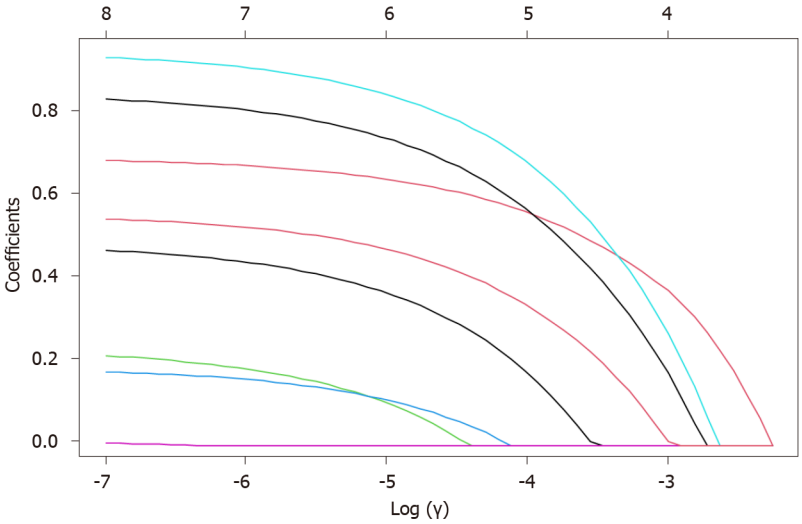
Indicator	AUC	SD	*P* value	95%CI	
				Lower	Upper
Lesion diameter	0.885	0.036	< 0.001	0.814	0.955
Clopidogrel medication history	0.651	0.052	0.003	0.549	0.753
Mucosal discoloration	0.648	0.048	0.004	0.554	0.742
Convergent folds	0.693	0.047	< 0.001	0.601	0.785
Prediction model	0.916	0.026	0.000	0.865	0.967

AUC: Area under the curve.

DISCUSSION

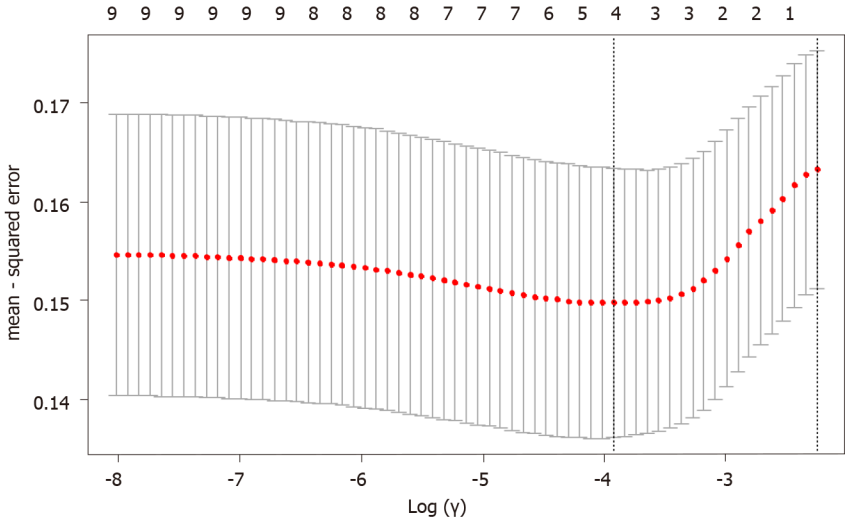
With advances in endoscopic techniques, ESD has become widely used in EGC treatment. ESD can provide a higher quality of life than surgical resection in terms of long-term outcomes[18]. To select ESD patients who may benefit from this treatment, personalized prediction of the outcome of EGC treatment is needed; therefore, previous studies have analyzed various clinicopathological factors and imaging modalities for personalized prediction[19]. Compared with non-ulcer EGCs, the incidence of lymph node micro-metastases in ulcerative EGC is significantly increased, so the presence or absence of ulcers has been identified as the key to a personalized treatment strategy for EGC.

However, currently, the presence of ulcers in the current ER criteria does not refer to endoscopic ulcers but to histological ulcers, which are based on data from surgically resected specimens. It is difficult to assess histological ulcers from biopsy specimens prior to treatment. Although the histological



DOI: 10.4251/wjgo.v14.i9.1823

Figure 1 Coefficient curves of nine clinical features included in LASSO regression.



DOI: 10.4251/wjgo.v14.i9.1823

Figure 2 The most suitable clinical features were selected by LASSO regression and ten-fold cross-validation.

appearance of ulcers is considered to be an important factor in EGC treatment decisions and ESD curability, they should also be distinguished from biopsy-derived scars[20,21]. Mucosal rupture cannot be defined as an ulcer alone, but some clinicians believe that it could be described as an endoscopic ulcer, which may lead to overestimation of ulcerative EGC. To avoid unnecessary surgery, careful examination and personalized assessment of the ulcer under endoscopy is urgently needed in clinical practice[22].

In terms of endoscopic features, a previous study of endoscopic images of EGC patients showed that the diagnostic accuracy was 28.2% in the case of superficial mucosal ruptures without converging folds; in cases with confluent folds without mucosal ruptures and in patients with pathological ulcerative lesions, the diagnostic accuracy was only 35.9%[23]. The reason for this may be that most endoscopists tend to consider sunken lesions or lesions with mucosal ruptures as endoscopic ulcers. In another study [24], the lesion surface was irregular, and concentric folds of the diseased tissue were observed during the postoperative healing process of mixed EGC.

Converging folds of the EGC being a risk factor for ulceration was confirmed in our study. We believe that converging folds may originate from previous ulcers during the healing process, which indicates the presence of histological ulcers, and the presence of ulcer scars is negatively related to the effect of ESD. If converging folds are observed during endoscopy, it should be concluded that the lesion is accompanied by ulcer scars, and the probability of postoperative ulceration is high, so the procedure should be handled by a skilled endoscopist.

In addition, a recent study reported that white discoloration was associated with undifferentiated histology of EGC[25]. It was also shown that well-differentiated or moderately differentiated adenocarcinoma tumors have abundant and dense blood vessels, while low-grade adenocarcinoma tumors have sparse and loose blood vessels[26]. These findings are associated with cancerous mucosal redness in well-differentiated or moderately differentiated adenocarcinomas and pallor in undifferentiated carcinomas. A retrospective study showed that a color change (OR = 2.33) was an independent factor for predicting histological ulcers[27]. These results were also confirmed in our study.

The relationship between clinicopathological features and postoperative ulceration is also a hot topic in various studies, and previous studies have confirmed that the diameter of the lesion is a predictor of ulceration after ESD[28], because the larger the tumor diameter is, the greater the resection range. The larger the size, the longer the treatment time, which was also observed in this study.

In addition, some studies identified antithrombotic therapy as an independent risk factor for ESD ulcers[29]. A history of clopidogrel use was associated with the occurrence of ulcers after ESD[30]. In our study, a history of clopidogrel medication was also an independent risk factor for ulcers after ESD in EGC patients. The reason may be that the long-term use of clopidogrel before surgery may lead to changes in the patients' coagulation function and increase the risk of postoperative ulcers. However, it is worth noting that aspirin and clopidogrel are both antithrombotic drugs, and aspirin does not increase the risk of postoperative ulcers, which may be related to the relatively small sample size of this study, so the relationship between aspirin and the risk of postoperative ulcers should be examined in a future study.

However, there are still some shortcomings in our study. First, our study was a single-center study, which may have selection bias in the collection of clinical case data. A multicenter study should be performed in the future. Second, the sample size was relatively small, and the predictive model of ulcers after ESD in EGC patients needs to be confirmed in a much larger study. Third, although a risk prediction model for EGC was built, the model was not validated. A prospective study should be performed to further confirm these results.

CONCLUSION

In summary, clopidogrel medication history, lesion diameter, convergent folds, and mucosal discoloration can predict the occurrence of ulcers after ESD in patients with EGC. The LASSO regression-based ulcer risk prediction model for EGC may be feasible and meaningful, and its clinical application value can effectively help clinicians identify high-risk groups for ulcers after ESD for EGC and provide targeted treatment measures.

ARTICLE HIGHLIGHTS

Research background

With the development of endoscopic techniques, endoscopic submucosal dissection (ESD) has been widely used in the treatment of early gastric cancer (EGC); however, due to the wide range of ESD peeling, deep lesion peeling, difficult operations, and relatively high risk of complications such as bleeding and perforation, a personal predictive model of the outcome is necessary.

Research motivation

A personalized and effective prediction method of the outcomes of ESD for EGC is urgently needed in clinical practice.

Research objectives

This study aimed to build a personalized prediction model that may provide a theoretical basis for the prevention of ulcers among EGC patients after ESD.

Research methods

A total of 196 EGC patients who received ESD treatment in our hospital from March 2019 to March 2021 were enrolled in our study. The general information of the patients, pathological features and endoscopic features were analyzed, and multivariate logistic regression analysis was performed to evaluate their predictive value.

Research results

After LASSO regression analysis and validation, clopidogrel medication history, lesion diameter, convergent folds, and mucosal discoloration were the 4 independent variables that predicted postoperative ulceration. Receiver operating characteristic curve analysis showed that the AUC of the

risk prediction model for ulcers after ESD in patients with EGC was 0.916 (95%CI 0.865-0.967). Compared to each of the four indicators alone, their combined prediction model should have significantly increased accuracy for the prediction of ulcer occurrence after ESD for EGC patients.

Research conclusions

A LASSO regression-based ulcer risk prediction model that included clopidogrel medication history, lesion diameter, convergent folds, and mucosal discoloration was built for EGC.

Research perspectives

A large sample size should be used to validate the prediction model in future studies.

FOOTNOTES

Author contributions: Gong SD and Wang XH designed the study; Gong SD and Li H performed the research; Gong SD, Li H, Xie YB and Wang XH analyzed the date; Gong SD wrote the paper; Wang XH revised the manuscript for final submission; Gong SD and Li H contributed equally to this study; Xie YB and Wang XH are the corresponding authors; and all authors read and approved the final version.

Supported by The CAMS Initiative for Innovative Medicine, No. 2016-I2M-1-007.

Institutional review board statement: The study was reviewed and approved by the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College.

Informed consent statement: Written informed consent was exempted, because all patients had already signed the informed consents before treatment according to the institutional guideline, and all the information used in present study were obtained the raw data documented in the database.

Conflict-of-interest statement: The authors declare no conflict of interest.

Data sharing statement: No additional data are declared to be shared.

Country/Territory of origin: China

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S-Editor: Wang JL
L-Editor: A
P-Editor: Wang JL

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1833-1843

DOI: 10.4251/wjgo.v14.i9.1833

ISSN 1948-5204 (online)

ORIGINAL ARTICLE

Observational Study

Percutaneous insertion of a novel dedicated metal stent to treat malignant hilar biliary obstruction

Francesco Cortese, Fabrizio Acquafredda, Andrea Mardighian, Maria Teresa Zurlo, Valentina Ferraro, Riccardo Memeo, Stavros Spiliopoulos, Riccardo Inchingolo

Specialty type: Radiology, nuclear medicine and medical imaging

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification
Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Havre RF, Norway; Piltcher-da-Silva R, Brazil; Sugimoto M, Japan

Received: April 8, 2022
Peer-review started: April 8, 2022
First decision: May 11, 2022
Revised: June 11, 2022
Accepted: August 21, 2022
Article in press: August 21, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Percutaneous bilateral biliary stenting is an established method for the management of unresectable malignant hilar biliary obstruction.

AIM

To evaluate the efficacy and safety of a novel uncovered biliary stent, specifically designed for hilar reconstruction.

METHODS

This, single-center, retrospective study included 18 patients (mean age 71 ± 11 years; 61.1% male) undergoing percutaneous transhepatic Moving cell stent (MCS) placement for hilar reconstruction using the stent-in-stent technique for malignant biliary strictures, between November 2020 and July 2021. The Patients were diagnosed with cholangiocarcinoma (12/18; 66.6%), gallbladder cancer (5/18; 27.7%), and colorectal liver metastasis (1/18; 5.5%). Primary endpoints were technical (appropriate stent placement) and clinical (relief from jaundice) success. Secondary endpoints included stent patency, overall survival, complication rates and stent-related complications.

RESULTS

The technical and clinical success rates were 100% (18/18 cases). According to Kaplan-Meier analysis, the estimated overall patient survival was 80.5% and 60.4% at 6 and 12 mo respectively, while stent patency was 90.9% and 68.2% at 6

mo and 12 mo respectively. The mean stent patency was 172.53 ± 56.20 d and median stent patency was 165 d (range 83-315). Laboratory tests for cholestasis significantly improved after procedure: mean total bilirubin decreased from 15.2 ± 6.0 mg/dL to 1.3 ± 0.4 mg/dL ($P < 0.001$); mean γGT decreased from 1389 ± 832 U/L to 114.6 ± 53.5 U/L ($P < 0.001$). One periprocedural complication was reported. Stent-related complications were observed in 5 patients (27.7%), including 1 occlusion (5.5%) and 1 stent migration (5.5 %).

CONCLUSION

Percutaneous hilar bifurcation biliary stenting with the MCS resulted in excellent clinical and technical success rates, with acceptable complication rates. Further studies are needed to confirm these initial positive results.

Key Words: Malignant hilar biliary obstructions; Hilar cholangiocarcinoma; Self-expandable metallic stent; Stent-in-stent technique; Percutaneous approach; Bilateral Y-stenting

Core Tip: This single-center, retrospective study investigated eighteen patients with unresectable malignant hilar biliary obstructions treated with a novel uncovered biliary metallic stent [Moving Cell Stent (MCS); BCM Co., Ltd., Gyeonggi-do, South Korea], specifically designed for hilar reconstruction, using stent-in-stent technique *via* percutaneous approach. Primary endpoints were clinical and technical success. The study results indicate that percutaneous MCS placement using stent-in-stent technique is feasible and safe. Comparison with other stents demonstrated superiority in both stent patency and technical success.

Citation: Cortese F, Acquafredda F, Mardighian A, Zurlo MT, Ferraro V, Memeo R, Spiliopoulos S, Inchingolo R. Percutaneous insertion of a novel dedicated metal stent to treat malignant hilar biliary obstruction. *World J Gastrointest Oncol* 2022; 14(9): 1833-1843
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1833.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1833

INTRODUCTION

Malignant hilar biliary obstructions (MHBO) are very difficult to treat because most patients are diagnosed at an unresectable stage[1]. Hilar Cholangiocarcinoma (HiCC) is the most frequent cause of MHBO. Other malignant strictures may be due to pancreatic, gallbladder and liver tumors, to metastatic hilar lesions or to lymphadenopathies[2]. The primary principle behind the criteria for unresectability is the requirement for biliary and vascular reconstruction options with adequate future remnant hepatic parenchyma, as well as the presence of distant metastases or comorbidity of the patient[3,4]. Since only 10% to 20% of patients are suitable for resection, most of them receive palliative treatment[5]. The main aim of palliation is to re-create a connection between the biliary system and bowel to allow physiological drainage, in order to reduce pain, relieve biliary obstruction, significantly decreasing the incidence of cholangitis and allowing the administration of chemotherapy[6].

Due to the complexity of MHBO management, an organized multidisciplinary approach is paramount to deliver best quality care[7]. The main palliative treatments are biliary drainage and biliary stent implantation which can be performed with percutaneous or endoscopic approach, but there is no clear evidences of the superiority of one over the other. According to currently available data and the ESMO guidelines, percutaneous is the recommended approach in cases in which the endoscopic methods are not possible, commonly noted in advanced hilar Bismuth IV obstructions[8-10]. Moreover, percutaneous approach enables precise lobar selection for drainage[6].

With regard to bilateral *vs* unilateral drainage/stenting in cases of advanced HiCC, the goal is to drain at least 50% of the liver volume, which usually requires more than one stent when bile ducts are dissociated[8]. A self-expandable metallic stent (SEMS) rather than a plastic one is preferred in patients with unresectable cancer and a life expectancy longer than 3 mo[9].

Bilateral stent implantation can be achieved using side-by-side (SBS) or stent-in-stent (SIS) technique, but there is no large consensus concerning which procedure is better[11,12]. Some studies have shown that SIS technique may offer a lower adverse events rate[13] and longer stent patency[12]. On the other hand, some authors have found no significant differences in clinical outcomes between SIS and SBS techniques[14,15]. However, SIS procedure is technically more difficult and complex due to the necessity of introducing the second SEMS through the mesh of the previously placed SEMS[16-18]. To

overcome this issue, a novel uncovered SEMS, the HILZO Moving Cell Stent (MCS) (BCM Co., Gyeonggi-do, South Korea) was created.

The purpose of the present study was to evaluate the efficacy and safety of a novel uncovered biliary stent, specifically designed for hilar reconstruction, in patients with MBHO.

MATERIALS AND METHODS

Patients

This, single-center, retrospective study was conducted at "F.Miulli" Hospital in the Inteventional Radiology Unit. A total of 18 patients (mean age 71 ± 11 years; 61.1% male) with MHBO undergoing percutaneous MCS (BCM Co., Ltd., Gyeonggi-do, South Korea) placement using SIS technique were enrolled within a 12-mo period (November 2020 and November 2021). The study was approved by the ethics committee of M Hospital and the patients provided written informed consent prior to enrolment. The study protocol conformed to the ethical guidelines of the 2013 Declaration of Helsinki (most recent version).

The diagnosis of MHBO was based on standard clinical and radiological criteria [following computed tomography (CT) and/or magnetic resonance imaging (MRI)], and was confirmed by percutaneous needle biopsy or percutaneous endobiliary forceps biopsy[19]. All patients were evaluated by a multidisciplinary team including oncologists, surgeons, gastroenterologists, radiotherapists, and interventional radiologists. Inclusion criteria were: MHBO caused by a biopsy-confirmed hilar malignancy, not suitable for surgery (due to unresectability, metastatic disease or severe comorbidities) and an estimated survival of over 3 mo. Exclusion criteria were patients with uncorrectable coagulopathy (INR >1.8; Platelets < 50.000) and presence of an atrophic lobe.

In the patient group, the causes of hilar obstruction included cholangiocarcinoma (12/18; 66, 6%), gallbladder cancer (5/18; 27, 7%), and colorectal liver metastasis (1/18; 5, 5%). Patients' baseline demographical data are outlined in Table 1.

Stent features

The Hilzo Biliary MCS (BCM Co., Ltd., Gyeonggi-do, South Korea) (Figure 1) is a novel uncovered metallic stent with a small cell size (4 mm) and a high radial force, dedicated for biliary SIS technique. The small cell size is expected to reduce ingrowth, and the high radial force results in higher expansion potential. The special design of this novel stent allows each cell to expand from 4 mm to 10 mm to enable a passage of the second stent through the stent struts. The MCS has radiopaque markers at each end, and two in the midsection and requires an 8Fr percutaneous access[20].

Procedure

This was a two-stage procedure. The first stage was percutaneous transhepatic biliary drainage (PTBD) and the second stage was MCS placement. All procedures were performed in the angiography suite, according to the CIRSE Standards of Practice on Percutaneous Transhepatic Cholangiography, Biliary Drainage and Stenting[21] using local anesthesia (2% Lidocaine), and conscious sedation (Fentanyl and Midazolam). A single-dose of iv antibiotic prophylaxis (Cefprozil 1g) was administrated before each procedure.

Under ultrasound guidance (Philips CX50) combined with fluoroscopy (Philips Allura FD20 Clarity), both right and left intrahepatic bile ducts were punctured with 21-gauge Chiba needles (Cook, Bloomington, IN, United States) and two 8.5-Fr drainage catheters (Cook Medical, Bloomington, IN, United States) were inserted (Figure 2A).

In 11 cases in which histological diagnosis was not already available, a percutaneous transluminal biopsy[19] was performed using a dedicated, transluminal biliary access and biopsy forceps set (Cook Medical, Bloomington, IN, United States) during the same PTBD session.

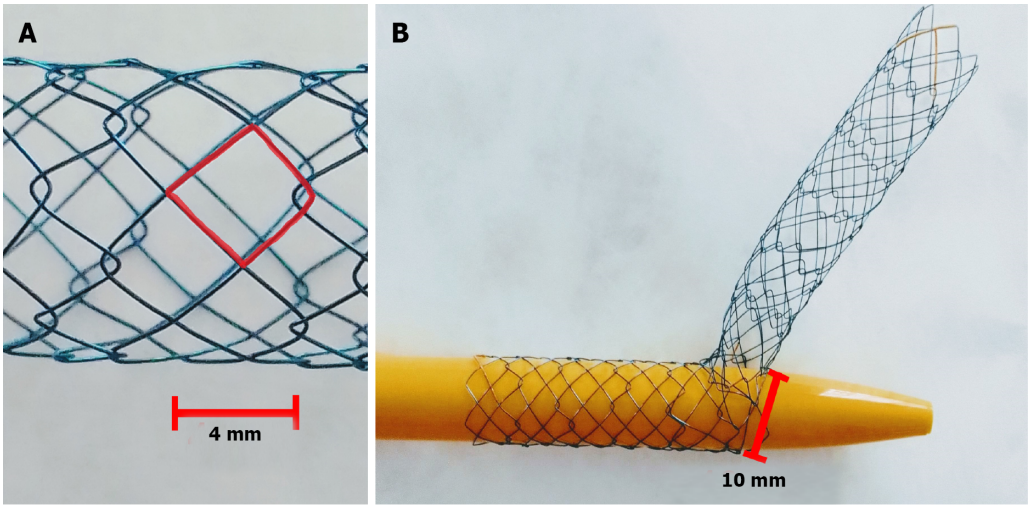
After approximately 7 to 21 d, and following improvement of obstructive jaundice symptoms, biliary stents placement was performed. Under fluoroscopic guidance, two hydrophilic guidewires (0.035 in.; Terumo Corporation, Tokyo, Japan) were introduced *via* the previously placed drainage catheters that were removed and two bilateral 8-Fr sheaths were placed within the biliary ducts over the hydrophilic guidewires.

Following cholangiography for the evaluation of the position and length of the biliary obstruction, the hydrophilic guidewire on one side was changed with an Amplatz Super Stiff™ 0.035 in. guidewire (Boston Scientific Corporation, Boston, MA, United States) using a 5-fr catheter KMP Beacon Tip (Cook Medical, Bloomington, IN, United States), and the corresponding type of MCS (10 or 8 mm × 10 or 8 or 6 cm) was implanted over the guidewire and dilated with a standard balloon catheter (Armada 35 PTA Catheter, Abbott Vascular, Santa Clara, CA, United States).

Analogously, on the other side, the hydrophilic guidewire was inserted through a mesh of the first MCS and exchanged (Figure 2B) with the stiff guidewire. Subsequently the second MCS (10 or 8 mm × 10 or 8 or 6 cm) was implanted and dilated. At this time, from the upper part of the first stent, the mesh of the controlateral MCS was engaged with the wire and, over the two stiff guidewires, two balloon

Table 1 Patient's baseline characteristics

Characteristics	Value
Total number of patients, *n*	18
Median age, yr	71
Range age, yr	37-84
Male sex, *n* (%)	11 (61.1)
Etiology, *n* (%)	
Cholangiocarcinoma	12 (66.6)
Gallbladder carcinoma	5 (27.7)
Colorectal liver metastases	1 (5.5)
Chemotherapy	17 (94.4)



DOI: 10.4251/wjgo.v14.i9.1833

Figure 1 The Hilzo Biliary Moving Cell Stent. A: The Hilzo Biliary Moving Cell Stent developed with small cell size (4 mm), with radiopaque markers at each end and two X-shape markers in the midsection; B: Each cell can expand from 4 mm to 10 mm to allows easier passage of the second stent through the cell.

catheters were placed inside the MCSs and a kissing balloon dilatation was performed (Figure 2C).

A final contrast check was performed to depict appropriate stent placement according to the SIS technique, thus the apex of the longest stent should be positioned within the duodenum, while the apex of the shorter stent should end within the first MCS (Figure 2D).

Pre-scheduled follow up protocol was set at 3 and 6 mo and every 6 mo thereafter and included clinical evaluation, laboratory tests and restaging CT (Figure 3).

Definitions and statistical analysis

The study's primary endpoints were technical and clinical success. Technical success was defined as appropriate placement of a bilateral MCS using the SIS technique (as described above). Clinical success was defined as a reduction of bilirubin values to normal (< 1.3 mg/dL) or to < 50% of the pre-PTDB value within 14 d. Secondary endpoints included stent patency, overall survival, peri-procedural adverse events, procedural duration and stent-related complications. Stent patency was defined as the time between stent placement and stent dysfunction, determined by the relapse of cholestasis and/or cholangitis according to clinical, laboratory and imaging findings. Stent patency and patient survival were estimated by the Kaplan-Meier method. Adverse events were graded according to the CIRSE Classification System for Complications[22]. Procedural duration was considered as the amount of elapsed time between local anaesthesia and removal of the sheaths.

mean ± SD were used to describe continuous variables, while counts and percentages were used for categorical variables. The statistical analysis was conducted using the SPSS statistical software (version 17.0; SPSS Inc., Chicago, IL, United States) and a *P* value of < 0.05 was considered significant.

