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WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc*.

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EDITORIAL

Challenges to addressing the unmet medical needs for immunotherapy targeting cold colorectal cancer

Keun-Yeong Jeong

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Abstract

With the establishment of the immune surveillance mechanism since the 1950s, attempts have been made to activate the immune system for cancer treatment through the discovery of various cytokines or the development of antibodies up to now. The fruits of these efforts have contributed to the recognition of the 3rd generation of anticancer immunotherapy as the mainstream of cancer treatment. However, the limitations of cancer immunotherapy are also being recognized through the conceptual establishment of cold tumors recently, and colorectal cancer (CRC) has become a major issue from this therapeutic point of view. Here, it is emphasized that non-clinical strategies to overcome the immunosuppressive environment and clinical trials based on these basic investigations are being made on the journey to achieve better treatment outcomes for the treatment of cold CRC.

Key Words: Colorectal cancer; Immunotherapy; Cell therapy; Checkpoint inhibitor; Cancer vaccine; Cytokine therapy

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Core Tip: There have been continuing attempts to treat colorectal cancer (CRC) with immunotherapies, and various methods of converting cold into hot tumors have gone through trial and error up to now. Based on this background, this editorial introduces the concept of cold CRC and various strategies across non-clinical and clinical for enhancing immunotherapeutic efficacy and further encourages the journey to an advanced level of immunotherapies targeting cold CRC.

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INTRODUCTION

Cancer immunotherapy provides a basis for activating the components of the immune system of cancer patients. Recently spotlighted methods of cancer immunotherapy utilize antibodies and peptides that bind to and inhibit the proteins related to immune evasion (*e.g.*, immune checkpoint inhibitors and cytokine therapies), DNA and RNA vaccines, and immune cell therapies such as chimeric antigen receptor natural killer (CAR-NK) and CAR-T cells (Figure 1)[1]. The idea of using immunotherapy for cancer treatment emerged with the first proposed theory of cancer immunosurveillance in the 1950s, which suggested that lymphocytes act as a monitoring system to identify and eliminate cells harboring somatic mutations[2]. However, due to a lack of non-clinical data to support these theories, it took a long time to establish a bridgehead for clinical applications[2]. Eventually, the identification of T-cell growth factor interleukin 2 (IL-2) in the 1970s allowed improved T-cell production through IL-2 exposure and led to positive results in patients with metastatic cancer[2,3]. Milstein and Köhler pioneered the production of monoclonal antibodies by fusion of lymphocytes around the same time, and antibody-based therapies led to the development of rituximab, which targets immature B cells-based NK cell activation[2,4]. After this discovery, development was stagnant because it was difficult to devise clinically effective cancer immunotherapy strategies until 2010. Ipilimumab [targeting cytotoxic T lymphocyte antigen 4 (CTLA-4)], nivolumab, and atezolizumab [targeting programmed cell death-1 (PD-1) or its ligand (PD-L1), respectively] have been approved in the 2010s as a result of ongoing research for the discovery of immune checkpoint molecules[1,5]. More recently, six CAR-T cell therapies have been approved for the treatment of lymphoma, some forms of leukemia, and multiple myeloma [6]. Such rapid development over the past decade established immunotherapy as the mainstream of cancer treatment as third-generation cancer treatment next to second-generation targeted therapies.

Meanwhile, it would be an erroneous attempt to follow in the footsteps of approaches focusing on only its potential while looking at the rapid development of immunotherapy. Given the extensive non-clinical research and clinical investigation efforts dedicated to advancing different immunotherapy approaches, such efforts should be accompanied by those focusing on the various prominent issues that emerge. A discussion may be required on the optimal model that can accurately reflect the human immune system by replacing the immunodeficient mouse used in the non-clinical efficacy evaluation studies or on the concerns about synthetic rather than endogenous immunity. However, here I would like to highlight organ-specific tumor immunity, especially in colorectal cancer (CRC), as a key concern among the multiple issues involved in the resistance to immunotherapies. The widely accepted concept of 'cold tumor' focuses on tumors that are unlikely to elicit a strong immune response due to the heterogeneity of the tumor microenvironment (TME)[7].