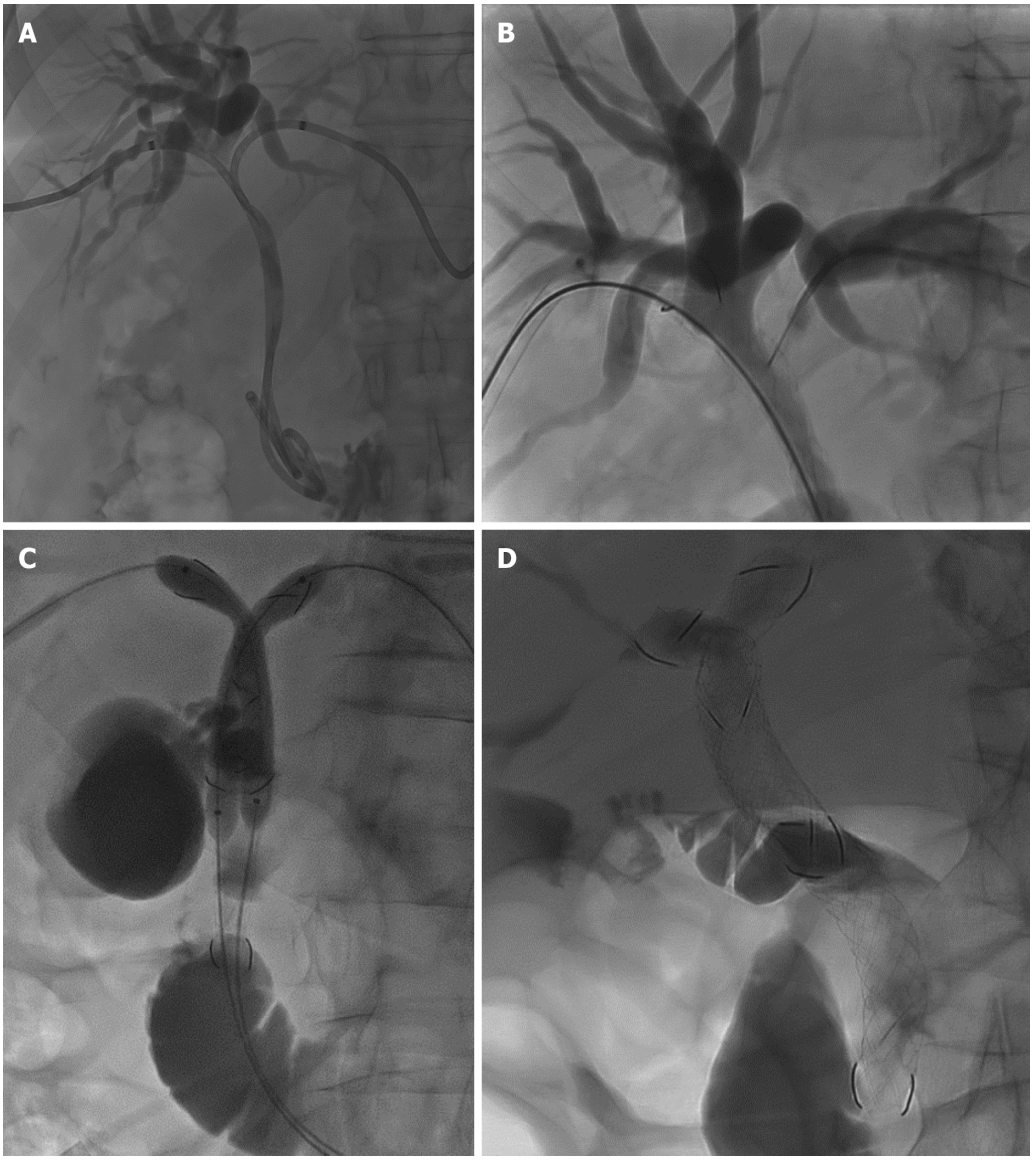


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Figure 2 Percutaneous transhepatic cholangiography. A: Percutaneous transhepatic cholangiography (PTC) showing hilar biliary obstructions with two bilateral bilateral 8.5-Fr drainage catheters; B: A hydrofilic guidewire (0.035 in.; Terumo Corporation, Tokyo, Japan) was inserted through a mesh of the Moving Cell Stent (MCS); C: PTC showing a kissing baloon dilatation over the stiff guidewires inside MCS placed using sten-in-stent technique; D: PTC showing the appropriate stents placement with the apex of the longest stent lies in the duodenum, while the apex of the shorter stent ends inside the first.

RESULTS

The clinical outcomes of bilateral MCS placement using the SIS technique are summarized in Table 2. Technical success and clinical success were 100% (18 out of 18 patients). The median procedural duration was 81.5 min ± 32.2 min. A single (5.5%) periprocedural adverse event occurred: Hemobilia due to porto-biliary fistula, treated during the same procedure with absorbable gelatin sponge (Spongostan) injection within the affected portal branch. This complication occurred during bile duct PTBD, and not during stent placement, and was judged as grade 1 according to the CIRSE Classification System for Complications[22].

The mean follow-up time was 169 d (range 83-315 d). Stent-related complications occurred in five (27.7%) patients (Table 3). Three (16.5%) patients who developed cholangitis without stent obstruction were treated with antibiotic therapy. Two patients (11%) presented with jaundice. For the first patient, the symptoms appeared 85 d after stent placement and the jaundice was caused by stent migration (5.5%) into common bile duct, treated with an additional MCS implantation. For the second patient, the jaundice appeared 151 d after stent placement and was caused by neoplastic ingrowth (5.5%). Due to the progression disease and the poor performance status of patients, it was decided to perform PTBD instead of an additional MCS placement. During the follow-up period, 4 patients (22.2%) died due to liver failure and/or progression disease.

Table 2 Clinical outcomes

Endpoint	Value
Technical success, *n* (%)	18 (100)
Clinical success, *n* (%)	18 (100)
Periprocedural complications, *n* (%)	1 (5.5)
Stent-related complications, *n* (%)	5 (27.7)
Stent occlusion, *n* (%)	1 (5.5)
Stent migration, *n* (%)	1 (5.5)
Mean procedural duration min	81.5 ± 32.2
Median stent patency days (range)	169 (93-315)
Overall mortality, *n* (%)	4 (22.2)

Table 3 Patients with stent-related complications

Age/sex	Etiology	Clinical manifestations	US findings	PTC findings	Treatment
75/F	GC	Jaundice	Left intrahepatic biliary dilatation	Stent migration	Additional MCS using SIS technique
77/M	CC	Jaundice	Bilateral intrahepatic biliary dilatation	Stent occlusion	PTBD
68/F	CC	Cholangitis	Aerobilia and no biliary dilatation	Not performed	Antibiotic therapy
81/M	CC	Cholangitis	Aerobilia and no biliary dilatation	Not performed	Antibiotic therapy
75/F	CC	Cholangitis	Aerobilia and no biliary dilatation	Not performed	Antibiotic therapy

GC: Gallbladder carcinoma; CC: Cholangiocarcinoma; US: Ultrasound; PTC: Percutaneous transhepatic cholangiography; MCS: Moving Cell Stent; PTBD: Percutaneous transhepatic biliary drainage; SIS: Stent-in-stent.

According to the Kaplan-Meier analysis, the estimated overall patient survival rate was 80.5% and 60.4% at 6 mo and 12 mo respectively, while stent patency was 90.9% and 68.2% at 6 and 12 mo respectively (Figure 4). The mean stent patency was 172.5 ± 56.2 d and median stent patency was 165 d (range 83-315). Laboratory tests for cholestasis significantly improved after procedure: mean total bilirubin decreased from 15.2 ± 6.0 mg/dL to 1.3 ± 0.4 mg/dL ($P < 0.001$); mean γGT decreased from 1389 ± 832 U/L to 114.6 ± 53.5 U/L ($P < 0.001$) (Table 4).

DISCUSSION

MHBO are often unresectable at presentation, thus palliative biliary decompression play a crucial role in improving the patients' quality of life[6].

Although outcomes of endoscopic US-guided biliary drainage techniques for hilar obstructions are very satisfactory[23-25], bilobar drainage with Y-configured SEMS using percutaneous approach is a well-established method for the palliative management of unresectable advanced MHBO in patients with estimated lifetime of more than 3 mo[9,10].

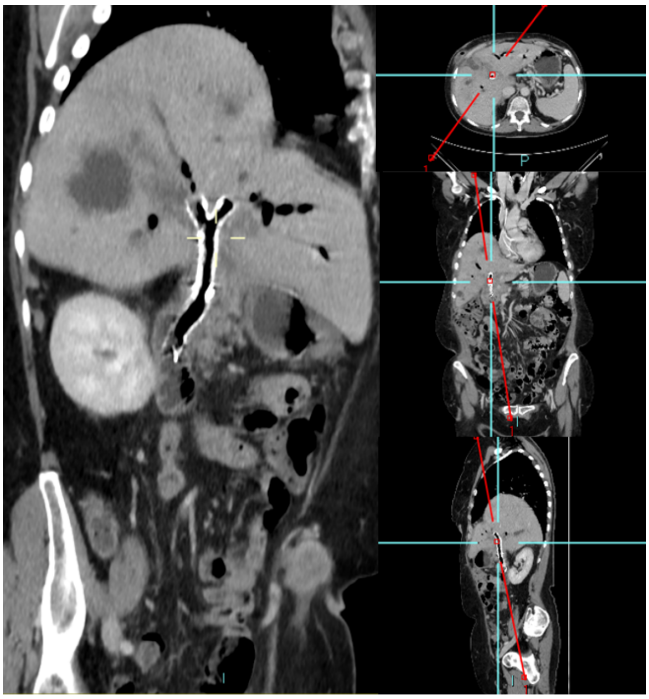
Bilateral SEMS placement can be achieved with SBS or SIS techniques (Figure 5). The SBS technique, considered technically easier[12], consists of the implantation of two parallel and close SEMS at and below the hepatic confluence, draining both hepatic lobes. Theoretically, the SBS technique has its inherent problems. The two SEMS cannot be fully expanded with major probability of partial collapse. Furthermore, the strong radial force caused by the parallel stent placement might be too strong to cause portal vein compression, bile duct rupture, or tumor ingrowth/tissue hyperplasia through the stent mesh[26,27].

On the other hand, in the SIS technique, after placing the first SEMS across the hilar stricture, a second SEMS is inserted into the contralateral hepatic duct through the mesh of first SEMS. Thereby, the single radial forces of both stents are added together opposing the biliary stricture, with a lower

Table 4 Laboratory tests

	PRE-PTBD	PRE-stent	POST-stent	*P* value
Total bilirubin (mg/dL)	15.2 ± 6.0	4.04 ± 1.50	1.31 ± 0.40	< di 0.001
Direct bilirubin (mg/dL)	13.5 ± 5.5	3.32 ± 1.30	0.86 ± 0.30	< di 0.001
γGT (U/L)	1389.2 ± 832.2	393.6 ± 321.7	114.6 ± 53.5	< di 0.001
Alkaline phosphatase (mU/mL)	321.7 ± 250.0	200.3 ± 179.4	115.7 ± 117.8	0.037
AST (UI/L)	243.9 ± 136.4	93.5 ± 47.6	50.6 ± 21.8	< di 0.001
ALT (UI/L)	319.3 ± 242.7	104.3 ± 53.3	71.7 ± 40.7	< di 0.001
WBC (10^3/μL)	10.2 ± 3.1	9.82 ± 4.00	7.16 ± 1.70	< di 0.001
PCR (mg/dL)	3.1 ± 1.5	3.9 ± 6.5	1.2 ± 1.2	< di 0.002

PTBD: Percutaneous transhepatic biliary drainage; ALT: Alanine aminotransferase; WBC: White blood cell; PCR: Polymerase chain reaction.



DOI: 10.4251/wjgo.v14.i9.1833

Figure 3 Three-months follow-up contrast-enhanced computed tomography. Sagittal oblique MPR showing two Y-shape Moving Cell Stent placed at the hilar bifurcation biliary with no intrahepatic biliary dilatation.

probability of stent migration or collapse; so the entire length of stricture is expanded by a single stent caliber[26]. Moreover, the SIS technique provides a more physiological Y-conformation stent to bile outflow, but it is still technically challenging[27].

The Hilzo Biliary MCS was designed specially for the SIS technique. According to the literature, there are only two previously published studies both investigating endoscopic bilateral Y-stenting using the MCS[17,18], therefore this is the first study investigating percutaneous placement of MCS.

The herein presented results are in accordance with those of Ogura *et al*[17] and Kawai *et al*[18] Specifically, similar technical success (100.0% *vs* 95.6%[17] *vs* 100.0%[18]), clinical success (100.0% *vs* 95.6 %[17] *vs* 89.9%[18]), periprocedural complications (5.5% *vs* 4.4%[17] vs 7.4%[18]) and 6-months stent patency rate (90.9% *vs* approx. 85.0% *vs* approx. 75.0%) were noted. However, dissimilar stent occlusion rates were noted [1/18 (5.5%) *vs* 4/23 (17.0%)[17] *vs* 12/27 (44.4%)[18]] The authors speculate that this discrepancy could be attributed to the only substantial technical difference: routine balloon post-dilatation was performed in all procedures in this study, whereas post-dilation was not performed in the two previously published studies. This could have contributed in the increased procedural duration noted in this study (81.5 ± 32.0 min *vs* 36.6 min, range 18-62[16] *vs* 23.7 ± 8.1 min[17]), but interestingly did not result in an increase of periprocedeural complications.

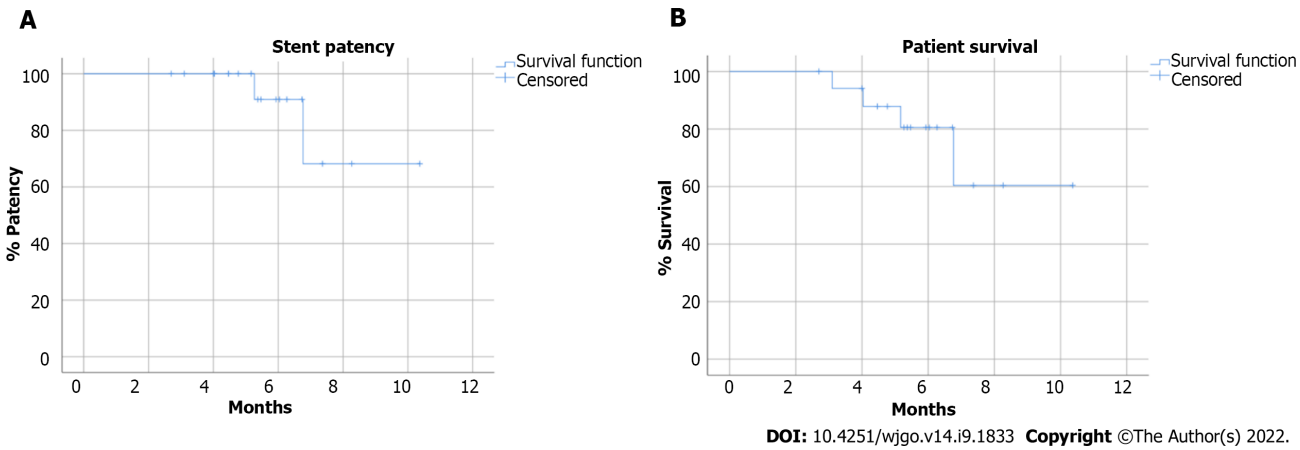


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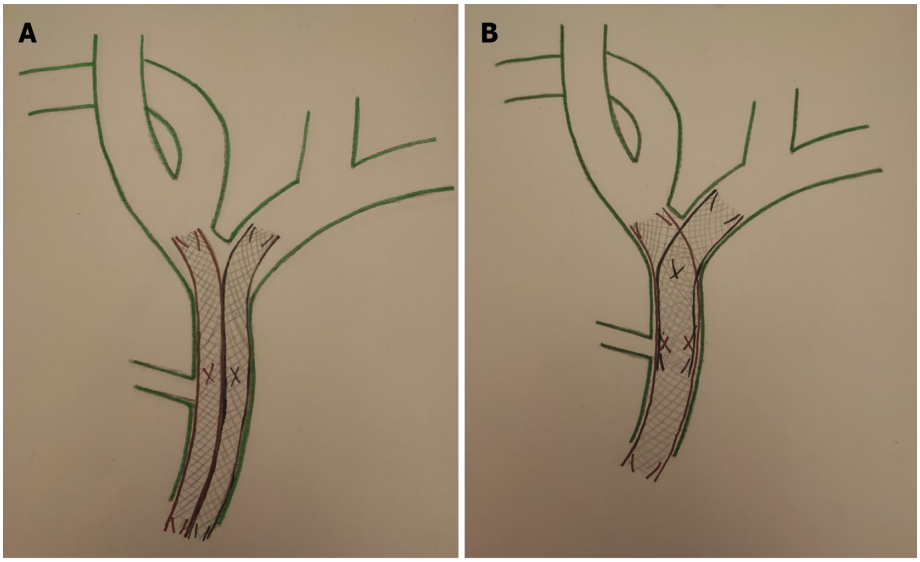
Figure 4 Kaplan-Meier analysis. A: The estimated stent patency; B: Overall patient survival.



DOI: 10.4251/wjgo.v14.i9.1833

Figure 5 Bilateral self-expandable metallic stent placement can be achieved with side-by-side or stent-in-stent techniques. A: Stent-by-stent technique: Two parallel and close self-expandable metallic stent (SEMS) at and below the hepatic confluence to drain the bile duct of both hepatic lobes; B: Stent-in-stent technique: Bilateral SEMS placed in a Y-configuration, in which a second stent across through the mesh of the first stent.

Generally, SEMS can be classified as small closed-cell, large open-cell types and mixed form of closed-cell type[16]. Closed-cell type SEMS (Wallstent, Boston Scientific Corp., Marlborough, MA, United States; Bonastent, Standard SciTech, Inc., Seoul, South Korea; Hanarostent, MI Tech Co., Seoul, Korea) have small cells to prevent ingrowth. However, characteristic of the closed-cell type hinders the deployment of a second stent or revision after stent malfunction, particularly in high-grade strictures [16], therefore they are not suitable for the SIS technique.

Open-cell type SEMS (JOSTENT SelfX, Abbott Vascular Devices, Redwood City, CA, United States; Zilver stent, Wilson-Cook Medical, Inc., Bloomington, IN, United States; Niti-S Y-type or Niti-S large cell D-type, Taewoong Medical Inc., Seoul, South Korea) facilitate the second stent implantation. Theoretically open-cell-type SEMS could be more vulnerable to tumor ingrowth and also demonstrate less radial force[16]. Although there are no published studies directly comparing outcomes of the SIS technique using these different stent types, superior stent patency rates were achieved by the MCS in this study compared to that of open-cell stents (MCS: 90.9%-68.2% *vs* large cell Niti-D biliary stent: 60%-20%[28] *vs* Sentinol stent: 65%-0%[29], at 6 mo and 12 mo; respectively).

Finally, the BONASTENT M-Hilar (Standard Sci Tech Inc., Seoul, South Korea) is a dedicate hilar reconstruction mixed form of closed-cell type stent, with a cross-wired structure only at the 25-mm-long central portion to facilitate placement of the contralateral stent[16,29]. However, the reported technical success rate was low (78.6 %), as the insertion of the second stent *via* the 25-mm central portion, is technical demanding unlike the MCS in which all the cells are dilatable and are therefore potential insertion sites for the second stent[30].

This study has several limitations. First, the number of patients is relatively low, so the statistical validity of the results is limited. Moreover, there was no control group, so comparative data are not available, while the single-center design limits the external validity of the results.

CONCLUSION

In conclusion, palliative treatment of patients with unresectable MHBO using percutaneous MCS placement with the SIS technique is safe and feasible and resulted in excellent clinical and technical success rates. Periprocedural and stent-related complications were acceptable. Prospective, multicentre, randomized trials are needed to verify these initial promising results.

ARTICLE HIGHLIGHTS

Research background

The treatment of malignant hilar biliary obstruction is very difficult because patients are often not suitable for surgery, therefore palliative care plays a pivotal role.

Research motivation

According to the literature, there are only two previously published studies both investigating endoscopic bilateral Y-stenting using the, therefore this is the first study investigating percutaneous placement of Moving Cell Stent (MCS).

Research objectives

To evaluate the efficacy and safety of a novel uncovered biliary stent, specifically designed for hilar reconstruction in patients with unresectable malignant hilar biliary obstructions.

Research methods

A retrospective, single-centre study was performed, investigating 18 patients with unresectable malignant hilar biliary obstructions treated with a novel uncovered biliary metallic stent (MCS; BCM Co., Ltd., Gyeonggi-do, South Korea), specifically designed for hilar reconstruction, using stent-in-stent technique *via* percutaneous approach. Primary endpoints were clinical and technical success.

Research results

The technical and clinical success rates were 100%. One periprocedural complication was reported. Stent-related complications were observed in 5 patients. According to Kaplan-Meier analysis, the estimated overall patient survival was 80.5% and 60.4% at 6 and 12 mo respectively, while stent patency was 90.9% and 68.2% at 6 mo and 12 mo respectively.

Research conclusions

For patients with unresectable malignant hilar biliary obstruction using percutaneous placement with the stent-in-stent technique was a feasible and safe and resulted in excellent technical and clinical success rates. Periprocedural and stent-related complications were acceptable.

Research perspectives

Since MCS is a recently introduced stent, prospective, multicentre, randomized trials are needed to verify these initial promising results.

FOOTNOTES

Author contributions: Cortese F, Acquafredda F, Mardighian A, Zurlo MT, Ferraro V, Memeo R, Spiliopoulos S, Inchingolo R equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Institutional review board statement: The study was reviewed and approved by the independent ethics committee of University Hospital Company "Consorziale Policlinico" of Bari, No 7083.

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

Conflict-of-interest statement: There are no conflicts of interest to report.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at riccardoin@hotmail.it. Consent was not obtained but the presented data are anonymized and risk of identification is low.

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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S-Editor: Chen YL
L-Editor: A
P-Editor: Chen YL

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1844-1855

DOI: 10.4251/wjgo.v14.i9.1844

ISSN 1948-5204 (online)

EVIDENCE-BASED MEDICINE

Prediction of gastric cancer risk by a polygenic risk score of *Helicobacter pylori*

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Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Keikha M, Iran; Wang W, China

Received: March 14, 2022
Peer-review started: March 14, 2022
First decision: April 17, 2022
Revised: April 29, 2022
Accepted: August 15, 2022
Article in press: August 15, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Genetic variants of *Helicobacter pylori* (*H. pylori*) are involved in gastric cancer occurrence. Single nucleotide polymorphisms (SNPs) of *H. pylori* that are associated with gastric cancer have been reported. The combined effect of *H. pylori* SNPs on the risk of gastric cancer remains unclear.

AIM

To assess the performance of a polygenic risk score (PRS) based on *H. pylori* SNPs in predicting the risk of gastric cancer.

METHODS

A total of 15 gastric cancer-associated *H. pylori* SNPs were selected. The associations between these SNPs and gastric cancer were further validated in 1022 global strains with publicly available genome sequences. The PRS model was established based on the validated SNPs. The performance of the PRS for predicting the risk of gastric cancer was assessed in global strains using quintiles and random forest (RF) methods. The variation in the performance of the PRS among different populations of *H. pylori* was further examined.

RESULTS

Analyses of the association between selected SNPs and gastric cancer in the global dataset revealed that the risk allele frequencies of six SNPs were significantly higher in gastric cancer cases than non-gastric cancer cases. The PRS model constructed subsequently with these validated SNPs produced significantly

higher scores in gastric cancer. The odds ratio (OR) value for gastric cancer gradually increased from the first to the fifth quintile of PRS, with the fifth quintile having an OR value as high as 9.76 (95% confidence interval: 5.84-16.29). The results of RF analyses indicated that the area under the curve (AUC) value for classifying gastric cancer and non-gastric cancer was 0.75, suggesting that the PRS based on *H. pylori* SNPs was capable of predicting the risk of gastric cancer. Assessing the performance of the PRS among different *H. pylori* populations demonstrated that it had good predictive power for cancer risk for hpEurope strains, with an AUC value of 0.78.

CONCLUSION

The PRS model based on *H. pylori* SNPs had a good performance for assessment of gastric cancer risk. It would be useful in the prediction of final consequences of the *H. pylori* infection and beneficial for the management of the infection in clinical settings.

Key Words: Polygenic risk scores; *Helicobacter pylori*; Gastric cancer; Single nucleotide polymorphism

Core Tip: Prediction of cancer risk is of importance in the clinical management of populations with a high risk of gastric cancer. This study constructed a polygenic risk score (PRS) model based on *Helicobacter pylori* (*H. pylori*) single nucleotide polymorphisms (SNPs) to predict the risk of gastric cancer. Associations between previously reported *H. pylori* SNPs and gastric cancer were validated in global strains. A PRS model constructed with validated SNPs had a high predictive power for gastric cancer at a global level and for individuals infected with hpEurope strains. It has potential for clinical use in the management of the *H. pylori* infection.

Citation: Wang XY, Wang LL, Liang SZ, Yang C, Xu L, Yu MC, Wang YX, Dong QJ. Prediction of gastric cancer risk by a polygenic risk score of *Helicobacter pylori*. *World J Gastrointest Oncol* 2022; 14(9): 1844-1855
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1844.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1844

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection affects more than half of the world's population[1,2]. The outcomes of *H. pylori* infection vary among individuals. The consequences of most infections are benign. However, a minority of infected individuals may eventually develop gastric cancer[3,4]. Predicting the outcomes of *H. pylori* infection is a major concern in the management of the infection. Substantial genetic variation has been found in the pathogen. Mutations cause increased virulence in certain strains, enhancing their carcinogenic potential[5,6]. It has been demonstrated that typing *H. pylori* strains based on the genetic variations of virulent genes has the potential to predict the risk of gastric cancer[7,8].