CONCEPT OF COLD TUMOR AND IMMUNE SIGNATURE OF CRC

The advancements in the knowledge of the interactions among different types of cells in the TME have enabled the establishment of the basis of therapeutic strategies focused on the immune system. Patient stratification with an immune score can be performed according to the types or densities of immune cells within the tumor, and it could be possible to make a more accurate prediction of prognosis compared with TNM staging[8]. This concept is based on the quantification of CD3+ and CD8+ T-cells abundance in and around the TME. The immune score ranges from I0 (immune score 0) to I4, where I0 denotes the absence of both CD3+ and CD8+ T-cell types, and I4 indicates a high density of immune cells positive for the expression of the T-cell types[9,10]. Such a system was proposed for immune-based tumor classification and allowed the discrimination between high-invasive immune score I4 (hot tumor) and non-invasive immune score I0 (cold tumor)[8-10]. The feasibility of the immune score has been proved in CRC and is recognized as having a greater relative prognostic value compared with pathological staging, lymphatic invasion, tumor differentiation, and microsatellite status[8,11]. Currently, the definition of cold tumor is routinely used to refer to tumors with little or no T-cell infiltration, inflamed but non-T-cell infiltrated, or non-inflammatory tumors[12]. In addition to the analysis of tumor-infiltrating lymphocytes, it is characterized by the regulation of antigen-presenting machinery markers, such as low expression levels of PD-L1 or reduced presentation of neoantigens[13]. At this point, it is possible to characterize the immune signature in CRC represented by the propensity for cold tumors. A neoantigen is an abnormal peptide mainly generated by a genetic mutation or gene fusion and is encoded by mutant genes in tumor cells[14]. Tumor-associated antigens, a type of neoantigen, are

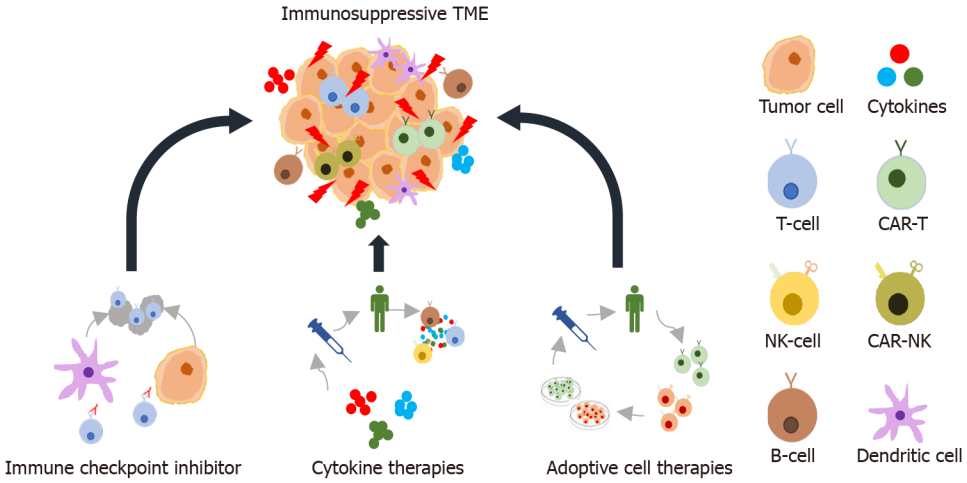

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Figure 1 The basic categories of immunotherapy. Different forms of cancer immunotherapy, including immune checkpoint inhibitors, cytokine therapies, adoptive cell transfer, and oncolytic virus therapies, target the immunosuppressive tumor microenvironment. CAR: Chimeric antigen receptor; NK: Natural killer; TME: Tumor microenvironment.

proteins that are overexpressed in tumor cells but also expressed in normal cells, limiting specific immune responses[14]. For example, carcinoembryonic antigen (CEA) is an important tumor-associated antigen in CRC and is frequently found on the surfaces of most metastatic CRC cells, but it induces immune tolerance since CEA is also expressed at the embryonic stage[14,15]. Further, the presence of various mechanisms that interfere with antigen presentation is a hallmark of metastatic CRC, typically with low microsatellite instability (MSI)/DNA mismatch repair deficient molecular characteristics[16,17]. Such mechanisms interfere with antigen presentation and proteasome processing of antigens, impede transporter functions involved in antigen processing and inhibit the expression of major histocompatibility complex (MHC) structural components through genetic mutations[16]. In particular, loss of β-2-microglobulin heterozygosity may affect antigen presentation of the MHC-I, which is known to induce resistance to T-cell invasion[18]. Furthermore, the immune signature of CRC indicates that it could interfere with the recruitment or activation of T-cells through various molecular biological changes as a result of the inter-communication between the constituents of the TME[19]. It has been reported that activation of Wnt/β-catenin signaling is associated with T-cell exclusion and inversely proportional to T-cell infiltration in CRC tissues[20]. Signal transducer and activator of transcription 3 (STAT3) can reduce the expression of interferon-gamma (IFN-γ) in CD8-positive T-cells[21]. This, in turn, inhibits chemokine (C-X-C motif) ligand secretion by tumor-associated myeloid cells and interferes with T-cell recruitment[21,22]. The mitogen-activated protein kinase (MAPK) signaling cascade upregulates the expression levels of the immunosuppressive cytokines such as vascular endothelial growth factor (VEGF) and IL-8, suppressing T-cell function and its infiltration into the TME[23-25]. These immune signatures in cold CRC by their genetic and molecular complexity may be a major cause of resistance to cancer therapies (especially in immunotherapy). Therefore, a variety of attempts are currently being made to overcome these obstacles through non-clinical and clinical studies.