Two studies have been recently conducted to investigate the association between *H. pylori* genomic variations and gastric cancer within the hpEurope and hpEastAsia populations, respectively[9,10]. The first study contained 173 hpEurope strains and found 11 cancer risk-associated variants, including gene loss variants and single nucleotide polymorphisms (SNPs). Risk scores calculated based on the status of the *cag*11, *cag*12 and *cag*20 genes were increased during the progression from inflammation to gastric cancer. The other study identified 11 SNPs and three DNA motifs associated with gastric cancer through examination of 240 hpEastAsia strains. It is unclear whether the association between these variations and gastric cancer exists for all *H. pylori* strains. However, the findings from these studies suggest that SNPs from the *H. pylori* genome have the potential to predict the risk of gastric cancer.

To explore the combined effect of multiple SNPs on disease susceptibility, the polygenic risk score (PRS) model has been developed[11]. A PRS is calculated as a sum of the effects of multiple SNPs on disease. PRS models composed of SNPs from the human genome have been successfully used to predict the risk of cancers such as gastric cancer, colorectal cancer, and breast cancer[12-15]. Few studies, however, have been conducted to explore the capacities of PRS model constructed with SNPs from bacterial genomes in predicting the risk of cancer. Our study aimed to construct a PRS model based on validated risk alleles of *H. pylori* to predict the risk of gastric cancer.

MATERIALS AND METHODS

Strains and SNP selection

A total of 2022 *H. pylori* genome sequences deposited in GenBank at the National Center for Biotechnology Information by December 8, 2021 (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/169/), and the figshare website (https://figshare.com/s/2174da1fa20ae71c71e0)[10] were downloaded for further analyses. Of them, 1187 *H. pylori* strains had relevant clinical information of patients. Subsequently, duplicate strains and strains isolated from peptic ulcer disease, mucosa-associated lymphoma or stromal tumors were excluded from further analyses. This led to a final dataset of 1022 global strains included in the study. They were divided into gastric cancer (n = 253) and non-gastric cancer (n = 769) groups. Patients in the latter group were diagnosed with functional dyspepsia (n = 46), or chronic gastritis with or without intestinal metaplasia (n = 143 and n = 580, respectively). A total of 15 *H. pylori* SNPs or genetic variants from the two previous genome-wide association studies (GWASs) were selected for further analyses (Figure 1, Table 1)[9,10]. We cited high-quality articles in *Reference Citation Analysis* (https://www.referencecitationanalysis.com). The selection criteria were as follows: (1) The length of the variants was no longer than five contiguous nucleotides; and (2) The SNP selected was located in a protein-coding region.

Construction of the neighbour-joining tree

Based on the 1022 *H. pylori* genomes, the SNPs in the core genome (present in > 99% isolates) were identified by aligning the assembled genomes against the reference genome (26695-1MET, accession number: CP010436.1) using MUMmer as previously described[16,17]. A neighbour-joining tree was then constructed based on the sequences of concatenated SNPs using TreeBeST software (http://treesoft.sourceforge.net/treebest.shtml) with default parameters.

Statistical analyses

The chi-square test was used to test the difference in the prevalence of risk alleles in strains isolated from gastric cancer and non-gastric cancer. Student's t test was used to compare the PRS values between the gastric cancer and non-cancer groups. These tests were performed using SPSS 18.0 software. Odds ratios (ORs) and 95% confidence intervals (CIs) of the selected SNPs were calculated using logistic regression analysis in R (version 3.6.3).

A PRS was created for each strain using the following equation: PRS = β1 + β2 + … βk… + βn. Briefly, in this equation, βk is the value obtained from the regression analysis of the risk allele and disease, and n is the total number of SNPs included in the PRS[18]. Logistic regression analysis was performed to evaluate the association between PRS and gastric cancer risk and by quintiles of the PRS risk distribution, standardized by the controls, and using the 3rd quintile, 40%-60%, as the reference[18].

Random forest algorithm

A random forest (RF) model was built using the AUC-RF algorithm[19]. The input variables were the scores of each of the validated SNPs. A 20-times repeated 10-fold cross-validation of the RF model was performed. The performance of the RF model was demonstrated by receiver operating characteristic curve analysis[20].

RESULTS

Validation of SNPs in the global dataset

Previous studies have identified two sets of *H. pylori* SNPs that are associated with gastric cancer[9,10]. The association between these SNPs and gastric cancer has been verified only in strains from the hpEurope or hpEastAsia populations, respectively. We selected 15 SNPs to validate the association between selected SNPs and gastric cancer in global strains (Table 1). The risk alleles were defined as those with a higher prevalence in strains from gastric cancer. Statistical analyses revealed that the risk alleles of six SNPs showed a significant increase in prevalence in the gastric cancer group compared with the non-gastric cancer group. These SNPs, validated in the global dataset, were used for subsequent analyses.

Establishment of the six-SNP PRS model

To construct a PRS model for predicting the risk of gastric cancer, the logOR values of each validated SNP were calculated (Table 1). A PRS model was subsequently constructed with the sum of the logOR values of six validated SNPs. The mean PRS value was 8.64 ± 1.71 and 6.99 ± 1.27 in the gastric cancer and non-gastric cancer groups, respectively. The PRS value in the gastric cancer group was significantly higher (P = 5.6E-36).

Table 1 Selected and validated single nucleotide polymorphisms associated with gastric cancer in the global dataset

SNP	Corresponding locus in the strain 26695	Gene name	Description	Position in gene	Position in chromosome	Risk allele	Amino acid change	Prevalence of risk allele	*P* value	LogOR -value
1[1]	HP0082[1]	*tlpC*[1]	Chemotaxis sensor[1]	163[1]	88029[1]	A[1]	K217E,Q[1]	16.2%[1]	2.88E-15[1]	1.29[1]
2[1]	HP0130[1]	*triH*[1]	BIR, Dps/NapA, RAD21 similarity[1]	345[1]	140797[1]	C[1]	Synonymous[1]	16.2%[1]	2.88E-15[1]	2.26[1]
3[1]	HP0231[1]	*dsbG/K*[1]	Thiol:disulfide interchange protein[1]	433[1]	241625[1]	A[1]	T145A[1]	23.2%[1]	5.34E-15[1]	1.24[1]
4	HP0468		Unknown	729	489762	A	Synonymous	34.6%	0.110	
5	HP0468		Unknown	705-708	489783-489786	CGCC	A236T	1.2%	0.313	
6	HP0709		Adenosyl-chloride synthase	145	762953	A	N49D	14.6%	0.08	
7	HP0709		Adenosyl-chloride synthase	159	762967	A	Synonymous	90.0%	0.274	
8[1]	HP0747[1]	*trmB*[1]	tRNA ([guanine-N(7)-]-methyltrans-ferase[1]	(934-937)[1]	(803467-803470)[1]	GGAA[1]	G312K,G,R+T313A,T,S[1]	38.7%[1]	1.17E-14[1]	1.20[1]
9	HP0797	*hpaA*	Neuraminyllactose-binding hemagglutinin	334	854406	T	S112A	26.8%	0.567	
10[1]	HP0797[1]	*hpaA*[1]	Neuraminyllactose-binding hemagglutinin[1]	325[1]	854415[1]	C[1]	L109F[1]	40.1%[1]	1.12E-14[1]	2.16[1]
11	HP0807	*fecA-2*	Iron importer in outer membrane	2010	861345	C	Synonymous	96.7%	0.158	
12[1]	HP1055[1]		Outer membrane protein[1]	798[1]	1117402[1]	A[1]	Synonymous[1]	34.6%[1]	2.93E-14[1]	2.58[1]
13	HP1250	*csd5*	Cell shape determinant	370	1325727	A	N116H,D	65.8%	1	
14	HP1440	*isp*	Inactive Ser protease	533	1513405	G	G173E	92.0%	0.505	
15	HP1467	*ompA101*	Outer membrane protein of OmpA family	53	1538114	T	I18T	98.4%	0.246	

[1]The single nucleotide polymorphisms validated in this work.
Risk allele: Allele associated with gastric cancer. SNP: Single nucleotide polymorphism; OR: Odds ratio.

Evaluation of the association between PRS and gastric cancer risk

To evaluate the performance of the 6-SNP PRS model for predicting the risk of gastric cancer, the PRS values for each of the selected 1022 strains were grouped according to the quintile method. With the third quintile as the reference, the estimated OR value gradually increased from the first quintile (< 20%) to the fifth quintile (> 80%) (Figure 2, Table 2). The fifth quintile had an OR value as high as 9.76 (95%CI: 5.84-16.29).

To further confirm the combined effect of the validated SNPs for prediction of gastric cancer risk, an RF model was constructed with logOR values from each SNP as input. The classification potentials of the combined logOR values of validated SNPs were then analysed. The importance of each SNP is shown in Figure 3. The AUC value was 0.75 (DeLong 95%CI: 0.71-0.78), suggesting a good classifying capacity of the combined SNPs.

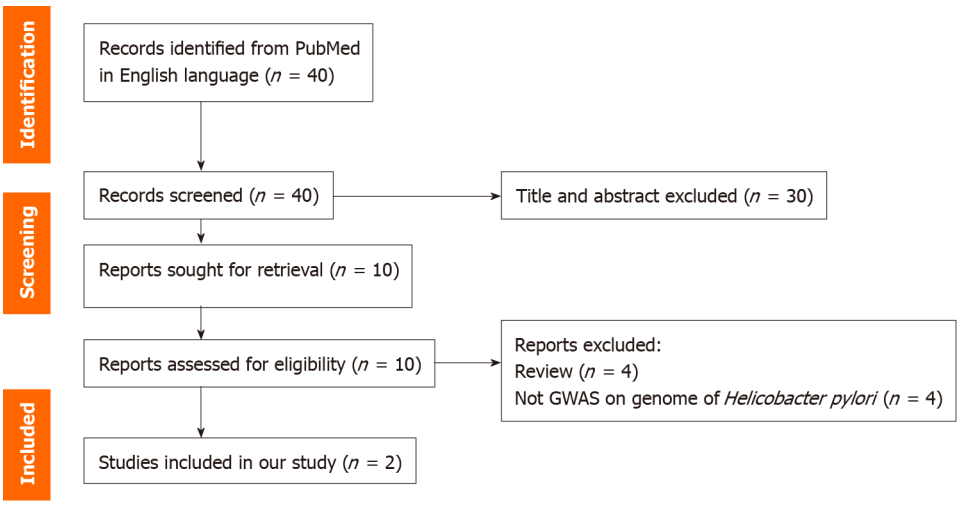
Performance of risk prediction by PRS for different H. pylori populations

Considering the remarkable genomic variations among strains from different *H. pylori* populations, the performance of PRS for predicting the risk of gastric cancer was subsequently assessed in different *H. pylori* populations. The results of the phylogenetic analyses divided the 1022 global strains into five groups, namely, the hpEastAsia, hpAsia2, hpEurope, America-related and Africa-related populations (Figure 4). Due to the small number of gastric cancer cases (2 cases in hpAsia2 and no cases in Africa-related populations), hpAsia2 and Africa-related populations were excluded from subsequent analyses.

Table 2 Associations between polygenic risk score and gastric cancer risk

Quintile	Non-GC	GC	OR (95%CI)	*P* value
1	315 (41.0%)	44 (17.3%)	0.79 (0.46-1.34)	0.405
2	98 (12.7%)	7 (2.8%)	0.40 (0.17-0.97)	0.052
3	141 (18.3%)	25 (9.9%)	1	-
4	141 (18.3%)	49 (19.4%)	1.96 (1.15-3.35)	0.013
5	74 (9.6%)	128 (50.6%)	9.76 (5.84-16.29)	1.25E-14

CI: Confidence interval; OR: Odds ratio; GC: Gastric cancer



DOI: 10.4251/wjgo.v14.i9.1844

Figure 1 PRISMA flow diagram. GWAS: Genome-wide association studies.

In analysing the performance of the established PRS model in different populations, the PRS value was higher in the gastric cancer group for all populations. Statistical analyses revealed a significant difference in PRS between the gastric cancer and non-gastric cancer groups in the hpEastAsia, hpEurope and America-related populations (Figure 5).

To further verify the combined effects of these SNPs for prediction of gastric cancer risk for different *H. pylori* populations, a RF classification model was built. The results of RF model analyses demonstrated that the AUC value was highest (0.78, DeLong 95%CI: 0.70-0.85) in the hpEurope population, suggesting a good ability of the combined SNPs to predict the risk of gastric cancer (Figure 6). However, the performance of the combined SNPs for risk prediction in other *H. pylori* populations was poor (Figure 6).

DISCUSSION

In this study, we constructed a PRS model based on validated *H. pylori* SNPs to predict the risk of gastric cancer. To our knowledge, our study is the first to evaluate a PRS model for cancer risk prediction constructed with genomic variants of *H. pylori*. *H. pylori* shows substantial genetic variations, resulting in remarkable interstrain differences in carcinogenetic potential[5,21]. The presence/absence or large sequence variation of virulence genes and *H. pylori* SNPs have been shown to promote gastric carcinogenesis. Few studies have been conducted to assess the predictive power of these cancer-related genetic variations for gastric cancer[9,10]. Moreover, the combined effect of multiple variations on the predictive power for cancer risk has not been explored. Findings from this study demonstrate that a PRS model combining six *H. pylori* SNPs had a moderate capacity for prediction of gastric cancer risk. This is similar to the findings in studies on PRS model constructed with cancer-associated SNPs from the human genome[14,15].

To assess the combined effects of SNPs on gastric cancer risk prediction, we first selected 15 cancer-associated *H. pylori* SNPs from two previous GWAS studies. Their association has been validated in strains from specific geographical regions but not in a global strain collection. Our results demonstrated

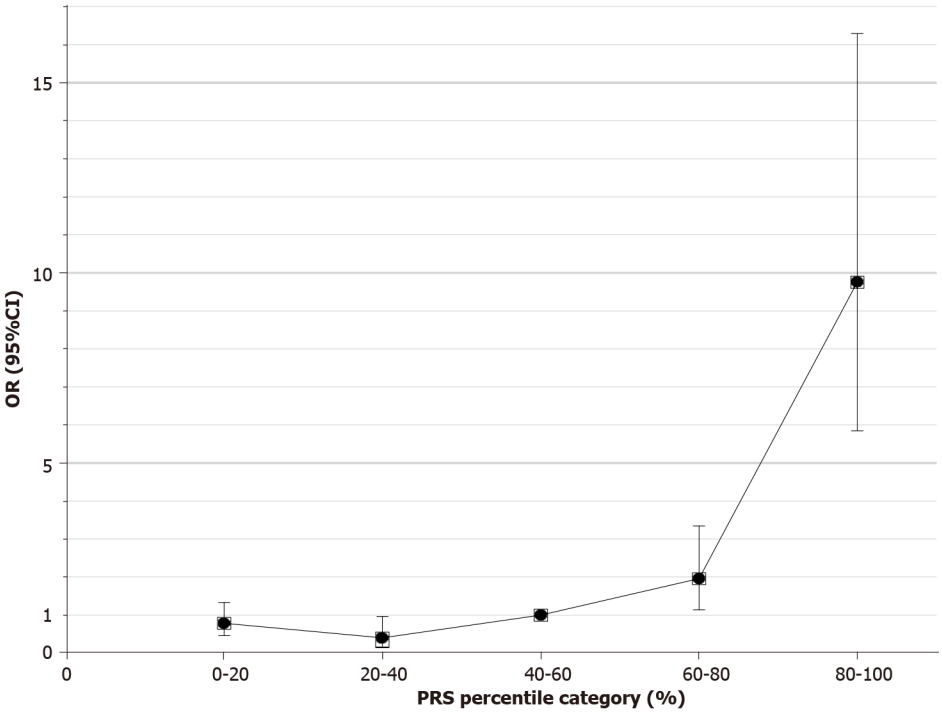


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Figure 2 Odds ratio and 95% confidence intervals (error bars) for percentiles of polygenic risk relative to the middle quintile. OR: Odds ratio; CI: Confidence interval; PRS: Polygenic risk score.

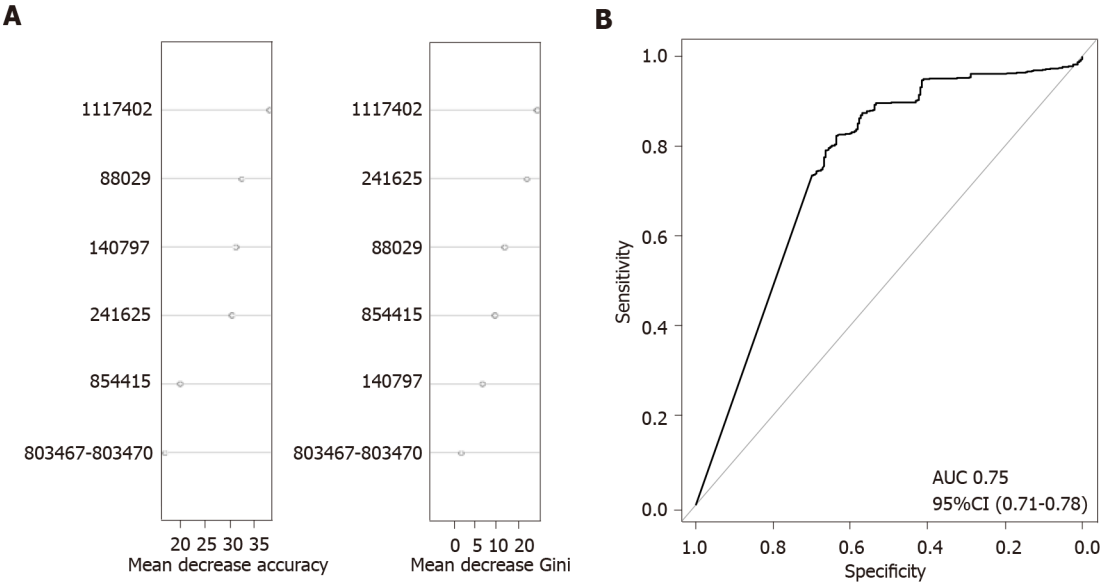

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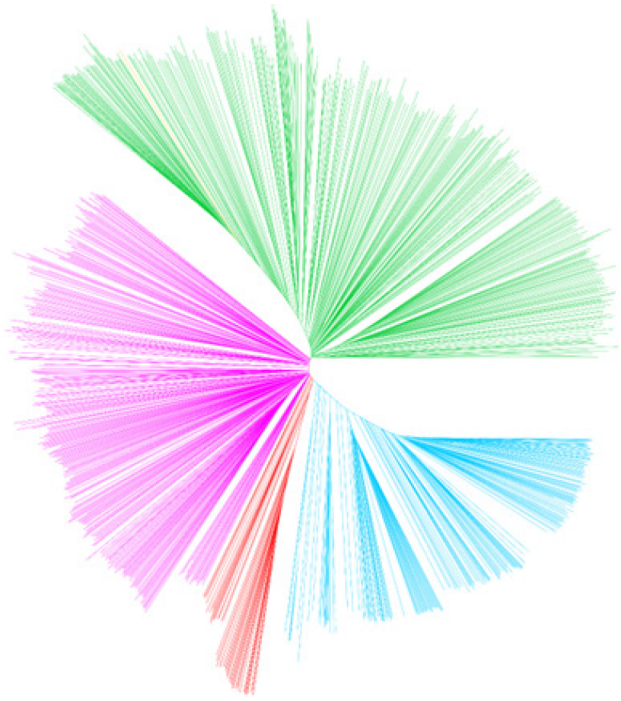
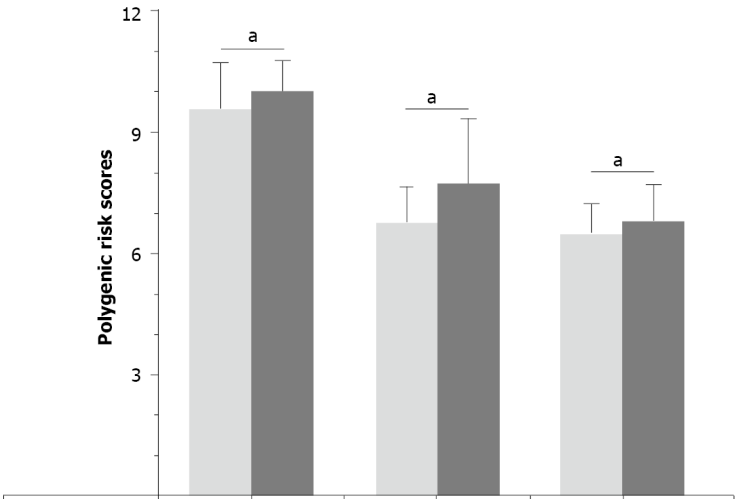
Figure 3 The importance of each validated single nucleotide polymorphisms and receiver operating characteristic curve for the polygenic risk score model on global strains. A: The median decrease in accuracy and median decrease in Gini coefficient of validated single nucleotide polymorphisms; B: Receiver operating characteristic curve of global strains. AUC: Area under the curve; CI: Confidence interval.

that only six of the SNPs showed a close association with gastric cancer in the global dataset. The SNPs at 88029, 241625, 803467 and 854415 in the reference strain 26695 caused nonsynonymous changes in the corresponding amino acid sequence, whereas the SNPs at 140797 and 1117402 in the reference strain 26695 produced synonymous variations. The *hpaA* gene, harbouring the SNP at 854415, encodes an adhesion gene of *H. pylori*[22]. This gene is essential for colonization and is associated with the occurrence of gastric cancer[23-25]. The SNP at 88029 was located on the *tlpC* gene. *TlpC* encodes a chemoreceptor that affects the chemotaxis of strains in the mouse gastric environment. It is associated with the induction of mucosal inflammation of the stomach[26,27]. The SNP at 241625 was located in



DOI: 10.4251/wjgo.v14.i9.1844

Figure 4 Neighbour-joining tree constructed from concatenated single nucleotide polymorphism sequences. Blue: hpEastAsia; Red: hpAsia2; Pink: hpEurope; Green: America-related populations; Yellow: Africa-related populations (strain hp_151, which was isolated in Morocco, was excluded from further analyses).



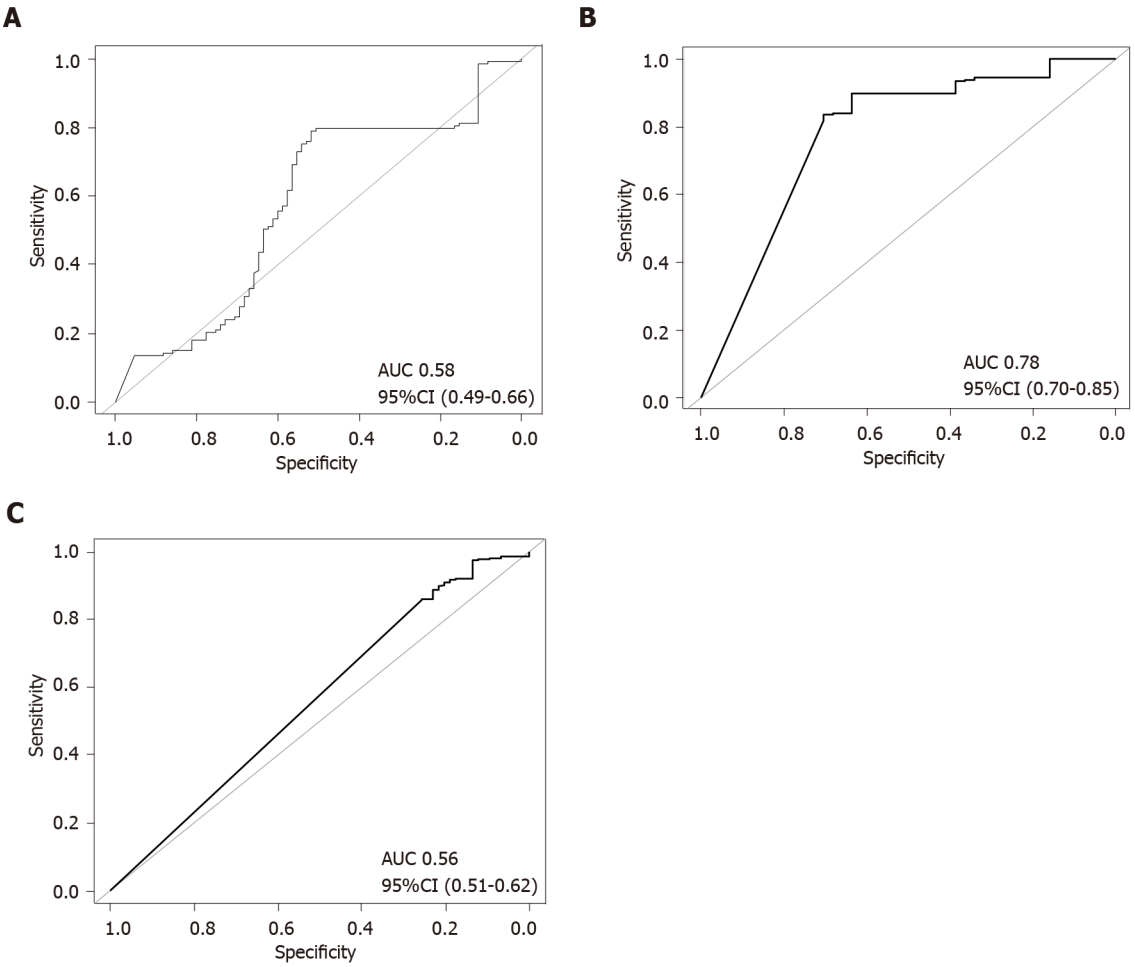
Disease	NC	GC	NC	GC	NC	GC
Number of strains	85	133	274	44	365	74
Groups	hpEastAsia		hpEurope		America-related populations	

DOI: 10.4251/wjgo.v14.i9.1844

Figure 5 Distribution of polygenic risk score for various groups. NC: Non-gastric cancer; GC: Gastric cancer. $^{a}P < 0.05$.

dsbG/K, which has protein disulfide isomerase activity. *DsbG/K* interacts with a virulence-related factor *in vitro*[28,29]. *In vitro* studies have shown that a lack of *dsbG/K* may cause the loss of T4SS function and inhibit *VacA* secretion, which are considered the main pathogenic factors in *H. pylori*[30].

In this study, we constructed a PRS model with six SNPs validated in a global dataset. Assessments of the performance of the PRS model demonstrated that the PRS value was significantly higher in the gastric cancer group than in the non-gastric cancer group. A significant increase in the risk of gastric cancer was found across the quintiles of the PRS. These findings demonstrate that the six-SNPs PRS model is capable of predicting the risk of gastric cancer. In support of this finding, RF analyses demonstrated that the combination of the six SNPs has a high predictive power for gastric cancer, with an AUC value of 0.75. In a recent report, a PRS model constructed with SNPs from the human genome



DOI: 10.4251/wjgo.v14.i9.1844

Figure 6 Receiver operating characteristic curve of the polygenic risk score model for various groups. A: Receiver operating characteristic curve (ROC) of hpEastAsia; B: ROC curve of hpEurope; C: ROC curve of America-related populations. AUC: Area under the curve; CI: Confidence interval.

showed unsatisfactory power in classifying gastric cancer from healthy controls, with an AUC value of 0.56[31]. It has been shown that a PRS model derived from 112 SNPs in the human genome and lifestyle factors possesses good predictive capacity for gastric cancer risk[32]. For individuals infected with *H. pylori*, assessment of their gastric cancer risk is of great concern in the clinical settings. Previous reports have demonstrated that certain genetic variants are associated with increased gastric cancer risk[9,10]. Our study, for the first time, demonstrated the combined effect of *H. pylori* genomic variations in the assessment of cancer risk. The PRS model derived from *H. pylori* SNPs would have a high capacity in predicting gastric cancer risk for patients infected with the pathogen. This will benefit the clinical management of the prognosis of the *H. pylori* infection. It is well known that age, gender and lifestyle factors, including alcohol consuming, smoking, diet habits and economic status, are closely associated with gastric cancer[33-35]. In the future, a PRS model constructed with *H. pylori* SNPs and those gastric cancer associated risk factors in this study would have substantially increased power in predicting the risk of gastric cancer. The *H. pylori* genome shows great variations between strains[36,37]. Genetic information differs greatly among *H. pylori* populations, and their carcinogenic potential is also different [5,21]. We thus evaluated the performance of the PRS model across *H. pylori* populations. Our results demonstrated a good predictive power of PRS for hpEurope strains.

A limitation of this study is that the performance of the PRS model was not assessed in hpAsia2 and Africa-related *H. pylori* populations because the number of strains with clinical information available was insufficient. Moreover, we could not consider age, gender, nutrition and other risk factors in the construction of the PRS model, as information on all of these risk factors was not consistently available across databases. A comprehensive risk model enclosing other risk factors of gastric cancer is indicated in future studies. Further *in vitro* and *in vivo* exploration of the roles of the combination of *H. pylori* SNPs identified in this study in gastric cancer would be much helpful in supporting our findings.

CONCLUSION

In summary, we constructed a PRS model based on *H. pylori* SNPs, which showed great potential in the prediction of gastric cancer risk globally, especially for individuals infected with hpEurope strains. Findings from this study demonstrated that the PRS model constructed from bacteria genomic variations, in addition to the PRS model established with human SNPs, can be of great value for disease risk prediction. In clinical practice, it is usually difficult to assess gastric cancer risk in patients infected with *H. pylori*. The model constructed in this study would be beneficial for solving this issue.

ARTICLE HIGHLIGHTS

Research background

Multiple single nucleotide polymorphisms (SNPs) of *Helicobacter pylori* (*H. pylori*) associated with gastric cancer have been identified through bacterial genome-wide association studies. Polygenic risk score (PRS) calculated as a sum of effect of SNPs provides a tool for assessing genetic impact on diseases.

Research motivation

Predicting risk of gastric cancer is a major concern in the management of the *H. pylori* infection.

Research objectives

This study constructed a PRS model based on *H. pylori* SNPs to predict the risk of gastric cancer.

Research methods

Associations between previously reported *H. pylori* SNPs and gastric cancer were validated in global strains. The PRS model based on the validated SNPs was evaluated by quintiles and random forest (RF) methods.

Research results

A PRS model was constructed with six validated SNPs. Quintiles and RF methods demonstrated the combination of six SNPs has a high predictive power for gastric cancer.

Research conclusions

PRS model constructed from bacterial genomic variations can be of great value for gastric cancer risk prediction.

Research perspectives

Comprehensive risk models including personal and genomic information need to be established in future studies.

FOOTNOTES

Author contributions: Yang C and Liang SZ collected sequencing data; Xu L and Yu MC analyzed the data; Wang XY wrote the manuscript; Wang LL and Wang YX wrote the discussion part of the manuscript; Dong QJ designed the research and supervised the manuscript; and all authors reviewed the manuscript and approved the final version of the manuscript.

Supported by the National Natural Science Foundation of China, No. 31870777.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Wang JJ

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World Journal of Gastrointestinal Oncology

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World J Gastrointest Oncol 2022 September 15; 14(9): 1856-1873

DOI: 10.4251/wjgo.v14.i9.1856

ISSN 1948-5204 (online)

META-ANALYSIS

Dissecting novel mechanisms of hepatitis B virus related hepatocellular carcinoma using meta-analysis of public data

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bredt LC, Brazil; Kao JT, Taiwan

Received: January 20, 2022
Peer-review started: January 20, 2022
First decision: April 17, 2022
Revised: April 26, 2022
Accepted: August 7, 2022
Article in press: August 7, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Hepatitis B virus (HBV) is a cause of hepatocellular carcinoma (HCC). Interestingly, this process is not necessarily mediated through cirrhosis and may in fact involve oncogenic processes. Prior studies have suggested specific oncogenic gene expression pathways were affected by viral regulatory proteins. Thus, identifying these genes and associated pathways could highlight predictive factors for HCC transformation and has implications in early diagnosis and treatment.

AIM

To elucidate HBV oncogenesis in HCC and identify potential therapeutic targets.

METHODS

We employed our Search, Tag, Analyze, Resource platform to conduct a meta-analysis of public data from National Center for Biotechnology Information's Gene Expression Omnibus. We performed meta-analysis consisting of 155 tumor samples compared against 185 adjacent non-tumor samples and analyzed results with ingenuity pathway analysis.

RESULTS

Our analysis revealed liver X receptors/retinoid X receptor (RXR) activation and farnesoid X receptor/RXR activation as top canonical pathways amongst others. Top upstream regulators identified included the Ras family gene rab-like protein 6 (RABL6). The role of RABL6 in oncogenesis is beginning to unfold but its specific role in HBV-related HCC remains undefined. Our causal analysis suggests RABL6 mediates pathogenesis of HBV-related HCC through promotion of genes related to cell division, epigenetic regulation, and Akt signaling. We conducted survival analysis that demonstrated increased mortality with higher RABL6 expression. Additionally, homeobox A10 (HOXA10) was a top upstream regulator and was strongly upregulated in our analysis. HOXA10 has recently been demonstrated to contribute to HCC pathogenesis *in vitro*. Our causal analysis suggests an *in vivo* role through downregulation of tumor suppressors and other mechanisms.

CONCLUSION

This meta-analysis describes possible roles of RABL6 and HOXA10 in the pathogenesis of HBV-related HCC. RABL6 and HOXA10 represent potential therapeutic targets and warrant further investigation.

Key Words: Hepatitis B virus; Hepatocellular carcinoma; Genomics; Meta-analysis

Core Tip: Hepatitis B virus (HBV) is a cause of hepatocellular carcinoma (HCC). Interestingly, this process is not necessarily mediated through cirrhosis and may in fact involve oncogenic processes. Prior studies have suggested specific oncogenic gene expression pathways were affected by viral regulatory proteins. Thus, identifying these genes and associated pathways could highlight predictive factors for HCC transformation, and has implications in early diagnosis and treatment. Our manuscript leverages big data to offer key insights to oncogenesis of HBV infection in HCC. We were able to dissect key genetic drivers to disease and namely demonstrate a newfound role for rab-like protein 6 and homeobox A10.

Citation: Aljabban J, Rohr M, Syed S, Cohen E, Hashi N, Syed S, Khorfan K, Aljabban H, Borkowski V, Segal M, Mukhtar M, Mohammed M, Boateng E, Nemer M, Panahiazar M, Hadley D, Jalil S, Mumtaz K. Dissecting novel mechanisms of hepatitis B virus related hepatocellular carcinoma using meta-analysis of public data. *World J Gastrointest Oncol* 2022; 14(9): 1856-1873

URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1856.htm

DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1856

INTRODUCTION

Hepatitis B virus (HBV) is a major cause of liver disease, significantly contributing to global morbidity and mortality. Recent estimates show there are approximately 240-300 million people chronically infected with HBV worldwide[1,2]. Chronic HBV infection leads to cirrhosis in 30% of patients, of which 53% later develop hepatocellular carcinoma (HCC)[3]. The Global Burden of Disease Study estimated that HBV-related cirrhosis and liver cancer annually causes 786000 deaths worldwide[4]. HBV utilizes both direct and indirect means to promote HCC. For example, HBV-induced HCC, without cirrhosis suggests involvement of oncogenic pathways independent of chronic liver inflammation. Some studies have implicated viral regulatory proteins such as HBV X protein affecting gene expression pathways[5]. In addition, mutations resulting in growth advantages may be conferred to infected cells by virtue of host chromosomal HBV DNA integration - a phenomenon known as insertional mutagenesis[5]. In the decades since Garcia *et al*[6] and Wang *et al*[7] identified cyclin (CCN) A and retinoic acid receptor-beta genes, respectively, as targets of HBV integration, other points of vulnerability have been identified. Li *et al*[8] found HBV integration into telomerase reverse transcriptase-promoter genes results in sex hormone-dependent responsiveness, providing a possible explanation for the threefold male-to-female preponderance of HBV-related HCC[6-8].

Despite advancements in nucleoside nucleotide analog (NA)-based treatment, and adequate viral suppression resulting in undetectable HBV DNA, patients are still at risk for developing HCC. This may be due to NAs ability to suppress viremia but not eliminate infection and leading to oncogenesis. Importantly, chronic HBV infection, irrespective of cirrhosis represents a lifetime risk for HCC development 10-25 times greater than non-infected patients[1,5,9]. These data highlight the importance of identifying predictive factors for HCC transformation, non-resolving acute infections, and chronic disease development. Understanding the evolution of HCC following HBV infection, and genetic signatures of HBV oncogenicity, will pave the way for improved risk assessment, treatment options, and patient outcomes. In this meta-analysis, we aim to identify transcriptomic correlates of HCC development in patients with HBV infection.

MATERIALS AND METHODS

Search Tag Analyze Resource

We developed the Search Tag Analyze Resource (STARGEO) platform to utilize the wealth of genomics data featured in the National Center for Biotechnology Information's Gene Expression Omnibus. STARGEO allows for meta-analysis of transcriptomic signatures between sample sets, such as between disease and normal tissue, through tagging of biological samples from public data. Briefly, through stargeo.org, we searched for studies that studied HBV-related HCC. We then manually curated samples through the "Tagging" interface built into STARGEO based on interactive regular expressions. We gathered liver tumor samples under the "HBV_HCC" tag and control samples from adjacent tumor samples under the "HCC_Control" tag. More information on STARGEO and can be found in our previous paper[10]. To investigate HBV-specific HCC, we tagged 155 tumor and 185 adjacent non-tumor samples as a control. Samples were paired 1:1 within each study. Data was sourced from the GSE19665, GSE44074, GSE55092, GSE62232, and GSE67764 series[10-15]. HCC patients in these studies had confirmed chronic HBV infection with no other co-morbid hepatic infections and had biopsied taken at time of diagnosis. Genetic analysis focused on hepatocytes. Stargeo.org mappings are based on mygene.info gene annotation service to map all probe identifiers to Entrez gene identifiers[16]. The mean difference of contrasts for expressions and the standard deviation of that mean difference were calculate for each gene in every study. Standard meta-analysis with fixed and random effects model were used to combine these estimates across studies to generate both meta *P* values and effects size across studies. Study weight percentages were calculated using the inverse variance method *via* the DerSimonian-Laird estimate[17]. We use Python to achieve the analyses explained above in stargeo.org. More information on this particular analysis of HBV-related HCC can be found on http://stargeo.org/analysis/669/. Individual genes can be searched and the number of patient samples in which we observed change in genetic expression is available, along with other information (see Figure 1). Lastly, to best contextualize our results we cited high quality articles in *Reference Citation Analysis* (https://www.referencecitationanalysis.com).

Ingenuity pathway analysis

In order to dissect potential mechanism of disease, potential biomarkers, and therapeutic targets, we extracted more than 21000 genes for our meta-analysis and analyzed the output using the ingenuity pathway analysis (IPA) tool[18]. Analysis was restricted to genes that showed statistical significance ($P < 0.05$) in both fixed and random effects models with an absolute experimental log ratio greater than 0.7 between experimental (HCC) and control samples. A total of 1035 genes were included in the IPA analysis. Top up- and downregulated genes determined by STARGEO are featured in Table 1. Genes analyzed by IPA are summarized in Supplementary Table 1 with *P* values and experimental log ratios.

Table 1 Summary of the most up and down-regulated genes from the meta-analysis of primary tumor samples from hepatocellular carcinoma patients, experimental log ratios indicating magnitude of change from control samples are shown

Top upregulated		Top downregulated	
GPC3	2.426	CLEC4G	-4.212
ANLN	2.398	CLEC1B	-4.122
CCNB1	2.239	LINC01092	-3.834
ASPM	2.220	SLCO1B3	-3.788
CDK1	2.215	CLEC4M	-3.689
NEK2	2.121	HAMP	-3.657
EPS8L3	2.116	STAB2	-3.604
PBK	2.017	OIT3	-3.575
DTL	2.010	MT1M	-3.508
PRC1	1.993	HHIP	-3.417

GPC3: Glypican 3; ANLN: Anillin; CCNB1: Cycline B1; ASPM: Abnormal spindle-like microcephaly-associated protein; CDK1: Cyclin-dependent kinase 1; NEK2: NIMA related kinase 2; EPS8L3: Epidermal growth factor receptor kinase substrate 8-like protein 3; PBK: PDZ binding kinase; DTL: Denticleless E3 ubiquitin protein ligase homolog; PRC1: Protein regulator of cytokinesis 1; CLEC4G: C-type lectin domain family 4 member G; CLEC1B: C-type lectin domain family 1 member B; LINC01092: Long intergenic non-protein coding RNA 1092; SLCO1B3: Solute carrier organic anion transporter family member 1B3; CLEC4M: C-type lectin domain family 4 member M; HAMP: Hepcidin; STAB2: Stabilin 2; OIT3: Oncoprotein induced transcript 3; MT1M: Metallothionein 1M; HHIP: Hedgehog interacting protein.

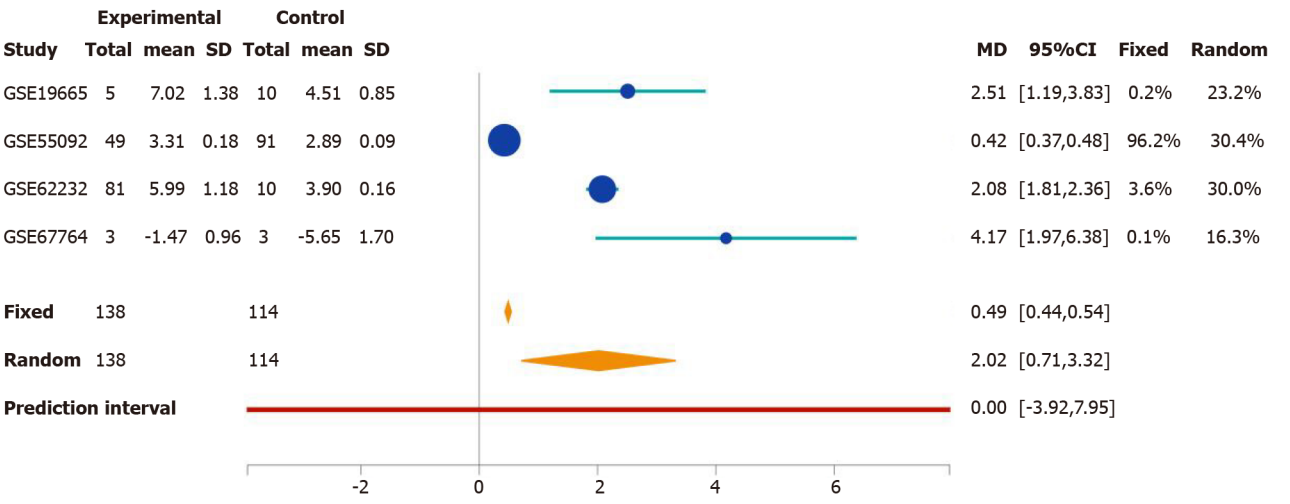

Figure 1 Screenshot from http://stargeo.org/analysis/669/ detailing the expression patterns of PDZ-binding kinase across different studies as shown. Fixed and random treatment effects are illustrated. MD: Mean difference; CI: Confidence interval. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

HCC is a complex disease that is a result of several pathological drivers. We used IPA Upstream Regulator analysis to elucidate upstream transcription regulators that best reflect our observed genetic expression dataset[18]. The *P* values are based on the degree of overlap of known effector targets and our gene list submission. The activation z-score illustrates the upstream regulator activation state, the magnitude of which represents likely activation states of upstream regulators.

Survival analysis

The association between rab-like protein 6 (RABL6) expression in HCC patients and survival was found using GPEIA2[19]. GPEIA2 uses samples from The Cancer Genome Atlas (TCGA) HCC cohort and analyzes survival based on the Kaplan-Meier survival method. Expression was split into the top 75% and bottom 25% of RABL6 expression. There were no interactions with human subjects or interventions involved in this study. Additionally, as all content presented is sourced from publicly available data and no patient-protected information was used. Therefore, no institutional review board approval was

deemed necessary.

RESULTS

Top canonical pathways and gene candidates from HBV-HCC analysis

IPA analysis demonstrated liver X receptor (LXR)/retinoid x receptor (RXR) activation, lipopolysaccharide-interleukin-1 (LPS-IL-1) mediated RXR inhibition, acetone degradation, melatonin degradation, and farnesoid X receptor (FXR)/RXR activation as top canonical pathways in HBV-associated HCC (see Supplementary Table 2 for more information on top canonical pathways). FXR (NR1H4) was downregulated in our analysis suggesting its inhibition (Supplementary Table 2).

Top upregulated genes in our analysis are implicated in several signaling processes (Table 1). For example, glypican-3 (GPC3), a cell surface glycoprotein that interacts with Wnt/β-catenin, Yes associated protein, and Hedgehog pathways[20]. Additionally, Akt signaling was activated through the epidermal growth factor receptor kinase substrate 8-like protein 3, a substrate for the epidermal growth factor receptor[21]. Furthermore, we found upregulation of the serine/threonine kinase PDZ-binding kinase (PBK), a mitotic kinase related to mitogen-activated protein kinase kinase (MAPKK)[22]. Other top regulated genes are involved in cell cycle division and control including anilin, CCNB1, assembly factor for microtubules, cyclin dependent kinase 1, and protein regulator of cytokinesis 1. Moreover, we found upregulation of the serine/threonine kinase NIMA related kinase a mediator of centrosome separation in mitosis and meiosis, and deniticleless, an adaptor of the E3-ubiquitin ligase that targets p21 to drive cell division[23].

Top downregulated genes are involved in liver metabolism and other processes. Several members of the C-type lectin family CLEC4G, CLEC1B, and CLEC4M were found among the most repressed genes. C-type lectins participate in adhesion and can act as signaling receptors for inflammation and immune-related processes[24]. Other downregulated genes are indicative of impaired liver function including the liver specific anion transporter solute carrier organic anion transporter family member 1B3, iron regulator hepcidin (HAMP), and the metallothionen (MT1M)[25]. Lastly, we found downregulation of Hh-interacting protein (HHIP), a negative regulator of Hedgehog signaling[26].

Network analysis of HBV-HCC

To elucidate top disease functions from our results we employed the IPA Network analysis function [18]. IPA ranks networks from the Global Molecular Network based on the number of focus genes from given networks that match with our analysis. Significance is given by the p-score [p-score = -log10(*P* value)]. We identified 25 networks with most being involved in cancer, cell cycle, gastrointestinal disease, and other disease functions. The top 6 networks are summarized in Table 2 and the top network is illustrated in Figure 2 (see Supplementary Table 3 for all networks).

Analysis of potential drivers of HBV-HCC pathogenesis

To investigate genes that most greatly influenced our gene dataset and oncogenesis we used IPA to identify top upstream regulators (see methods). We searched for genes that were the identified as the most activated up-regulators in IPA and were upregulated in our dataset (see Supplementary Table 4). The most activated upstream regulator was RABL6, a member of the Ras family of GTPases[27]. We also found activation of transcription factors T-box transcription factor 2 (TBX2) involved in hepatocyte proliferation, migration, and invasion[28]. We also noted activation of E2F transcription factor 1 (E2F1), which has a mixed but predominantly proliferative role in HCC[29]. Additionally, we found activation of the transcription factor forkhead box M1 (FOXM1), which promotes cell turnover through CCNB1 and CCND1 upregulation and through other mechanisms[30,31]. Other activated transcription factors implicated in tumor activity included RUNX transcription factor 1, melanocyte inducing transcription factor, and homeobox A10 (HOXA10)[32-35]. Moreover, other top upstream regulators demonstrated varied biologic activity. For example, E1A binding protein P400 (EP400), a component of the NuA4 histone acetyltransferase complex, is associated with epigenetic activity[36]. Other upstream regulators are proteins involved in signaling pathways including actin like 6a, an actin binding protein involved in Notch1/SOX2 signaling, LIN9-MYBL2 [interacts with the tumor suppressors such as retinoblastoma (Rb) protein], SHC adaptor protein 1 (SHC1, a signaling adaptor for growth factor receptors), protein inhibitor of activated STAT 4 (PIAS4, a protein inhibitor of STAT), and Tumor necrosis factor (TNF) receptor associated factor 2 [TRAF2, role in TNF and nuclear factor-kappaB (NF-κB)][37-42]. We also found activation of RNA binding proteins including ELAVL1, a regulator of ferroptosis in hepatic stellate cells, as well as oncogenic members of the negative elongation factor family NELFE, NELFA, and NELFCD[43]. Lastly, we found activation of oncogenesis-promoting kinases such as mitogen activated kinase 4 and neurotrophic receptor kinase 2 (NTRK2), which aid in cell adhesion[44,45].

We next focused on inhibited upstream regulators (Supplementary Table 5), several of which are implicated in the innate immune response including the innate receptor DExD/H-Box helicase 58 (DDX58), or RIG-I, and the pattern recognition and toll-like receptors (TLR)2, TLR3, TLR4, and TLR9[46,47]. There was predicted inhibition and downregulation of inflammatory mediators NF-κB and

Table 2 Top disease and functions identified by ingenuity pathway analysis network analysis

Top disease and function	P-score	Focus genes	Genes in network
Cell cycle; cellular assembly and organization; DNA replication, recombination and repair	46	33	BUB1, BUB1B, C4, C4BP, CENPA, CENPH, CENPK, CENPL, CENPM, CENPW, CNDP1, DSN1, ESR1, FCN2, FCN3, GGT5, HIST1H2BF, HJURP, HPS5, KNL1, LILRB5, MASP1, MASP2, MBL2, MND1, MPC1, NDC80, NUF2, OIP5, OVOS2, SLC1A4, TENM1, TUFT1, WHRN, XK
Cancer; cell death and survival; organismal injury and abnormalities	42	31	ANKS6, Ap1, AURKA, B9D1, BCKDHB, BOLA2/BOLA2B, CA5A, CBX5, CCT3, CDK1, CDKN2A, CEMIP, EPB41L5, estrogenreceptor, ETFRF1, EZH2, H2AFX, HIST1H2AM, HMGA1, KIF11, MCM2, MFAP4, MKI67, NAT2, NGFR, NT5DC2, PRKDC, Rnr, SETDB1, Smad2/3, TCF19, TK1, TMEM131L, TUBE1, ZSWIM5
Cancer; cell cycle; cellular movement	42	31	ATAD2, ATPase, BMP, BMP5, CCBE1, CEP55, CTH, DTL, ECT2, GORASP2, IGF2BP1, IL12 (family), IL18R1, IL1RAP, IPO9, KIF14, KIF23, LUM, MAP1LC3, MSH2, NAAA, NUP62, OLA1, PBLD, PLSCR4, RAD54B, SIGLEC1, STAU2, TEX37, TRAF5, TRIP13, VSIG2, VWA8, WDYHV1, XPO5
Cell morphology, cellular assembly and organization, DNA replication, recombination, and repair	39	30	ACAA2, ARMC6, BCHE, C4BPA, CDCA3, CDCA8, CENPE, CENPF, Ciap, CRNDE, ENO3, Enolase, FOXM1, KALRN, KIF20A, KIF2C, KIF4A, LRAT, MAGEA3/MAGEA6, MZT1, NAV1, NEB, PRC1, RAS, RASGRP2, RASSF4, SESTD1, SGO2, SRD5A1, SRD5A2, Steroid 5 alpha-Reductase, TARBP1, TGM3, transglutaminase, TRIO
Cancer; organismal injury and abnormalities; reproductive system disease	39	30	ANGPTL6, BMPER, Cysteine Protease, DPF3, EGLN, FNIP2, GCDH, GDF2, Granzyme B-Perforin-SRGN, GREM2, HMGCL, HOXA13, KIF15, LYVE1, MS4A7, MT1G, NOSTRIN, PCDH9, PDE7B, PLVAP, RNF125, RNF165, RRAGD, SERPINB9, SESN3, SLC7A2, Smad1/5/8, SPARCL1, SRGN, STC1, TPX2, TRIM16, Vegf, VSIG4, ZFP
Cancer, gastrointestinal disease, hepatic system disease	37	29	AKR1D1, ALDH, ALDH1A3, ALDH6A1, ALDH8A1, ANK3, CA2, COBLL1, CYP39A1, ENAH, ESM1, FOS, GBA, GLS2, GPM6A, GPSM2, GRHPR, GUCY1A1, HIST1H3H, histone-lysine N-methyltransferase, HOOK1, MECOM, NCKAP1L, NSD2, PALM2, PXMP2, Rab11, RAB11FIP4, sGC, SLC1A1, SMYD3, Sos, TSKU, UXS1

P-score indicates statistical significance [p-score = -log10(*P* value)] and the number of focus genes indicates the number of genes in our analysis that are a part of the respective network. Genes that are labeled red are upregulated in our analysis and genes that are green are downregulated. Genes in black are a part of the network but were not featured in our results. For *P* values and experimental log ratios of genes see Supplementary Table 1. TENM1: Teneurin transmembrane protein 1; GGT5: Gamma-glutamyltransferase5; SLC1A4: Solute carrier family 1 member 4; OVOS2: Alpha-2-macroglobulin like 1 pseudogene; XK: X-linked Kx blood group; WHRN: Whirlin; MPC1: Mitochondrial pyruvate carrier 1; LILRB5: Leukocyte immunoglobulin like receptor B5; CNDP1: Carnosine dipeptidase 1; CENPM: Centromere protein M; CENPL: Centromere protein L; ESR1: Estrogen receptor 1; C4BP: C4b-binding protein; TUFT1: Tuftelin 1; DSN1: MIND kinetochore complex component; KNL1: Kinetochore scaffold 1; BUB1: Mitotic checkpoint serine/threonine-protein kinase BUB1; HPS5: Hermansky-Pudlak syndrome 5 protien; FCN3: Ficolin 3; MASP1: MBL associated serine protease 1; MBL2: Mannose binding lectin 2; HIST1H2BF: Histone H2B type 1; OIP5: Opa interacting protein 5; NDC80: Kinetochore protein NDC80; BUB1B: BUB1 mitotic checkpoint serine/threonine kinase B; MND1: Meiotic nuclear divisions 1; CENPW: Centromere protein W; CENPH: Centromere protein H; CENPK: Centromere protein K; CENPA: Centromere protein A; ANKS6: Ankyrin repeat and SAM domain-containing protein 6; Ap1: Activator protein 1; AURKA: Aurora A kinase; B9D1: B9 domain containing 1; BCKDHB: 2-Oxoisovalerate dehydrogenase subunit beta; BOLA2/BOLA2B: BolA family member 2; CA5A: Carbonic anhydrase 5A; CBX5: Chromobox protein homolog 5; CCT3: Chaperonin containing TCP1 complex; CDK1: Cyclin-dependent kinase 1; CDKN2A: Cyclin dependent kinase inhibitor 2A; CEMIP: Cementum protein 1; EPB41L5: Erythrocyte membrane protein band 4.1 like 5; ETFRF1: Electron transfer flavoprotein regulatory factor 1; EZH2: Enhancer of zeste 2 polycomb repressive complex 2 subunit; H2AFX: H2A histone family, member X; HIST1H2AM: Histone cluster 1, H2am; HMGA1: High mobility group AT-hook 1; KIF11: Kinesin family member 11; MCM2: Mini-chromosome maintenance complex component 2; MFAP4: Microfibril associated protein 4; MKI67: Marker of proliferation Ki-67; NAT2: N-acetyltransferase 2; NGFR: Nerve growth factor receptor; NT5DC2: 5'-nucleotidase domain containing 2; PRKDC: Protein kinase, DNA-activated, catalytic subunit; Rnr: Ribonucleotide reductase; SETDB1: SET domain bifurcated histone lysine methyltransferase 1; Smad2/3: SMAD family member 2/3; TCF19: Transcription factor 19; TK1: Thymidine kinase 1; TMEM131L: Transmembrane 131 like; TUBE1: Tubulin epsilon 1; ZSWIM5: Zinc finger SWIM-type containing 5; ATAD2: ATPase family AAA domain containing 2; BMP: Bone morphogenetic protein; BMP5: Bone morphogenetic protein 5; CCBE1: Collagen and calcium binding EGF domains 1; CEP55: Centrosomal protein 55; CTH: Cystathionine gamma-lyase; DTL: Denticleless E3 ubiquitin protein ligase homolog; ECT2: Epithelial cell transforming 2;

GORASP2: Golgi reassembly stacking protein 2; IGF2BP1: Insulin like growth factor 2 mRNA binding protein 1; IL12 (family): Interleukin-12; IL18R1: Interleukin-18 receptor 1; IL1RAP: Interleukin-1 receptor accessory protein; IPO9: Importin 9; KIF14: Kinesin-like protein 14; KIF23: Kinesin-like protein 23; LUM: Lumican; MAP1LC3: Microtubule-associated protein 1 light chain 3 beta; MSH2: MutS homolog 3; NAAA: N-acylethanolamine acid amidase; NUP62: Nucleoporin 62; OLA1: Obg like ATPase 1; PBLD: Phenazine biosynthesis like protein domain containing; PLSCR4: Phospholipid scramblase 4; RAD54B: RAD 54 homolog B; SIGLEC1: Sialic acid binding Ig like lectin 1; STAU2: Staufen double-stranded RNA binding protein 2; TEX37: Testis expressed 37; TRAF5: TNF receptor associated factor 5; TRIP13: Thyroid hormone receptor interactor 13; VSIG2: V-set and immunoglobulin domain containing 2; VWA8: Von Willebrand factor A domain containing 8; WDYHV1: WDYHV motif containing 1; XPO5: Exportin 5; ACAA2: Acetyl-CoA acyltransferase 2; ARMC6: Armadillo repeat containing 6; BCHE: Butyrylcholinesterase; C4BPA: Complement component 4 binding protein alpha; CDCA3: Cell division cycle associated 3; CDCA8: Cell division cycle associated 8; CENPE: Centromere protein E; CENPF: Centromere protein F; CRNDE: Colorectal neoplasia differentially expressed; ENO3: Enolase 3; FOXM1: Forkhead box M1; KALRN: Kalirin RhoGEF kinase; KIF20A: Kinesin family member 20A; KIF2C: Kinesin family member 2C; KIF4A: Kinesin family member 4A; LRAT: Lecithin-retinol acyltransferase; MAGEA3/MAGEA6: MAGE family member A3/A6; MZT1: Mitotic spindle organizing protein 1; NAV1: Neuron navigator 1; NEB: Nebulin; PRC1: Protein regulator of cytokinesis 1; RAS: RAS GTPase; RASGRP2: RAS guanyl releasing protein 2; RASSF4: Ras association domain family member 4; SESTD1: SEC14 and spectrin domain containing 1; SGO2: Shugoshin 2; SRD5A1: steroid 5 alpha-reductase 1; SRD5A2: Steroid 5 alpha-reductase 5; TARBP1: TAR RNA binding protein 1; TGM3: Transglutaminase 3; TRIO: Trio Rho guanine nucleotide exchange factor; ANGPTL6: Angiopoietin like 6; BMPER: BMP-binding endothelial regulator; DPF3: Double PHD fingers 3; EGLN: Endoglin; FNIP2: Folliculin interacting protein 2; GCDH: Glutaryl-CoA dehydrogenase; GDF2: Growth differentiation factor 2; GREM2: Gremlin 2; HMGCL: 3-hydroxy-3-methylglutaryl-CoA lyase; HOXA13: Homeobox A13; KIF15: Kinesin family member 15; LYVE1: Lymphatic vessel endothelial hyaluronan receptor 1; MS4A7: Membrane spanning 4-domains A7; MT1G: Metallothionein 1G; NOSTRIN: Nitric oxide synthase trafficking; PCDH9: Protocadherin 9; PDE7B: Phosphodiesterase 7B; PLVAP: Plasmalemma vesicle associated protein; RNF125: Ring finger protein 125; RNF165: Ring finger protein 165; RRAGD: Ras related GTP binding D; SERPINB9: Serpin family B member 9; SESN3: Sestrin 3; SLC7A2: Solute carrier family 7 member 2; Smad1/5/8: SMAD family member 1/5/8; SPARCL1: SPARC like 1; SRGN: Serglycin; STC1: Stanniocalcin; TPX2: TPX2 microtubule nucleation factor; TRIM16: Tripartite motif containing 16; Vegf: Vascular endothelial growth factor; VSIG4: V-set and immunoglobulin domain containing 4; ZFP: Zinc finger protein; AKR1D1: Aldo-keto reductase family 1 member D1; ALDH: Aldehyde dehydrogenase; ALDH1A3: Aldehyde dehydrogenase 1 family member A3; ALDH6A1: Aldehyde dehydrogenase 6 family member A1; ALDH8A1: Aldehyde dehydrogenase 8 family member A1; ANK3: Ankyrin 3; CA2: Carbonic anhydrase 2; COBLL1: Cordon-bleu WH2 repeat protein like 1; CYP39A1: Cytochrome P450 family 39 subfamily A member 1; ENAH: ENAH actin regulator; ESM1: Endothelial cell specific molecule 1; FOS: Fos proto-oncogene, AP-1 transcription factor subunit; GBA: Glucosylceramidase beta; GLS2: Glutaminase 2; GPM6A: Glycoprotein M6A; GPSM2: G protein signaling modulator 2; GRHPR: Glyoxylate and hydroxypyruvate reductase; GUCY1A1: Guanylate cyclase 1, soluble, alpha 1; HIST1H3H: Histone cluster 1 H3 family member h; histone-lysine N-methyltransferase; HOOK1: Hook microtubule tethering protein 1; MECOM: MDS1 and EVI1 complex locus; NCKAP1L: NCK associated protein 1 like; NSD2: Nuclear receptor binding SET domain protein 2; PALM2: Paralemmin 2; PXMP2: Peroxisomal membrane protein 2; Rab11: Rab 11 protein; RAB11FIP4: RAB11 family interacting protein 4; sGC: Soluble guanylyl cyclase; SLC1A1: Solute carrier family 1 member 1; SMYD3: SET and MYND domain containing 3; Sos: Son of Sevenless; TSKU: Tsukushi, small leucine rich proteoglycan; UXS1: UDP-glucuronate decarboxylase 1.

interleukins interleukin (IL)-5, IL-12, IL-18, and IL-33. We also found downregulation and predicted inhibition of the immune co-stimulatory molecule CD86, as well as forkhead head transcription factor FOXO3, a mediator of the antioxidant response and autophagy[48]. Several other inhibited upstream regulators are well-described tumor suppressors such as TP53, CDKN1A, Rb gene, and Rb transcriptional suppressor type 2[49]. Additionally, we found predicted inhibition of hepatocyte nuclear factors HNF4 and HNF41A, and the LXR NR1H3, which have been shown to exhibit anti-tumor activity[50-53]. We also found downregulation and predicted inhibition of the transcription factor CCAAT enhancer binding protein delta (CEBPD), a regulator of apoptosis and potential tumor suppressor[54]. Lastly, we observed downregulation and predicted inhibition of the SAM domain, SH3 domain, and nuclear localization signals 1 (SAMSN1), a lung cancer tumor suppressor that is hypermethylated in HCC[55, 56].

Causal networks of HBV-HCC

To elucidate the pathologic potential of the upstream regulators described above, we assessed downstream effector genes through IPA. We focused on upstream regulators with high activation z-scores. We first investigated RABL6 as it is the most activated upstream regulator in our analysis and its target genes play important roles in HBV-HCC (see Supplementary Table 4). Most of the activated genes are involved in promoting cell division. For example, the mitotic spindle checkpoint genes BUB1 and BUB1B, several cyclins including CCNA2, CCNB2, and CCNE2, and the M-phase inducer CDC25C were all upregulated[57]. Likewise, we observed upregulation of centromere protein F, helicase RAD54B, and topoisomerase TOP2A. We also found upregulation of the mitogen PBK, NEK2 (a regulator of mitotic progression), and the kinase TTK, all of which promote HCC cell proliferation and migration *via* Akt signaling[21,22,58]. Similarly, we found upregulation of minichromosome maintenance family (MCMs) members MCM2 and MCM10, which also promote cell division[59-61]. There are also downstream genes of RABL6 with recently described roles in cancer. For example, we found upregulation of the ubiquitin-conjugating enzyme E2C. Knockdown of this gene has been shown to suppress cellular proliferation, migration, and invasion in HCC[62]. Lastly, RABL6 may mediate HCC progression through downregulation of enhancer of zeste homolog 2, which regulates histone and DNA methylation and silences tumor suppressors[63,64]. Thus, our analysis suggests RABL6 promotes HCC through several pro-oncogenic mechanisms. A summary of the downstream effects of RABL6 are presented in Figure 3.

Since RABL6 has not been described in HCC, we conducted survival analysis using the GEP1A2 platform. GEP1A2 is a website that uses patient samples from TCGA, which is used for bioinformatic analysis on genes of interest among differ cancer types[19]. We examined prognosis based on quartile

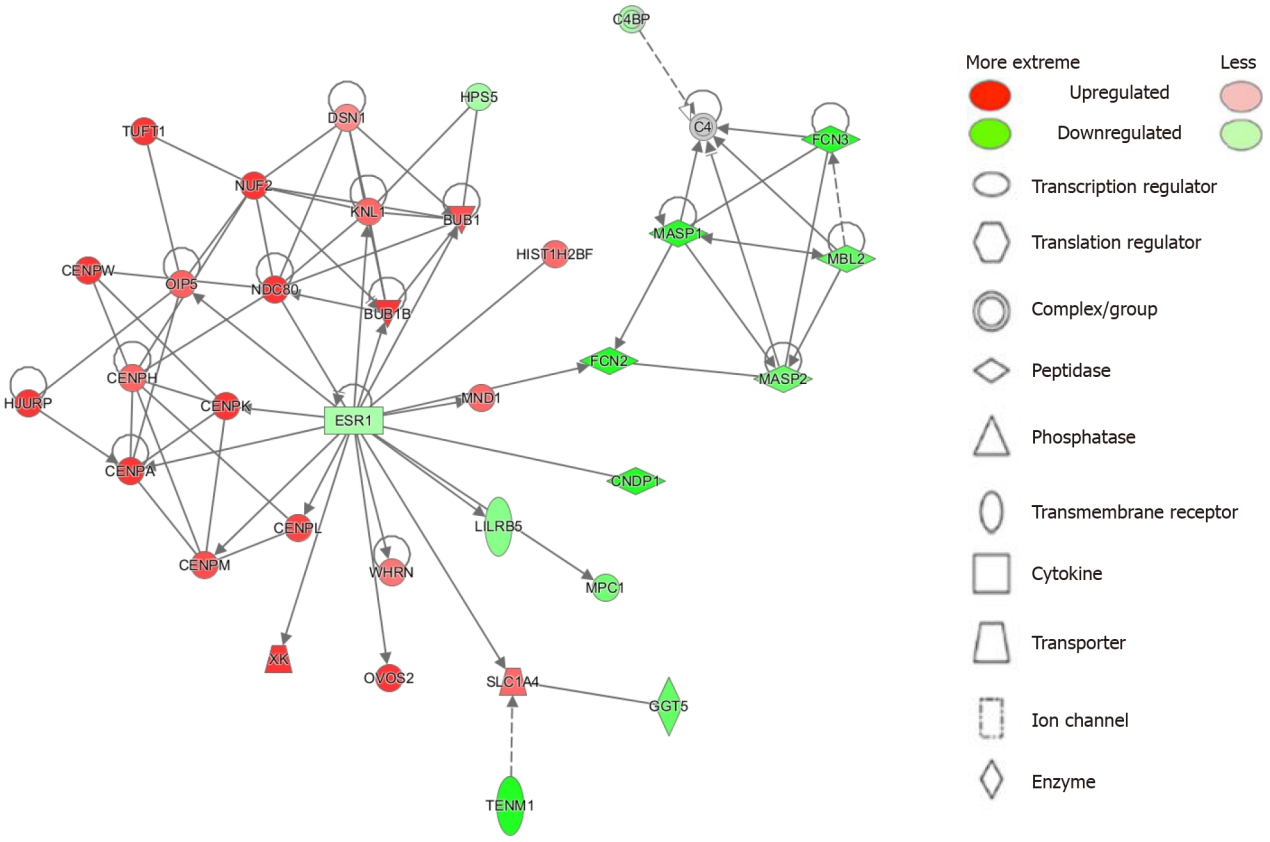

Figure 2 Top network (cell cycle; cellular assembly and organization; DNA replication, recombination and repair) identified by ingenuity pathway analysis Network analysis. Legend illustrates class of the gene. Red indicates upregulation and green downregulation, with shade depicting magnitude of change. Solid and dashed lines depict direct and indirect, respectively, relationship between genes. Figure was generated using ingenuity pathway analysis. TENM1: Teneurin transmembrane protein 1; GGT5: Gamma-glutamyltransferase5; SLC1A4: Solute carrier family 1 member 4; OVOS2: Alpha-2-macroglobulin like 1 pseudogene; XK: X-linked Kx blood group; WHRN: Whirlin; MPC1: Mitochondrial pyruvate carrier 1; LILRB5: Leukocyte immunoglobulin like receptor B5; CNDP1: Carnosine dipeptidase 1; CENPM: Centromere protein M; CENPL: Centromere protein L; ESR1: Estrogen receptor 1; C4BP: C4b-binding protein; TUFT1: Tuftelin 1; DSN1: MIND kinetochore complex component; KNL1: Kinetochore scaffold 1; BUB1: Mitotic checkpoint serine/threonine-protein kinase BUB1; HPS5: Hermansky-Pudlak syndrome 5 protien; FCN3: Ficolin 3; MASP1: MBL associated serine protease 1; MBL2: Mannose binding lectin 2; HIST1H2BF: Histone H2B type 1; OIP5: Opa interacting protein 5; NDC80: Kinetochore protein NDC80; BUB1B: BUB1 mitotic checkpoint serine/threonine kinase B; MND1: Meiotic nuclear divisions 1; CENPW: Centromere protein W; CENPH: Centromere protein H; CENPK: Centromere protein K; CENPA: Centromere protein A. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

expression of RABL6 (upper 75% *vs* lower 25%) in HCC patients. We found a statistically lower chance of survival with higher expression of RABL6.

The upstream regulators TBX2, E2F1, FOXM1, and EP400 share similar activated genes to those described for RABL6 (see Supplementary material), such as HOXA10, and thus may act synergistically to promote HBV-HCC. This prompted us to next study activated genes downstream of HOXA10 due to its stark upregulation and high activation z-score (see Supplementary Table 6). While role of HOXA10 is not well-defined in HCC, knockdown model has been recently shown to inhibit HCC cell tumorigenesis [35]. Additionally, another study by Shao *et al*[65] demonstrated the involvement of HOXA10 in the renewal and survival of liver tumor initiation cells. IPA identified several genes downstream of HOXA10 that may explain its pathogenic activity. For example, HOXA10 may induce tumor progression through downregulation of the tumor suppressor gene NDRG2 as well as glutathione S-transferase A3 or GSTA3, whose inactivity results in hepatocyte oxidative stress and liver injury[66,67]. We also found upregulation of dickkopf-1, a negative regulator of Wnt signaling and negative prognosticator for HCC[68]. Insulin-like growth factor binding protein-3 is a potential mediator of growth suppression signals and is downregulated in our dataset[69]. The hepatic enzyme CYP2E1 was similarly downregulated and is known to be repressed in HCC and linked with a poor prognosis[70]. Lastly, xanthine dehydrogenase, a rate-limiting enzyme in purine metabolism, was downregulated and its suppression has been linked to enhanced cancer stem-cell activity in HCC[71]. A suggested model of the potential multifactorial role HOXA10 and its interplay in HCC is shown in Figure 4.

Next, we investigated the downstream signaling of PIAS4 given its significant upregulation and activation z-score (see Supplementary Table 4). PIAS4 involvement in HCC has been recently described [41]. Downstream of PIAS4, we found upregulation of lymphoid enhancer factor 1 and downregulation

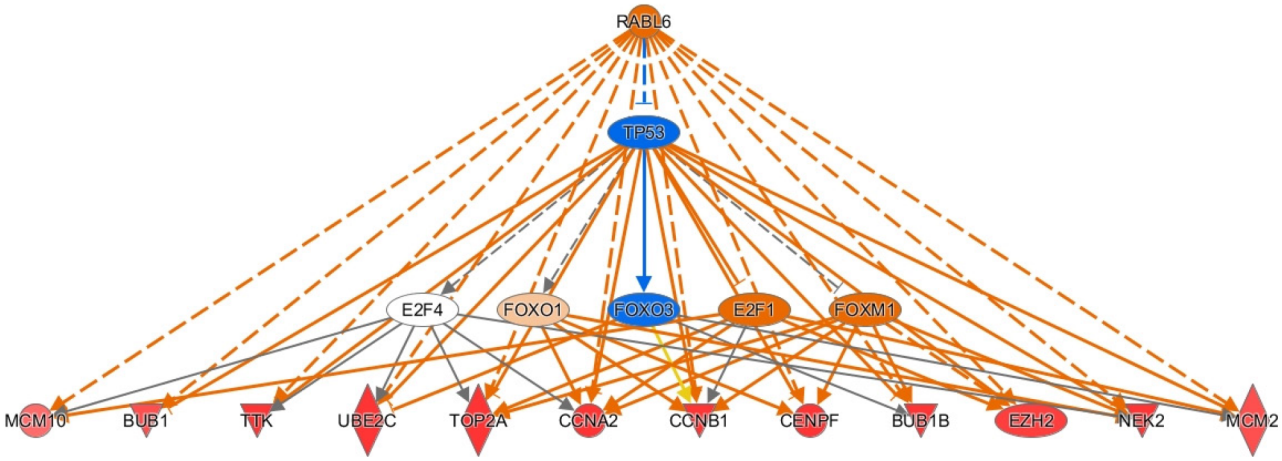

Figure 3 Ingenuity pathway analysis of rab-like protein 6 signaling in hepatitis B-related hepatocellular carcinoma tumors. Genes are implicated in several disease potential disease processes including inflammation, cell division, Akt signaling, and more. Legend illustrates relationship between genes. See Figure 2 legend for identification of shapes. RABL6: RAB, member RAS oncogene family like 6; TP53: Tumor protein p53; E2F4: E2F transcription factor 4; FOXO1: Forkhead box O1; FOXM1: Forkhead box M1; MCM10: Mini-chromosome maintenance 10; BUB1: Mitotic checkpoint serine/threonine-protein kinase BUB1; TTK: TTK protein kinase; UBE2C: Ubiquitin-conjugating enzyme E2 C; TOP2A: DNA topoisomerase IIα; CCNA2: Cyclin A2; CENPF: Centromere protein F; EZH2: Enhancer of zeste homolog 2; NEK2: Serine/threonine-protein kinase Nek2. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

of protocadherin 9, both of which promote epithelial-mesenchymal transition in HCC[72-74]. We also found downregulation of fatty acid binding protein 1, which has been shown to reduce oxidative stress, a major contributor to HCC development[75]. Its loss may also lead to microsatellite instability in colorectal carcinomas and may have similar effects in HCC[76]. Furthermore, we found downregulation of albumin, with evidence showing albumin itself suppressing HCC cellular proliferation[77]. These results suggest a mechanistic role for PIAS4 in HCC progression (Figure 5).

Lastly, we investigated how inhibition of SAMSN1 may contribute to HCC. As mentioned previously, SAMSN1 inhibition is linked to malignant HCC tumorigenesis[55]. From our IPA analysis, inhibition of SAMSN1 has immunologic implications including downregulation of the pattern recognition receptor TLR3 and macrophage receptor with collagenous structure. These changes are known to negatively impact HCC prognosis[46,78]. Furthermore, we found downregulation of the de-ubiquitinase USP12, which complexes with WD repeat protein WDR48 to suppress Akt signaling and tumor cell survival [79]. Our suggested model of molecular mechanisms linking SAMSN1 inhibition with HCC is showed in Figure 5.

DISCUSSION

Despite robust vaccination strategies in some countries, hepatitis B infection remains a leading global cause of liver cancer[1,3]. Therapeutic options for HBV-related HCC remain poor owing to an overall lack of understanding of pathways involved in HBV oncogenesis. Current literature suggests HCC development is a result of aberrant activation of cellular signaling processes such as Wnt/FZD/β-catenin, PI3K/Akt/mTOR, IRS1/IGF, and Ras/Raf/MAPK[80]. Even with such knowledge, directed therapy for HBV-related HCC cases requires a more detailed understanding of the interactome. In this meta-analysis, we found potential underlying cellular pathways that define HBV-related HCC and its disease mechanisms. Our results build upon known contributors to HBV-related HCC including LXR/FXR/RXR signaling, Akt signaling, and immunological changes within the tumor microenvironment that are favorable for both HBV infection and tumor progression. We also illustrate the possible activities of upstream regulators, whose role in HBV-related HCC are not well described, such as RABL6, HOXA10, PIAS4, and SAMSN1.

We began our analysis by studying the top canonical pathways identified by IPA, which included LXR/RXR activation, LPS-IL-1 mediated RXR inhibition, acetone degradation, melatonin degradation, and FXR/RXR activation. LXR heterodimerizes with RXR and bind to LXR response elements, directly regulating gene expression[81]. LXR primarily regulates expression of genes essential for lipid metabolism and is a known HCC tumor-suppressor[82]. Similarly, the bile acid regulator FXR (NR1H4) suppresses hepatocarcinogenesis and was starkly downregulated in our analysis[83]. In addition, inhibition of the acetone degradation pathway in HCC suggests alteration hepatic ketone metabolism, suggesting an increase in acetone levels may serve as a disease biomarker[84]. Lastly, we saw predicted inhibition of the melatonin degradation pathway, which would presumably lead to a rise in melatonin.

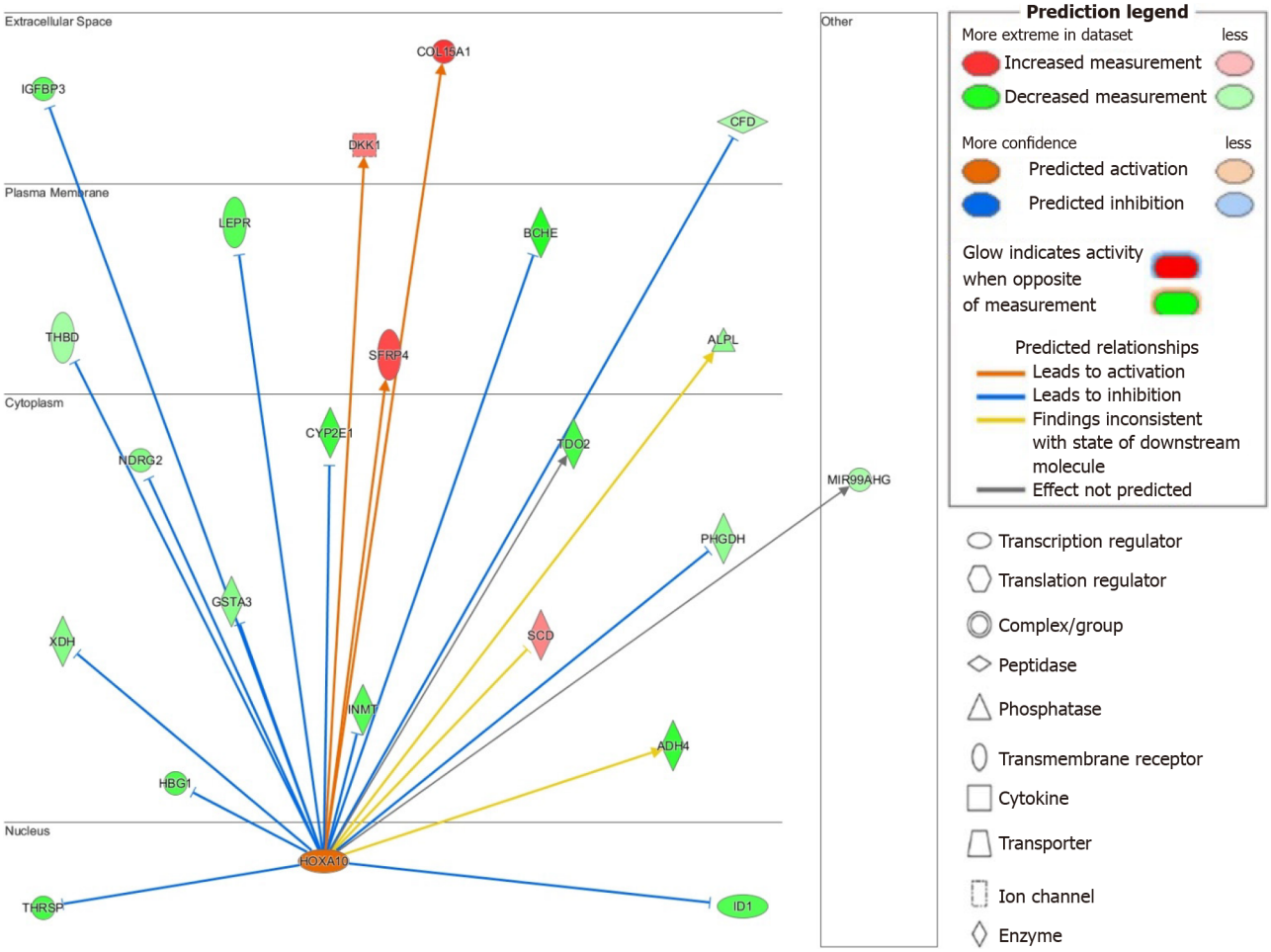

Figure 4 Ingenuity pathway analysis of homeobox A10 activity in hepatitis B-related hepatocellular carcinoma. Homeobox A10 signaling has potential implications on tumor suppression, liver metabolism, and other disease-related activity. Genes and location are shown above. Legend illustrates relationship between genes and gene classification. COL15A1: Collagen alpha-1(XV) chain; IGFBP3: Insulin-like growth factor binding protein 3; DKK1: Dickkopf-related protein 1; CFD: Complement factor D; LEPR: Leptin receptor; BCHE: Butyrylcholinesterase; THBD: Thrombomodulin; SFRP4: Secreted frizzled-related protein 4; ALPL: Alkaline phosphatase; CYP2E1: Cytochrome P450 2E1; TDO2: Tryptophan 2,3-dioxygenase; NDRG2: N-myc downstream-regulated gene family member 2; GSTA3: Glutathione S-transferase A3; PHGDH: Phosphoglycerate dehydrogenase; XDH: Xanthine dehydrogenase; INMT: Indolethylamine N-methyltransferase; SCD: Stearoyl-CoA desaturase; HBG1: Hemoglobin subunit gamma 1; ADH4: Alcohol dehydrogenase 4; HOXA10: Homeobox A10; THRSP: Thyroid hormone-inducible hepatic protein. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

Melatonin has been demonstrated to inhibit HCC progression through let7i-3p-mediated RAF1 suppression[85]. Overall, the top canonical pathways above reinforce the roles of LXR/RXR/FXR signaling in HCC pathogenesis and suggests a role for melatonin degradation in HBV-related HCC.

Top up- and downregulated genes identified in our analysis are implicated in oncogenic cellular signaling and other pathologic processes. For example, GPC3, the most upregulated gene in our analysis, functions as a co-receptor for Wnt proteins[86]. Wnt signaling is vital for hepatobiliary function and cell differentiation. Therefore it's no surprise that aberrations in activity are major contributors to HCC tumorigenesis and other liver disorders[87]. GPC3 is also implicated in hedgehog signaling[20], another important regulator of cell growth and differentiation; overactivation of which is associated with multiple cancer types including HCC[88]. *In vitro* studies suggest GPC3 mediates cell proliferation through hedgehog signaling[89]. Additionally, the negative hedgehog regulator HHIP was one of the most downregulated genes in our dataset[26]. This change may act synergistically with GPC3 to further promote hedgehog signaling and cellular proliferation. Lastly, we found the kinase PBK as one of the most upregulated genes. This kinase is related to the MAPKK family and has been recently been shown to promote HCC metastasis through ETV4a-uPar signaling[22,90]. ETV4 is part of the ETS family of transcription factors and directly regulates cell division to promote pancreatic cancer and other cancer types[91,92].

In addition to activation of cellular signaling pathways described above, our results also highlight changes in the tumor microenvironment and the immune response that may confer advantages to HBV infection and tumor progression. Our most downregulated genes included several members of the C-type lectin family including CLEC4G, CLEC1B, and CLEC4M. C-type lectins function in both the innate

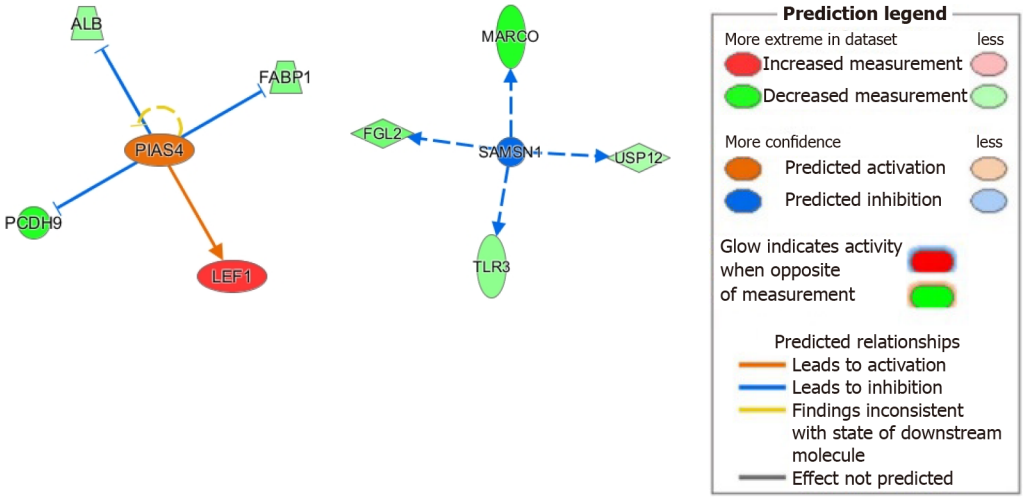

Figure 5 Protein inhibitor of activated STAT 4 and SH3 domain, and nuclear localization signals 1 potential role in homeobox A10 have only been recently described and much remains to be understood. Ingenuity pathway analysis analysis demonstrated activation of protein inhibitor of activated STAT 4, and activation of downstream genes implicated in epithelial-mesenchymal transition through LEF1 and protocadherin 9. Our analysis also demonstrated inhibition of SH3 domain, and nuclear localization signals 1 with downstream effects on viral recognition and regulation of cell survival. Legend illustrates relationship between genes. See legend of Figure 3 for classification of genes. ALB: Albumin; FABP1: Fatty acid binding protein 1; MARCO: Macrophage receptor with collagenous structure; FGL2: Fibrinogen like 2; SAMSN1: SAM domain, SH3 domain and nuclear localization signals 1; USP12: Ubiquitin-specific protease 12; PIAS4: Protein inhibitor of activated STAT protein gamma; PCDH9: Protocadherin 9; LEF1: Lymphoid enhancer-binding factor 1; TLR3: Toll-like receptor 3. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

and adaptive immune response and downregulation of has been demonstrated in HCC[24,93]. Lower expression of CLEC1B is associated with poorer outcomes in HCC[94]. In addition, we found downregulation of pattern recognition receptors, like TLR3 and TLR2, implying impairment of the innate immune response. For example, TLR3, a receptor that recognizes viral components and double-stranded RNA[95], has been associated with control of HBV infection and apoptosis of HCC cells[46]. Likewise, TLR2, whose activity limits HCC cellular proliferation, was downregulated[47]. We also noted downregulation of the innate immune receptor DDX58 or RIG-I. Elevated RIG-I expression limits HCC cellular proliferation and invasion[96]. Moreover, HBV limits RIG-I signaling through induction of the miRNA miR146a[97]. Lastly, other immunologic changes of note were repression of CD86 and IL-18. CD86 is a co-stimulatory molecule that has been well described as an anti-tumor response inducer through stimulation of cytotoxic T cells and other means[98]. While the role of IL-18 in cancer is unclear, expression of IL-18 exhibits anti-tumor effects through the recruitment of tumor-infiltrating T cells[99]. Thus, downregulation of IL-18 in the tumor microenvironment could be a prime contributor to HCC tumor progression.

Our analysis revealed FOXM1, E2F1, and EP400 as activated top upstream regulators in HBV-related HCC, each of which play a prominent role in facilitating cancer proliferation. FOXM1 has previously been shown to promote HCC progression *via* expression of genes KIF4A and CCNB1[22]. Additionally, FOXM1 promotes tumor cell proliferation *via* increasing expression of CCNB1 and CCND1, and decreasing expression of cell cycle checkpoint molecules p27 and p21[100]. E2F1 is a transcription factor that has been shown to have both proliferative and apoptotic effects in HCC although the proliferative effects seem to be more prominent[29]. E2F1 activates MYBL2 (another upregulated transcription factor in our analysis) and is involved in cell cycle progression[101]. EP400 is a component of NuA4 histone acetyltransferase complex and is associated with activation of various genes. Recent studies have revealed it to be a critical transcription factor associated with greater HCC relapse and lower overall survival[36].

In addition, our results showed RABL6, ESR1, NR0B2, and CEBPD as top upstream regulators that negatively regulate HCC tumor suppression. RABL6 is a member of Ras GTPase family that is overexpressed in HCC[102]. Survival analysis suggests overexpression leads to a poorer prognosis (Figure 6). ESR1 was downregulated in our analysis, concordant with prior findings implicating ESR1 as a potential HCC tumor suppressor gene[103]. Moreover, our results showed downregulation of NR0B2 and CEBPD. NR0B2 is a nuclear receptor and tumor suppressor; downregulation of which is associated with HCC and renal cell carcinoma[104,105]. Similarly, CEBPD has also been posited as a candidate HCC tumor suppressor gene primarily through modulating IL-1 signaling[106].

Interestingly, our results also revealed that HBV-related HCC progression may be intrinsically linked with repression of inflammatory and innate immune responses. Our analysis showed stark inhibition of the NF-κB pathway, a Myc-dependent driver of HCC tumorigenesis[107]. Indeed, other studies have proposed the NF-κB pathway is an important mediator of hepatic fibrosis and disease progression,

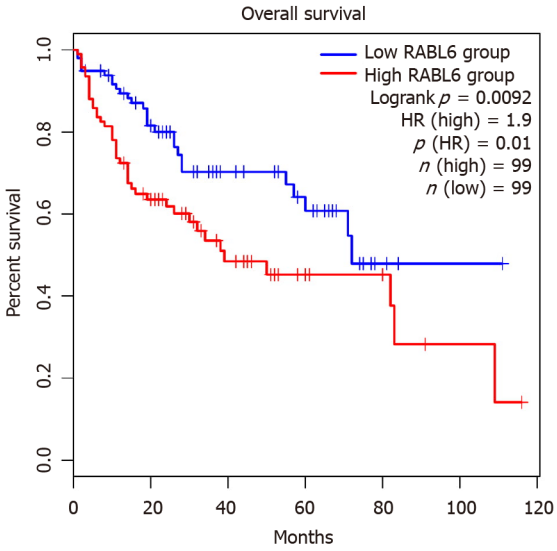

Figure 6 Survival analysis comparing high and low rab-like protein 6 expression in survival of hepatocellular carcinoma patients. RABL6: Rab-like protein 6; HR: Hazards ratio. Plot generated using GPEIA2. Citation: Figures produced from IPA are available under an open-access CC-BY 4.0 license for purposes of publication. The authors have obtained the permission for figure using from the QIAGEN Digital Insights (Supplementary material).

especially when inhibition is pronounced[108]. While our meta-analysis allows for robust results of larger datasets, it has certain limitations. Annotations of public samples are limited and can introduce confounding variables to our analysis. For one, samples were taken from patients at different stages of HBV-related HCC. There may be significant differences in genetic aberrations based on tumor stage and grade. While samples were taken at the time of diagnosis, patient characteristics, such as ethnicity, comorbidities, and medications, were not clarified and may affect the results. Additionally, there may be differences in how samples were processed and how omics were performed between the studies included in our analysis. Of note, the studies in our meta-analysis did not clarify if changes in gene expression were attributable to HBV-DNA integration to the hepatocyte genome. Analysis focused on hepatocytes and the genes identified have links to HBV infection epiphonema as detailed above so a portion of the changes we have seen are associated to HBV. As explained, analysis focused on hepatocytes so gene changes would not be related to infiltrates. We did identify several genes involved in cell cycle regulation, but cellularity is important to function of these genes and could not be described in this approach. For future directions, we aim to validate our top genetic candidates using patient samples and control for such factors as stage of disease. We also hope to compare results between different etiologies of HCC such as HCV and alcohol. Doing so will elucidate the similarities and differences between these etiologies, allowing for a greater understanding of the oncogenic process while aiding the development of directed therapeutics for patient-specific treatment.

CONCLUSION

HBV is a leading cause of HCC and treatment options are still limited. In this meta-analysis based on public data, we studied the pathogenesis of HBV and pave the way for novel therapeutic avenues. We illustrated genetic changes that contribute to pro-oncogenic signaling through such pathways as the Akt, hedgehog, ETV4, and Wnt pathways. We also illustrated changes in the tumor microenvironment and immune response that are contributory to HBV infection and tumor progression. Additionally, we clarify the role of key upstream regulators such as RABL6, HOXA10, PIAS4, and SAMNS1 and describe how their downstream effects contribute to disease. These observations need to be further confirmed in prospective studies on oncogenesis. There is also need for investigating HBV-related cirrhosis and progressive changes of HBV-related HCC to assess the stepwise activity that define HBV oncogenesis.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC) through several mechanisms including cirrhosis and direct oncogenic phenomena.

Research motivation

Studying HBV related HCC will offer novel insights to viral hepatic oncogenesis. It will also potentially lead to more directed therapy in HBV-related HCC.

Research objectives

Identify genetic changes and pathways that define HBV-related HCC. Identify novel therapeutic targets.

Research methods

Used our novel Search Tag Analyze Resource platform to mine liver biopsies from HBV-related HCC patients and used ingenuity pathway analysis to study the results of our meta-analysis.

Research results

Our meta-analysis highlighted several genes and pathways with oncogenic potential. Of note, we describe two potential novel mediators of oncogenesis in rab-like protein 6 (RABL6) and homeobox A10 (HOXA10).

Research conclusions

This meta-analysis describes possible roles of RABL6 and HOXA10 in the pathogenesis of HBV-related HCC. RABL6 and HOXA10 represent potential therapeutic targets and warrant further investigation.

Research perspectives

The next steps to our research is to validate RABL6 and HOXA10 relevance in HBV-related HCC using clinical samples and establish its mechanistic underpinnings in an animal model.

FOOTNOTES

Author contributions: Aljabban J and Rohr M contributed to the conception or design of the work; Aljabban J, Rohr M, and Hadley D involved in the data collection; Aljabban J, Rohr M, Syed S, Cohen E, Hashi N, Syed S, Khorfan K, Aljabban H, and Mumtaz K drafted manuscript; Aljabban J, Rohr M, Syed S, Cohen E, Hashi N, Syed S, Khorfan K, Aljabban H, Boateng E, Nemer M, Panahiazar M, Hadley D, Jalil S, and Mumtaz K involved in the critical revision of manuscript; Aljabban J, Rohr M, Syed S, Cohen E, Hashi N, Syed S, Khorfan K, Aljabban H, Boateng E, Nemer M, Panahiazar M, Hadley D, Jalil S, and Mumtaz K contributed to the final edits and approval.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Wang JJ
L-Editor: A
P-Editor: Yuan YY

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World Journal of Gastrointestinal Oncology

Submit a Manuscript: https://www.f6publishing.com

World J Gastrointest Oncol 2022 September 15; 14(9): 1874-1886

DOI: 10.4251/wjgo.v14.i9.1874

ISSN 1948-5204 (online)

META-ANALYSIS

Prognostic and clinicopathological value of Twist expression in esophageal cancer: A meta-analysis

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Specialty type: Oncology

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Bairwa DBL, India; Casella C, Italy

Received: March 16, 2022
Peer-review started: March 16, 2022
First decision: June 12, 2022
Revised: June 30, 2022
Accepted: July 31, 2022
Article in press: July 31, 2022
Published online: September 15, 2022

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Abstract

BACKGROUND

Twist is a repressor of E-cadherin transcription that induces epithelial-mesenchymal transition and cancer metastasis. However, the prognostic value of Twist expression in patients with esophageal cancer remains controversial.

AIM

To investigate the prognostic and clinicopathological value of Twist expression in esophageal cancer.

METHODS

Published literature in databases such as EMBASE, Web of Science, PubMed, China National Knowledge Infrastructure, Wanfang, and VIP databases was searched for eligible articles. Participants with esophageal cancer whose tumor tissues underwent immunohistochemistry to detect the expression of Twist were considered. Our meta-analysis was conducted using Stata version 12.0. The hazard ratio (HR) and relative ratio (RR) with their 95%CI were pooled. Heterogeneity was estimated by I^2 statistics.

RESULTS

Eleven articles published between 2009 and 2021 fulfilled the selection criteria. The pooled HR for overall survival was 1.88 (95%CI: 1.32-2.69, I^2 = 68.6%), and the pooled HR for disease-free survival/relapse-free survival/progression-free survival was 1.84 (95%CI: 1.12-3.02, I^2 = 67.1%), suggesting that high Twist expression is associated with poor prognosis in esophageal cancer patients. In addition, overexpression of Twist was correlated with T stage (T3 + T4 *vs* T1 + T2, RR = 1.38, 95%CI: 1.14-1.67), lymph node metastasis (yes *vs* no, RR = 1.34, 95%CI: 1.11-1.60), distant metastasis (yes *vs* no, RR = 1.18, 95%CI: 1.02-1.35), tumor, node and metastasis (TNM) stage (III + IV *vs* I + II, RR = 1.35, 95%CI: 1.14-1.60), and clinical stage (III + IV *vs* I + II, RR = 1.58, 95%CI: 1.34-1.87). However, no correlation between Twist expression and age, gender, tumor location, differentiation, or venous invasion was observed.

CONCLUSION

High expression of Twist is associated with poor esophageal cancer prognosis. Moreover, Twist overexpression is correlated with T stage, lymph node metastasis, distant metastasis, TNM stage, and clinical stage, which indicates that Twist might accelerate esophageal cancer progression and metastasis.

Key Words: Twist; Esophageal cancer; Prognosis; Epithelial-mesenchymal transition; Metastasis; Meta-analysis

Core Tip: Esophageal cancer is a leading cause of cancer mortality worldwide. Twist is a transcription factor involved in the process of epithelial-mesenchymal transition and esophageal cancer metastasis. However, the prognostic value of Twist expression in patients with esophageal cancer remains controversial. Therefore, we conducted a meta-analysis to investigate the prognostic and clinicopathological value of Twist expression in esophageal cancer in terms of overall survival, disease-free survival/relapse-free survival/progression-free survival, age, gender, tumor location, T stage, differentiation, lymph node metastasis, distant metastasis, tumor, node and metastasis stage, clinical-stage, and venous invasion.

Citation: Song WP, Wang SY, Zhou SC, Wu DS, Xie JY, Liu TT, Wu XZ, Che GW. Prognostic and clinicopathological value of Twist expression in esophageal cancer: A meta-analysis. *World J Gastrointest Oncol* 2022; 14(9): 1874-1886

URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1874.htm

DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1874

INTRODUCTION

According to the latest global cancer burden report, there were an estimated 572000 new esophageal cancer cases and 509000 deaths in 2020, ranking seventh and fifth in morbidity and mortality, respectively[1]. Among esophageal cancers, 90% of the histological types are esophageal squamous cell carcinoma (ESCC)[1-3]. Although a slew of breakthroughs in terms of the diagnosis and treatment of esophageal cancer has been achieved[4], the 5-year survival rate of ESCC is only 15%–20%[5] due to invasion and distant metastasis. Therefore, there is an urgent need for the identification of new prognostic biomarkers to address the poor prognosis of esophageal cancer.

Epithelial-mesenchymal transition (EMT) describes a key developmental program in which epithelial cells change to motile mesenchymal cells[6]. Tumor cells can undergo EMT to promote local invasion [7], which is the first step of tumor metastasis[8]. Twist is reported to be a helix-loop-helix transcription factor that can directly bind to the promoter of E-cadherin, a tumor suppressor gene associated with EMT, and downregulate E-cadherin expression[9,10]. Thus, Twist can induce EMT and tumor metastasis. The prognostic value of Twist in esophageal cancer has been investigated in many studies [11-21] with controversial results. Some studies[12,13,15,17] have shown that Twist overexpression is closely related to the poor prognosis of esophageal cancer, while others show that it is unrelated[11,14,16,18-21]. Therefore, we performed a meta-analysis to combine relevant studies and clarify whether Twist could be a promising biomarker for predicting prognosis in esophageal cancer.

MATERIALS AND METHODS

Data mining

Gene expression profiling interactive analysis 2[22] (GEPIA2) is a valuable and efficient web server with which we can perform gene expression analysis based on the The Cancer Genome Atlas and the Genotype-Tissue Expression databases. We used GEPIA2 to analyze the expression of Twist in esophageal cancer tissues and normal tissue. Scatter diagrams and box plots were generated to assess the expression of Twist in esophageal cancer tissues and normal tissues.

Literature retrieval

A systematic literature search of the EMBASE, Web of Science, PubMed, China National Knowledge Infrastructure, Wanfang, and VIP databases was conducted to identify relevant studies up to December 28, 2021. The following keywords were variably combined: "Twist", "esophageal", "esophagus", "tumor", "cancer", "carcinoma", and "neoplasm". Moreover, relevant meta-analysis articles, reviews, and references from the included studies were also screened.

Inclusion and exclusion criteria

The inclusion criteria in the present meta-analysis were as follows: (1) Twist expression was analyzed in human esophageal cancer tissues; (2) The hazard ratio (HR) with 95%CI was reported or available to be calculated indirectly; (3) Correlations between Twist expression and clinicopathologic characteristics were investigated; and (4) The reports were published in English or Chinese. The exclusion criteria were as follows: (1) Duplicate studies; (2) Reviews, animal experiments, case reports, and conference abstracts; and (3) The HR or 95%CI were unavailable.

Data extraction

Two of the authors (Wen-Peng Song and Su-Yan Wang) independently extracted the following data from each eligible study: the first author, year of publication, country, sample size, tumor location, positive proportion of Twist, tumor, node and metastasis (TNM) stage, clinical stage, venous invasion, detection method, cutoff value, antibodies against Twist, follow-up time, survival analysis, and HR estimates for positive or high expression of Twist *vs* negative or low expression of Twist, with their 95%CIs.

Quality assessment of included studies

Two of the authors (Wen-Peng Song and Su-Yan Wang) independently assessed the quality of the included studies with the Newcastle–Ottawa scale (NOS) criteria. Included studies with NOS scores ≥ 6 were considered high-quality studies[23].

Statistical analysis

Our meta-analysis was conducted using Stata version 12.0 (StataCorp, College Station, Texas 77845 United States). We derived pooled HRs and their 95%CIs for all types of survival outcomes [overall survival (OS), disease-free survival (DFS), relapse-free survival (RFS), progression-free survival (PFS)]. Heterogeneity of the effect across the included studies was estimated by I^2 statistics. We used a random-effects model if $I^2 > 50\%$ and/or $P < 0.10$, which indicated the presence of significant heterogeneity. Otherwise, we used a fixed-effects model[24]. Moreover, we further investigated the correlations between Twist expression and clinicopathologic characteristics. These clinicopathologic characteristics included age, gender, tumor location (*e.g.*, upper thorax, middle thorax, lower thorax), T stage, differentiation, lymph node metastasis, distant metastasis, TNM, clinical stage, and venous invasion. We performed sensitivity analyses to estimate the stability of the meta-analysis results. Publication bias was assessed with Egger's test and Begg's funnel plots[25,26]. *P* values less than 0.05 indicated the presence of significant publication bias[27]. In addition, we used the Reference Citation Analysis database (https://www.referencecitationanalysis.com/) to retrieve and supplement cutting-edge research results.

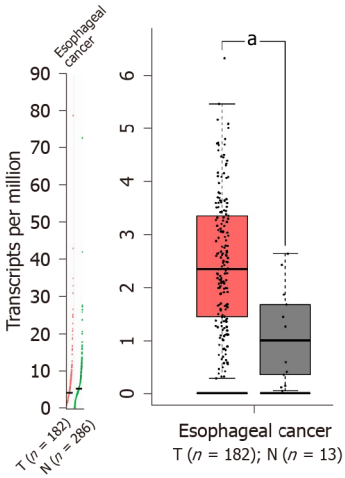
RESULTS

Data mining

We used the GEPIA2 web server to detect the expression of Twist in esophageal cancer tissues and normal tissues. The expression of Twist was significantly higher in esophageal cancer tissues than in normal tissues (Figure 1). Therefore, we further explored the prognostic value of Twist overexpression in esophageal cancer by meta-analysis.

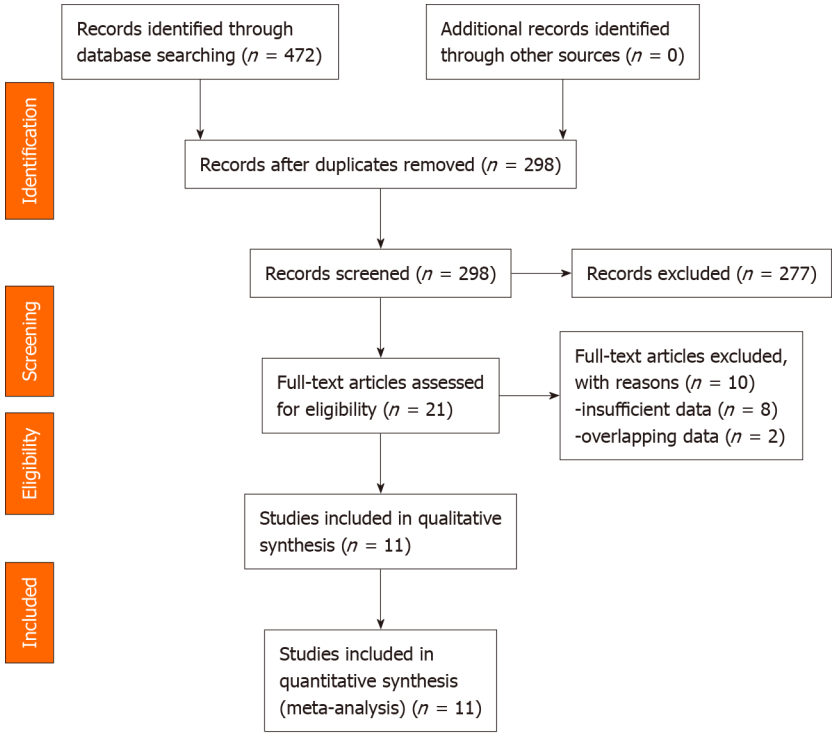
Literature retrieval

Figure 2 shows the flow diagram for the literature search and selection. We finally identified 11 eligible studies in this meta-analysis[11-21].



DOI: 10.4251/wjgo.v14.i9.1874

Figure 1 The expression of Twist in esophageal cancer (Gene expression profiling interactive analysis 2). [a]*P* < 0.05.



DOI: 10.4251/wjgo.v14.i9.1874

Figure 2 PRISMA flow diagram.

Study characteristics

The baseline characteristics of the included studies are shown in Table 1. Among all eligible studies, six studies were published in English[11-14,17,18], while five were published in Chinese[15,16,19-21]. All included studies examined the expression of Twist in esophageal cancer tissue with immunohistochemistry (IHC). Two metrics for IHC staining were used in some studies[11,12,18-21]: The percentage of positively stained cells and the staining intensity. However, some studies[13-17] evaluated Twist expression using only one metric for IHC staining, which resulted in assessing the expression of Twist at various cutoff values. In addition, HRs were directly reported in some studies[11-14,17,20], while others [15,16,18,19,21] were indirectly calculated from survival curves.

Meta-analysis

All included studies reported HRs of OS, and four reported DFS/RFS/PFS (Table 2, Figure 3). Both the pooled HR for OS (HR = 1.88, 95%CI: 1.32-2.69, I^2 = 68.6%) and the pooled HR for DFS/RFS/PFS (HR = 1.84, 95%CI: 1.12-3.02, I^2 = 67.1%) suggested that Twist overexpression was associated with poor

Table 1 Basic characteristics of included studies

Ref.	Country	Sample size	TNM stage	Detection method	Antibody	Method of quantification	Cut-off value	Positive proportion (%)	Outcome	Source of HR	Follow-up time (mo)	NOS score
Sasaki *et al*[11], 2009	Japan	166	I-IV	IHC	Anti-Twist (sc-15393, Santa Cruz)	Multiply percentage score and intensity score	Low: 0-5; High: 6-7	40.2	OS	R	24 (1-181)	8
Xie *et al* [12], 2009	China	112	I-IV	IHC	Anti-Twist (sc-15393, Santa Cruz)	Multiply percentage score and intensity score	Negative: 0-3; Positive: 4-5+; 6-8++; ≥ 9+++	79.5	OS	R	35.8 (3.4-87)	7
Lee *et al* [13], 2012	South Korea	165	I-IV	IHC/RT-PCR	Anti-Twist1 (ab50887, Abcam)	Intensity score	Negative: No expression; Positive: Weak, moderate, strong	50.9	OS/DFS	R/E	115 (2-155)	6
Nakajima *et al*[14], 2012	Japan	54	I-IVA	IHC	Anti-Twist (sc-15393, Santa Cruz)	Intensity score	Faint: 1; Moderate: 2; Strong: 3	37	OS/RFS	R	NA	7
Sun *et al* [15], 2013	China	164	I-III	IHC	Anti-Twist1 (ab50887, Abcam)	Percentage of stained cells	Negative: 0%-10%; Positive: > 10%	34.1	OS	E	96-120	7
Chen *et al* [16], 2016	China	50	NR	IHC	Anti-Twist1 (Abcam)	Percentage of stained cells	NA	50	OS	E	> 60	7
Yeo *et al* [17], 2017	Korea	169	I-IV	IHC	Anti-Twist1 (Abcam)	Intensity score	Negative: 1; Positive: 2-3	89.9	OS/DFS	R	NA	7
Xu *et al* [18], 2021	China	229	I-IV	IHC	Anti-Twist1 (ab175430; Abcam)	Multiply percentage score and intensity score	Negative: 0-5; Positive: ≥ 6	59	OS/PFS	E	NA	6
Du *et al* [19], 2021	China	72	I-III	IHC	Anti-Twist (bs-2441R, Bioss)	Multiply percentage score and intensity score	Negative: 0-2; Positive: ≥ 3	61.1	OS	E	14-90	6
Tang *et al* [20], 2021	China	40	II-IV	IHC	Anti-Twist1 (ab50581, Abcam)	Multiply percentage score and intensity score	Negative: 0-2; Positive: ≥ 3	15	OS	R	17 (13.9-20.1)	7
Wang *et al*[21], 2021	China	72	I-III	IHC	Anti-Twist1 (bs-2441R, Bioss)	Multiply percentage score and intensity score	Negative: 0-3; Positive: ≥ 4	61.1	OS	E	14-90	6

TNM: Tumor, node and metastasis; IHC: Immunohistochemistry; RT-PCR: Reverse transcription-polymerase chain reaction; OS: Overall survival; DFS: Disease-free survival; RFS: Relapse-free survival; PFS: Progression-free survival; HR: Hazard ratio; R: Reported; E: Estimated; NA: Not applicable; NOS: Newcastle-Ottawa quality assessment scale.

prognosis in esophageal cancer patients. Heterogeneity was explored by subgroup analysis based on the detection method. Immunoreactivity scored by multiplying the percentage score and intensity score (pooled OS; HR = 1.517, 95%CI: 0.869-2.649, I^2 = 79.5%) showed very high heterogeneity when compared with scoring by staining intensity (pooled OS; HR = 2.72, 95%CI: 1.84-4.03, I^2 = 0%) or percentage of stained cells (pooled OS; HR = 2.45, 95%CI: 1.43-4.19, I^2 = 0%) (Table 2 and Figure 3C).

Correlation between the expression of Twist and clinicopathologic characteristics

As shown in Table 3 and Figure 4, Twist overexpression was correlated with T stage (T3 + T4 *vs* T1 + T2, RR = 1.38, 95%CI: 1.14-1.67), lymph node metastasis (yes *vs* no, RR = 1.34, 95%CI: 1.11-1.60), distant metastasis (yes *vs* no, RR = 1.18, 95%CI: 1.02-1.35), TNM stage (III + IV *vs* I + II, RR = 1.35, 95%CI: 1.14-1.60), and clinical stage (III + IV *vs* I + II, RR = 1.58, 95%CI: 1.34-1.87), which indicated that Twist overex-

Table 2 Meta-analyses for the association of Twist expression with survival of esophageal cancer

Meta-analysis	Endpoints	HR (95%CI)	Heterogeneity test (I^2)	*P* value	Number of studies
TWIST (+) *vs* TWIST (−)	OS	1.88 (1.32-2.69)[a]	68.6%	0.000	11
	DFS/RFS/PFS	1.84 (1.12-3.02)[a]	67.1%	0.028	4
Method of quantification	Multiply percentage score and intensity score	1.52 (0.87-2.65)	79.5%	0.319	6
	Intensity score	2.72 (1.84-4.03)[a]	0.00	0.062	9
	Percentage of stained cells	2.45 (1.43-4.19)[a]	68.6%	0.199	4

[a]If $I^2 \geq 50\%$ and/or $P < 0.1$, random effects models are applied.
HR: Hazard ratio; OS: Overall survival; DFS: Disease-free survival; RFS: Relapse-free survival; PFS: Progression-free survival.

Table 3 Correlations of Twist expression with clinicopathological characteristics in esophageal cancer

Clinical features	RR (95%CI)	Heterogeneity test (I^2)	*P* value	Number of studies
Age (≥ 60 *vs* < 60)	1.07 (0.95-1.21)	5.88	0.319	6
Gender (male *vs* female)	1.02 (0.89-1.18)[a]	14.85	0.062	9
Location (upper + middle *vs* lower)	0.89 (0.80-1.00)	4.66	0.199	4
T stage (T3 + T4 *vs* T1 + T2)	1.38 (1.14-1.67)[a]	15.30	0.018	7
Differentiation (high + moderate *vs* low)	0.94 (0.81-1.09)[a]	21.26	0.003	8
Lymph node metastasis (yes *vs* no)	1.34 (1.11- 1.60)[a]	14.99	0.036	8
Distant metastasis (yes *vs* no)	1.18 (1.02-1.35)[a]	10.74	0.030	5
TNM stage (III + IV *vs* I + II)	1.35 (1.14-1.60)[a]	13.34	0.038	7
Clinical stage (III + IV *vs* I + II)	1.58 (1.34-1.87)	0.39	0.534	2
Venous invasion (yes *vs* no)	1.46 (0.83-2.56)[a]	4.49	0.034	2

[a]If $I^2 \geq 50\%$ and/or $P < 0.1$, random effects models are applied.
TNM: Tumor, node and metastasis.

pression might accelerate esophageal progression and metastasis. However, no correlation between Twist expression and age, gender, tumor location, differentiation, or venous invasion was observed.

Sensitivity analysis

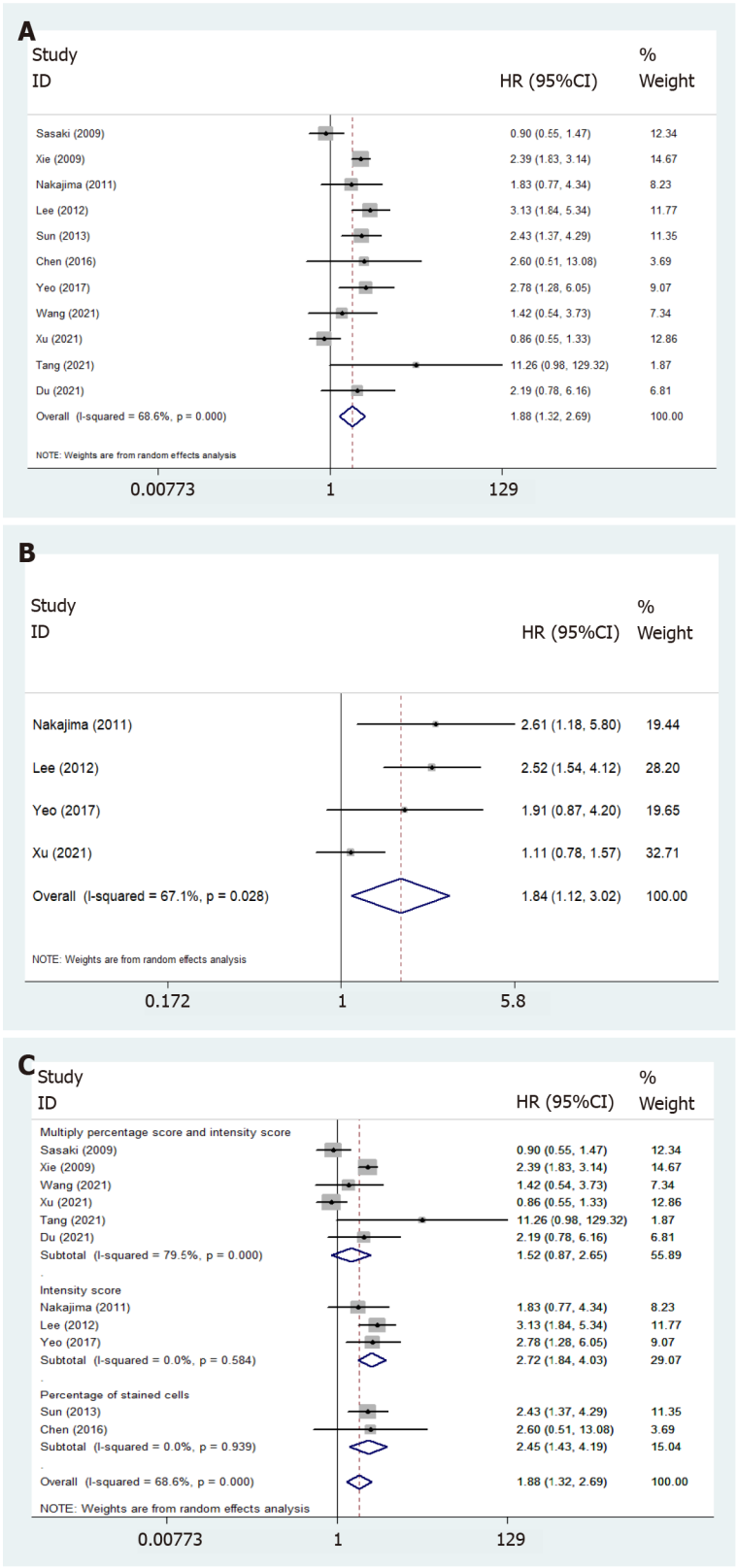
The sensitivity analyses for the association between Twist expression and esophageal cancer prognosis suggested that the results of this meta-analysis were stable and reliable (Figure 5).

Publication bias

Publication bias was assessed, and the results showed symmetrical Begg's funnel plots for OS with a *P* value of 0.78 (Figure 6), suggesting that no obvious publication bias existed.

DISCUSSION

This meta-analysis suggests that high expression of Twist is associated with poor prognosis in esophageal cancer. The subgroup analyses by the detection method of Twist expression imply that major heterogeneity is derived from evaluating Twist expression by different metrics for IHC staining. Several clinicopathological parameters, such as T stage, lymph node metastasis, distant metastasis, TNM stage, and clinical stage, were positively correlated with Twist expression. Some meta-analyses have investigated the relationship between Twist expression and prognosis in other cancers. For example, Zeng *et al*[28] investigated the prognostic value of Twist in lung cancer and found that high expression of Twist indicated a worse prognosis. Similarly, several meta-analyses revealed that Twist overexpression indicated poor prognosis in breast cancer[29], head and neck carcinoma[30], colorectal cancer [31], hepatocellular carcinoma, urinary cancer, and female reproductive cancer[32]. Our meta-analysis presents similar results and suggests that Twist might be a valuable prognostic biomarker in esophageal

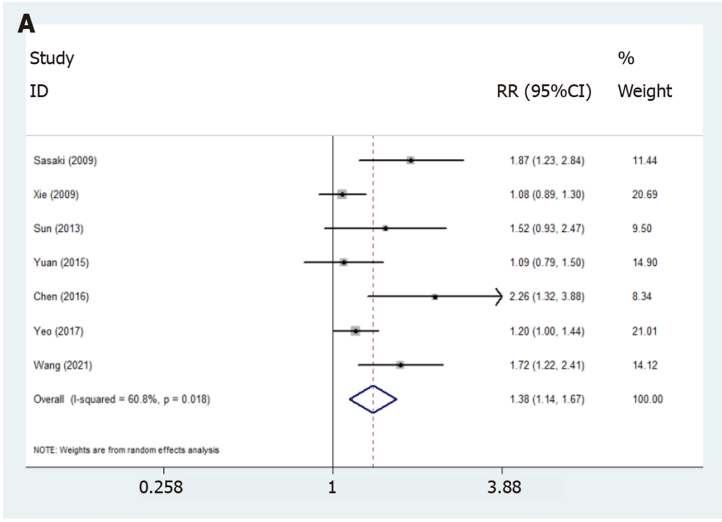


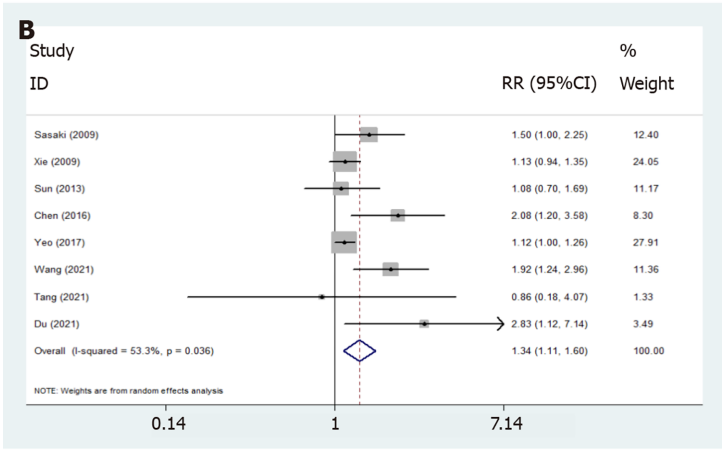
DOI: 10.4251/wjgo.v14.i9.1874

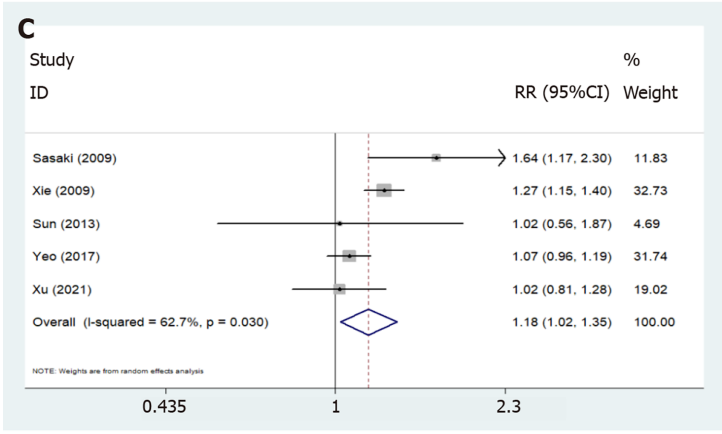
Figure 3 Forest plots of the association between Twist overexpression and poor overall survival and disease-free survival/relapse-free survival/progression-free survival of patients with esophageal cancer. A: Poor overall survival (OS); B: Disease-free survival/relapse-free survival/progression-free survival; C: Subgroup analysis of OS based on the detection method.

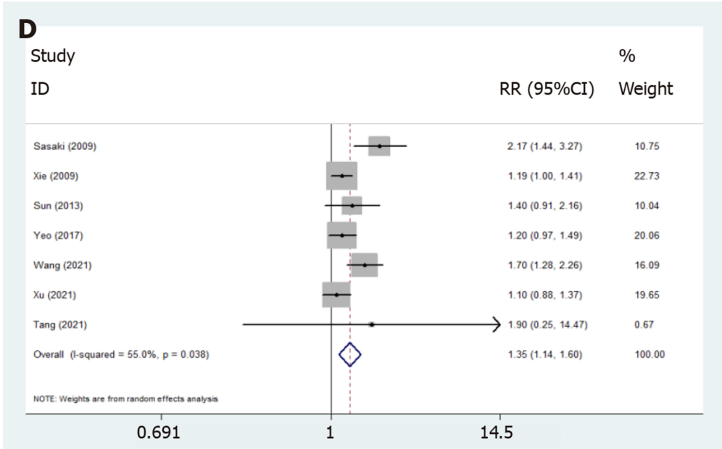
cancer.

The human Twist gene constitutes one intron and two exons localized on 7q21.2[33]. Twist is widely expressed in various cancers, such as lung cancer[34], breast cancer[35,36], esophageal cancer[37], and prostate cancer[38,39]. Twist not only plays an important role in mesodermal development but can also


A
Study
ID
RR (95%CI)
%
Weight
Sasaki (2009) 1.87 (1.23, 2.84) 11.44
Xie (2009) 1.08 (0.89, 1.30) 20.69
Sun (2013) 1.52 (0.93, 2.47) 9.50
Yuan (2015) 1.09 (0.79, 1.50) 14.90
Chen (2016) 2.26 (1.32, 3.88) 8.34
Yeo (2017) 1.20 (1.00, 1.44) 21.01
Wang (2021) 1.72 (1.22, 2.41) 14.12
Overall (I-squared = 60.8%, p = 0.018) 1.38 (1.14, 1.67) 100.00
NOTE: Weights are from random effects analysis
0.258
1
3.88


B
Study
ID
RR (95%CI)
%
Weight
Sasaki (2009) 1.50 (1.00, 2.25) 12.40
Xie (2009) 1.13 (0.94, 1.35) 24.05
Sun (2013) 1.08 (0.70, 1.69) 11.17
Chen (2016) 2.08 (1.20, 3.58) 8.30
Yeo (2017) 1.12 (1.00, 1.26) 27.91
Wang (2021) 1.92 (1.24, 2.96) 11.36
Tang (2021) 0.86 (0.18, 4.07) 1.33
Du (2021) 2.83 (1.12, 7.14) 3.49
Overall (I-squared = 53.3%, p = 0.036) 1.34 (1.11, 1.60) 100.00
NOTE: Weights are from random effects analysis
0.14
1
7.14


C
Study
ID
RR (95%CI)
%
Weight
Sasaki (2009) 1.64 (1.17, 2.30) 11.83
Xie (2009) 1.27 (1.15, 1.40) 32.73
Sun (2013) 1.02 (0.56, 1.87) 4.69
Yeo (2017) 1.07 (0.96, 1.19) 31.74
Xu (2021) 1.02 (0.81, 1.28) 19.02
Overall (I-squared = 62.7%, p = 0.030) 1.18 (1.02, 1.35) 100.00
NOTE: Weights are from random effects analysis
0.435
1
2.3


D
Study
ID
RR (95%CI)
%
Weight
Sasaki (2009) 2.17 (1.44, 3.27) 10.75
Xie (2009) 1.19 (1.00, 1.41) 22.73
Sun (2013) 1.40 (0.91, 2.16) 10.04
Yeo (2017) 1.20 (0.97, 1.49) 20.06
Wang (2021) 1.70 (1.28, 2.26) 16.09
Xu (2021) 1.10 (0.88, 1.37) 19.65
Tang (2021) 1.90 (0.25, 14.47) 0.67
Overall (I-squared = 55.0%, p = 0.038) 1.35 (1.14, 1.60) 100.00
NOTE: Weights are from random effects analysis
0.691
1
14.5

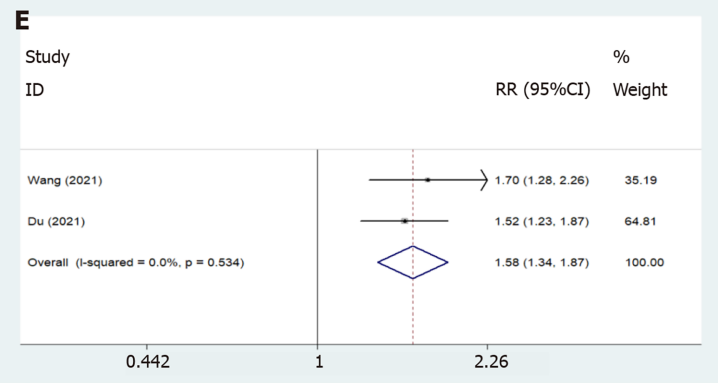


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Figure 4 Forest plots showed that Twist over-expression was correlated with T stage, N stage, M stage, tumor, node and metastasis stage, and clinical stage. A: T stage; B: N stage; C: M stage; D: Tumor, node and metastasis stage; E: Clinical stage.

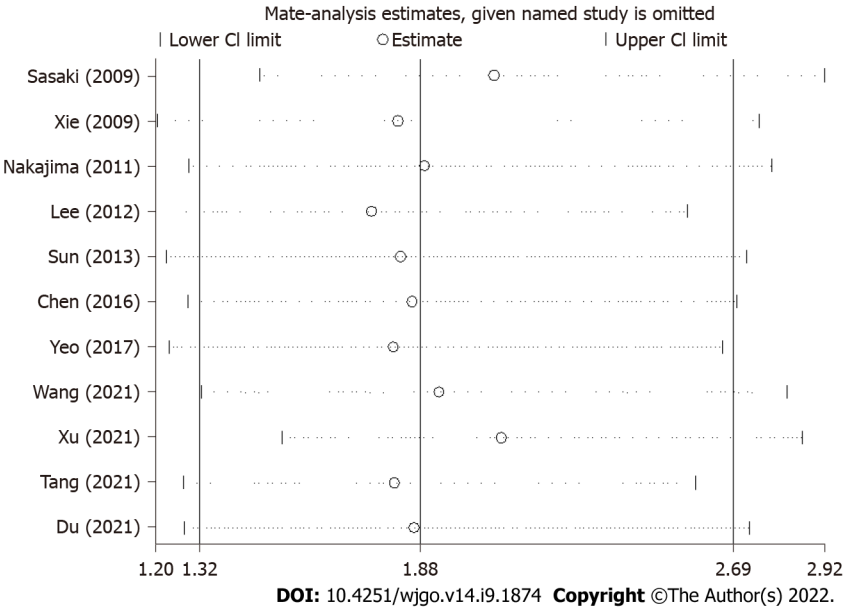

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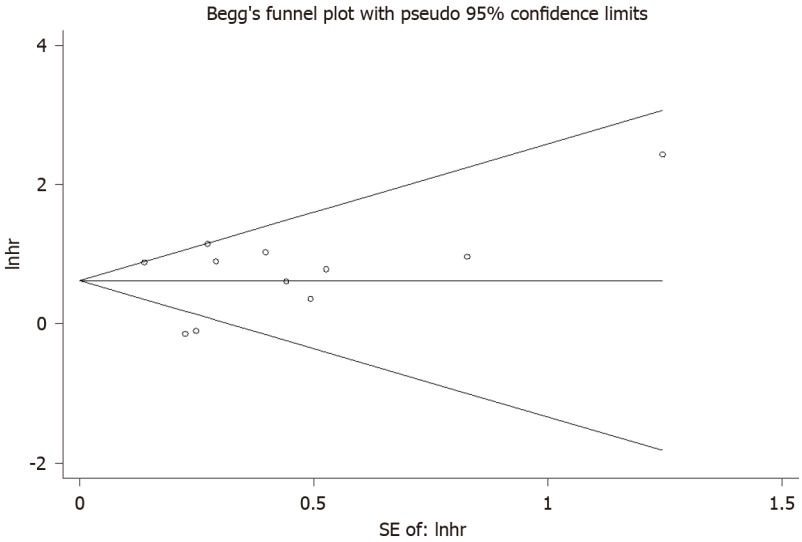
Figure 5 Sensitivity analysis of the association between Twist expression and overall survival.

participate in the EMT of some epithelium-derived tumor cells. Twist could interact with the Mi2/NuRD chromatin remodeling and gene repression complex (MTA2, RbAp46, Mi2, and HDAC2) [40]. Twist recruits MTA2 to the E-cadherin promoter and reduces the level of acetylation in the promoter region, thereby inhibiting the expression of E-cadherin and promoting the invasive progression of ESCC[41]. Moreover, integrin-mediated adhesion to interstitial matrix proteins may differentially regulate nuclear/cytoplasmic translocation and DNA binding of Twist1, thereby activating the transcription of N-cadherin[38]. In malignant melanoma, increased N-cadherin expression following the loss of E-cadherin mRNA expression has been shown to play an important role in the regulation of cell migration, invasion, and survival[42].

Although all eligible studies used IHC to detect Twist expression, the type of primary antibody used, the degree of antibody dilution, and the quantification of the method were not the same. Second, immunohistochemical scores were classified into three categories in the included studies: scored by intensity, scored by the percentage of stained cells, and multiplied by the percentage score and intensity score, which may be the main sources of heterogeneity. The subgroup analysis found that immunoreactivity scored by multiplying the percentage score and intensity score showed very high heterogeneity (I^2 = 79.5%), indicating that different scoring methods for IHC could contribute to potential publication bias. In addition, the scoring criteria and cutoff points for immunohistochemistry were subjective and not uniform in the included studies.

According to Sun *et al*[15], the positive expression of the Twist gene in ESCC stromal fibroblasts was associated with poor overall survival. Similarly, Yeo *et al*[17] found high Twist protein expression in cancer-associated fibroblasts of ESCC and concluded that Twist was an independent predictor of poor



DOI: 10.4251/wjgo.v14.i9.1874

Figure 6 The Begg's funnel plot for overall survival.

prognosis for OS. Therefore, more research is needed to explore the clinical significance of Twist expression in stromal fibroblasts. Nakajima *et al*[14] studied the expression of Twist in 54 patients who consecutively received 5-fluorouracil neoadjuvant chemotherapy followed by surgery. The results also showed that high Twist expression was positively associated with a worse esophageal cancer prognosis. In addition, Tang *et al*[20] detected tumor samples of 55 ESCC and 31 EAC obtained by endoscopy instead of surgery, while other included studies all detected Twist expression in tissues obtained from patients who underwent surgical treatment. Therefore, the conclusions of the studies discussed above are consistent with the results of our meta-analysis.

This study might have several limitations. First, only 11 studies including 1293 patients were included. Second, all of the patients were from Asian countries, and most were from China, which limited the application of our findings in other countries and regions. Third, the use of different anti-Twist antibodies in the included studies might cause heterogeneity in our meta-analysis. Hence, more evidence is urgently needed to assess the correlation between the expression of Twist and prognostic value in esophageal cancer patients.

Many aspects of Twist deserve further research. Except for the study of Tang *et al*[20], our meta-analysis only included ESCC patients who underwent surgery. We found few studies investigating the clinicopathological and prognostic significance of the Twist gene in other histological types of esophageal cancer. Furthermore, Lee *et al*[13] demonstrated that TWIST-positive circulating tumor cells (CTCs) were common in ESCC patients (75% of the total study population), and a proportion of TWIST (+) CTCs ≥ 0.5 was significantly associated with advanced histologic grade[43]. IHC staining is mostly used in studies on the clinical significance of TWIST in esophageal cancer, but this is not conducive to the application of Twist in the diagnosis and treatment of esophageal cancer. As a novel noninvasive biomarker for the diagnosis and prediction of tumor progression, CTCs are needed for more studies to evaluate the clinical prognostic value of TWIST (+) CTCs in esophageal cancer patients and overcome the challenges of standard CTC isolation and the diversity of CTC counting methods.

CONCLUSION

In summary, this meta-analysis suggests that Twist overexpression is associated with a poor esophageal cancer prognosis despite the limitations encountered by our study. Twist overexpression is correlated with T stage, lymph node metastasis, distant metastasis, TNM stage, and clinical stage, which indicates that Twist might accelerate esophageal cancer progression and metastasis. Furthermore, the sensitivity analyses implied that our meta-analysis yielded a stable and reliable estimate.

ARTICLE HIGHLIGHTS

Research background

Twist can induce epithelial–mesenchymal transition (EMT) and cancer metastasis. However, the

prognostic value of Twist expression in patients with esophageal cancer remains controversial.

Research motivation
To clarify whether Twist could be a promising biomarker for predicting prognosis in esophageal cancer.

Research objectives
To investigate the prognostic and clinicopathological value of Twist expression in esophageal cancer.

Research methods
Published literature in several databases was searched for eligible articles. Participants with esophageal cancer whose tumor tissues underwent immunohistochemistry to detect the expression of Twist were considered when they met the inclusion criteria. The hazard ratio (HR) and relative ratio (RR) with their 95%CI were pooled. Heterogeneity was estimated by I^2 statistics.

Research results
The pooled HR for overall survival was 1.88 (95%CI: 1.32-2.69, I^2 = 68.6%), and the pooled HR for disease-free survival/relapse-free survival/progression-free survival was 1.84 (95%CI: 1.12-3.02, I^2 = 67.1%). In addition, overexpression of Twist was correlated with T stage (T3 + T4 *vs* T1 + T2, RR = 1.38, 95%CI: 1.14-1.67), lymph node metastasis (yes *vs* no, RR = 1.34, 95%CI: 1.11-1.60), distant metastasis (yes *vs* no, RR = 1.18, 95%CI: 1.02-1.35), tumor, node and metastasis (TNM) stage (III + IV *vs* I + II, RR = 1.35, 95%CI: 1.14-1.60), and clinical stage (III + IV *vs* I + II, RR = 1.58, 95%CI: 1.34-1.87).

Research conclusions
Twist overexpression indicates poor esophageal cancer prognosis. Moreover, Twist overexpression is correlated with T stage, lymph node metastasis, distant metastasis, TNM stage, and clinical stage, which indicates that Twist might accelerate esophageal cancer progression and metastasis.

Research perspectives
Our meta-analysis suggests that Twist might be a valuable prognostic biomarker in esophageal cancer.

FOOTNOTES

Author contributions: Song WP, Wang SY, Zhou SC and Che GW designed the research; Song WP, Zhou SC, Wu DS, Wu XZ and Xie JY conducted the literature search; Song WP and Wang SY collected and retrieved the data; Song WP, Wang SY, Wu DS, Wu XZ, Liu TT and Xie JY analyzed the data; Song WP wrote and revised the manuscript; Liu TT and Che GW contributed to editing; All authors approved the final version.

Conflict-of-interest statement: No conflicts of interest.

PRISMA 2009 Checklist statement: The authors have read the PRISMA 2009 Checklist, and the manuscript was prepared and revised according to the PRISMA 2009 Checklist.

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S-Editor: Zhang H
L-Editor: A
P-Editor: Zhang H

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World J Gastrointest Oncol 2022 September 15; 14(9): 1887-1891

DOI: 10.4251/wjgo.v14.i9.1887

ISSN 1948-5204 (online)

LETTER TO THE EDITOR

Nutrition deprivation affects the cytotoxic effect of CD8 T cells in hepatocellular carcinoma

Chun-Ye Zhang, Shuai Liu, Ming Yang

Specialty type: Oncology

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Liao R, China; Lu H, China; Yang L, China

Received: June 18, 2022
Peer-review started: June 18, 2022
First decision: July 19, 2022
Revised: July 28, 2022
Accepted: August 16, 2022
Article in press: August 16, 2022
Published online: September 15, 2022

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the third leading cause of cancer-related death worldwide. Factors including carcinogens, infection of hepatitis viruses, alcohol abuse, and metabolic disorders such as non-alcoholic fatty liver disease mainly contribute to HCC initiation and progression. Immunotherapy is one of the most powerful tools for unresectable HCC treatment in patients. $CD8^+$ T cells are a major immune component in the tumor microenvironment with cytotoxic effects against cancer cells. However, these $CD8^+$ T cells commonly display an exhaustion phenotype with high expression of programmed cell death protein 1, T-cell immunoglobulin and mucin-domain containing-3, and/or lymphocyte-activation gene 3, producing low levels of perforin (PRF1) and granzyme B (GZMB), as well as anti-tumor cytokines, such as interferon gamma and tumor necrosis factor alpha. In the referenced study, the authors also showed that deprivation of glutamine decreased the antitumor function of $CD8^+$ T cells, as well as the production of PRF1 and GZMB. However, the role of each amino acid in T cell function and exhaustion may depend on tumor type and tumor microenvironment, including the source of other nutrients. Overall, amino acids or other nutrient metabolites in the tumor microenvironment play a pivotal role in both tumor growth and immune response.

Key Words: Hepatocellular carcinoma; Metabolism; Amino acids; Tumor microenvironment; T cell function

Core Tip: Immunotherapy is one of the most powerful tools for patients with unresectable hepatocellular carcinoma. $CD8^+$ T cells are a major immune component in the tumor microenvironment with cytotoxic effects against tumor cells. However, these $CD8^+$ T cells commonly display an exhaustion phenotype with high expression of immune checkpoints such as programmed cell death protein 1, producing less anti-tumor proteins and cytokines, such as perforin and granzyme B. Here, we show that the roles of amino acids such as glutamine in T cell activation and function are dependent on tumor types and nutrients in the tumor microenvironment. Overall, nutrient metabolism reprogramming in the tumor microenvironment plays a pivotal role in both tumor growth and immune response.

Citation: Zhang CY, Liu S, Yang M. Nutrition deprivation affects the cytotoxic effect of CD8 T cells in hepatocellular carcinoma. *World J Gastrointest Oncol* 2022; 14(9): 1887-1891
URL: https://www.wjgnet.com/1948-5204/full/v14/i9/1887.htm
DOI: https://dx.doi.org/10.4251/wjgo.v14.i9.1887

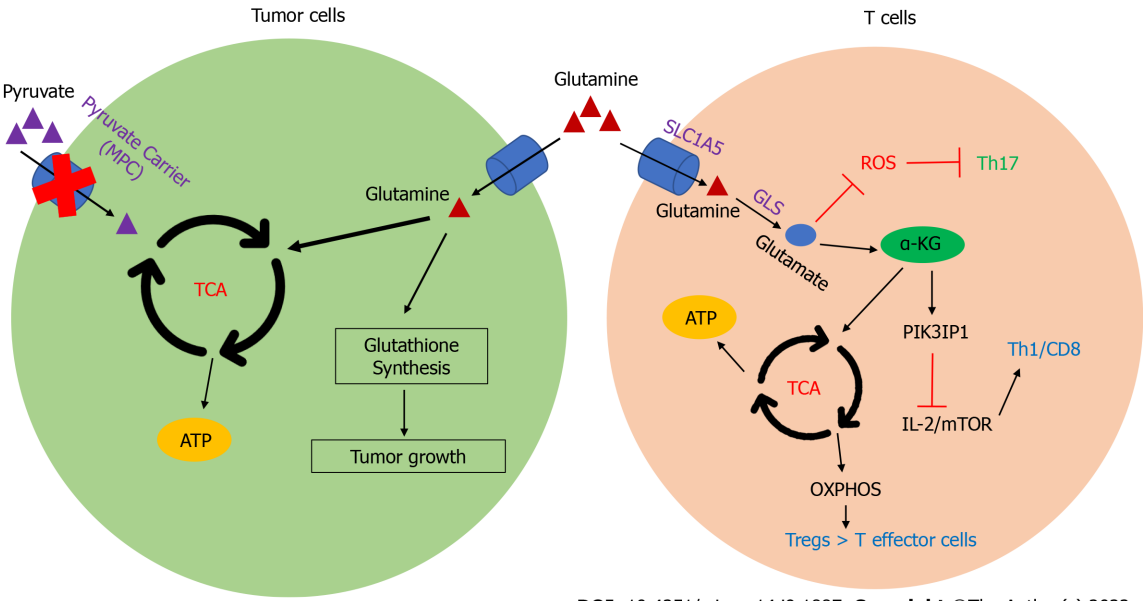
TO THE EDITOR

We read a basic study recently published by Wang *et al*[1] with great interest, which shows that glutamine deprivation impairs the cytotoxic function of tumor-infiltrating $CD8^+$ T cells in hepatocellular carcinoma (HCC) by inducing mitochondrial dysfunction and apoptosis. HCC is the primary liver cancer and the third leading cause of cancer-related death worldwide[2]. Factors including carcinogens, infection of hepatitis viruses, alcohol abuse, and metabolic disorders such as non-alcoholic fatty liver disease mainly contribute to HCC initiation and progression[3].

Immunotherapy is one of the most powerful tools for unresectable HCC treatment in patients[4]. $CD8^+$ T cells are a major immune component in the tumor microenvironment with cytotoxic effects against tumor cells. However, these $CD8^+$ T cells commonly display an exhaustion phenotype with high expression of programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3, and/or lymphocyte-activation gene 3, which produce low levels of anti-tumor cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α)[5,6]. In the referenced study, the authors also showed that deprivation of glutamine decreased the secretion of perforin and granzyme B in $CD8^+$ T cells in HCC[1]. Treatment of immune checkpoint inhibitors by targeting PD-1, programmed death protein-ligand-1 (PD-L1), or cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) has shown clinical effects in HCC patients[7,8]. For example, the U.S. Food and Drug Administration approved the use of nivolumab (anti-PD1) or in combination with ipilimumab or ipilimumab (anti-CTLA-4) for the treatment of patients with HCC in certain conditions[9-11]. Furthermore, the state-art chimeric antigen receptor-engineered T-cell therapy has displayed the promise for HCC treatment[12, 13].

Accumulating data indicate that tumor cells can compete with immune cells for nutrition in a nutrient-poor tumor microenvironment, especially for cytotoxic effective $CD8^+$ T cells to suppress their anti-tumor immunity[14]. For example, restriction of dietary asparagine (Asn), asparaginase administration, or inhibition of the asparagine transporter solute carrier family 1 member 5 (SLC1A5) impaired the function of $CD8^+$ T cells[15]. In contrast, increased Asn levels enhance $CD8^+$ T-cell activation and function against tumor cells (*e.g.*, B16-OVA) *in vitro* and *in vivo*[15]. Supplementation of creatine significantly inhibited tumor growth in multiple mouse tumor models (*e.g.*, B16-OVA melanoma) by activating T cells, which had a synergistic with a PD-1/PD-L1 blockade treatment[16]. Some non-essential amino acids such as serine are required for T cell proliferation by promoting nucleotide biosynthesis[17]. Additionally, nutrients are also required for $CD8^+$ T cell differentiation into effector and memory subsets, such as glucose, lactate, glutamine, methionine, and neutral amino acids[18]. Under a low-glucose tumor microenvironment, due to the consumption of glucose by tumor cells, the function of effector $CD8^+$ T cells was impaired and the expression of PD-1 was enhanced in regulatory T cells, resulting in treatment failure of PD-1 blockade[19]. In addition, tumor cell-derived metabolites such as lactate can also inhibit $CD8^+$ T cell cytotoxicity[20]. Another study also showed that accumulation of long-chain fatty acids (LCFAs) due to downregulation of regulating enzymes can impair $CD8^+$ T cell function by causing their mitochondrial dysfunction and reducing fatty acid catabolism[21]. Tumor cells can reprogram their metabolic pathways to compete with $CD8^+$ T cells for nutrients such as fatty acids [22]. Therefore, regulation of nutrient metabolism can impact the function of T cells. Inhibiting glutaminase, an amidohydrolase enzyme that can generate glutamate from glutamine, can also suppress $CD8^+$ T cell activation induced by anti-PD-1 immunotherapy[23].

Different nutrients show diverse functions in $CD8^+$ T cells. Regulation of tryptophan metabolism impacts the cytotoxic effect of $CD8^+$ T cells. For example, inhibiting tryptophan catabolism using indoleamine 2,3-dioxygenase inhibitors can activate $CD8^+$ T cells and suppress their expression of PD-1 by elevating intracellular tryptophan levels[24]. Meanwhile, tryptophan supplementation also



DOI: 10.4251/wjgo.v14.i9.1887

Figure 1 Glutamine metabolism impacts T cell differentiation and tumor growth. Glutamine metabolism can be transferred into cells by solute carriers, such as Solute carrier family 1 member 5 (also known as alanine-serine-cysteine transporter 2). It can be metabolized into glutamate through glutaminolysis (GLS) to impact T helper 17 (Th17) cells, Th1, and CD8 T cell differentiation by regulating the production of reactive oxygen species and expression of phosphoinositide-3-Kinase Interacting Protein 1, respectively. Increasing GLS leads to a proinflammatory effector phenotype, while restriction of GLS causes a slanted Treg differentiation by inhibiting oxidative phosphorylation. In addition, hepatocyte mitochondrial pyruvate carrier disruption redirects glutamine from glutathione synthesis into the tricarboxylic acid cycle, which impaired hepatocellular carcinoma by limiting glutathione synthesis. MPC: Mitochondrial pyruvate carrier; TCA: Tricarboxylic acid; SLC1A5: Solute carrier family 1 member 5; GLS: Glutaminolysis; OXPHOS: Oxidative phosphorylation; TOR: Target of Rapamycin; Th1: T helper 1; PIK3IP1: Phosphoinositide-3-Kinase Interacting Protein 1; ROS: Reactive oxygen species.

promoted the cytotoxic function of $CD8^+$ T cells against co-cultured B16F10 tumor cells *in vitro* and increased tumor-infiltration of $CD8^+$ T cells and their functions in mouse lung cancer model[24]. In contrast, another study also showed that depletion of dietary tryptophan decreased aryl hydrocarbon receptor activity in tumor-associated macrophages and increased tumor infiltration of tumor necrosis factor alpha $(TNF\alpha)^+IFN\gamma^+CD8^+$ T cells in pancreatic ductal adenocarcinoma, while supplementation of dietary indoles inhibited this effect[25].

In the reviewed study, the authors showed that mitochondrial damage and apoptosis caused $CD8^+$ T cell dysfunction. These findings shed light on the need for further investigation into the molecular mechanisms of glutamine metabolism impacting T cell functions. Glutamine metabolism has been shown to regulate the T helper 17 cell differentiation but restrict Th1 and $CD8^+$ T cell differentiation through glutaminolysis (GLS) by regulating the production of reactive oxygen species and expression of phosphoinositide-3-kinase interacting protein 1 (Figure 1), respectively. SLC1A5, also known as alanine-serine-cysteine transporter 2, mediates glutamine transportation, as well as other solute carriers (SLCs) including SLC6A14, 19, and SLC38A1-5[26]. Increasing GLS leads to a proinflammatory effector phenotype, while restriction of GLS results in a slanted Treg differentiation through the inhibition of oxidative phosphorylation[27]. In addition, hepatocyte mitochondrial pyruvate carrier disruption redirected glutamine from glutathione synthesis into the tricarboxylic acid cycle, which impaired HCC by limiting glutathione synthesis[28]. Another study showed that inhibition of glutamine metabolism can reduce T-cell exhaustion and increase the antitumor activity of tumor-specific $CD8^+$ T cells against mouse lymphoma[29]. Overall, the function of glutamine on $CD8^+$ T cells is dependent on tumor microenvironment and tumor type. Meanwhile, regulation of nutrient metabolism could be a synergetic strategy for cancer treatment.

FOOTNOTES

Author contributions: Zhang CY, Liu S, and Yang M designed, collected data, wrote, revised, and finalized the manuscript, contributed equally, and shared the first authorship.

Conflict-of-interest statement: All authors declare no conflicts of interest.

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S-Editor: Wang LL
L-Editor: A
P-Editor: Wang LL

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