NON-CLINICAL STRATEGIES TO TARGET COLD CRC

Non-clinical strategies are being designed to overcome the obstacles in cancer immunotherapy, and these strategies can be classified into key categories as follows: Increasing the number of antigen-specific T-cells, T-cell priming, and promoting T-cell trafficking and infiltration[26-29]. First, the method of increasing the number of antigen-specific T-cells and T-cell priming includes the application of adoptive cell therapy[30,31], adjuvant immunotherapy[32,33], epigenetic modification inhibitors[34,35], cancer vaccines[36,37], oncolytic viruses[38,39], and their combination with conventional therapies[40-43] (Table 1). Adoptive cell therapy enhances the immune response through CAR-T or CAR-NK cells. Utilization of CAR-T or CAR-NK cells involves the genetic modification of T lymphocytes or NK cells to express specific antigens to target the tumor cells. The activities of CAR-T or CAR-NK cells are not limited by the presence or absence of MHC and can further enhance the immune response against tumor cells through the addition of costimulatory molecules such as CD28, OX40, or 4-1BB[30,44]. The strategy utilizes the direct recognition of tumor antigens by CARs and has the potential to treat cold CRC[30]. Adjuvant immunotherapy is based on innate immune responses through the activity of the pattern recognition receptor family[45]. The pattern recognition receptor family includes Toll-like receptors, nucleotide oligomerization domain-like receptors, retinoic acid-inducible gene-I-like

Table 1 Key categories for non-clinical strategies to overcome obstacles in cancer immunotherapy

Strategy	Therapeutic approach	Ref.
Increase in antigen-specific T-cells and T-cell priming	Adoptive cell therapy (CAR-T and -NK)	[30-44,49]
	Adjuvant immunotherapy	
	Epigenetic modification inhibitors	
	Cancer vaccine	
	Oncolytic viruses	
	Combined with conventional therapies	
Promoting T-cell trafficking and infiltration	TGF-β suppression	[50-58,60,61]
	Oncogenic pathway inhibitors	
	Angiogenesis inhibitors	
	CXCR4 inhibitors	
	Immunocytokines	

CAR: Chimeric antigen receptor; NK: Natural killer; TGF: Transforming growth factor; CXCR: C-X-C motif chemokine receptor.

receptors, and type C lectin receptors. Agonistic activation of these receptors can generate a variety of proinflammatory cytokines including type I IFNs to promote T-cell priming[45]. Targeting DNA methyltransferase and histone deacetylase activities to inhibit epigenetic modifications has been shown to enhance the expression levels of tumor antigens and other immune-related genes, as a specific therapy for tumors with low antigen expression[46]. Cancer vaccines enhance the treatment efficacy and overcome the limitations of immunotherapy by increasing the number of specific effector T-cells. They include molecular-based vaccines using peptides, protein, DNA and mRNA prepared with isolated cancer cells and adenovirus for the expression of cancer-specific antigens[47]. Oncolytic viruses capable of selectively targeting and destroying cancer cells contribute to the maturation of antigen-presenting cells that carry out the activation of antigen-specific CD4+ and CD8+ T-cell responses and activate both innate and adaptive immune responses to convert a cold tumor into a hot tumor[38]. Chemotherapy and radiotherapy can exert anti-tumor effects by directly killing tumor cells while contributing to immune system stimulation[41,42]. Radiotherapy promotes the activation of dendritic cells and the expression of cell adhesion molecules that promote the attraction of immune cells[42,45]. Chemotherapy regulates immunogenicity and increases T-cell infiltration. 5-fluorouracil and oxaliplatin-based chemotherapies and MAPK and epidermal growth factor receptor inhibitors are some examples[41,48,49]. Methods for promoting T-cell trafficking and infiltration include the application of transforming growth factor (TGF)-β suppression[50,51], oncogenic pathway inhibitors[52,53], angiogenesis inhibitors, CXC chemokine receptors (CXCR) inhibitors[54,55], and immune cytokines[56] (Table 1). TGF-β is associated with a lack of immune responses in the noninflamed T-cell phenotype with a deterioration in the ability to produce type I IFNs in tumor-associated dendritic cells, leading to STAT3 up-regulation and an imbalance in T-cell infiltration. Non-clinical studies have shown that a combination of TGF-β blocking antibodies induces T-cell penetration into tumors, allowing for anti-tumor immunity and tumor regression[50,51]. Targeting oncogenic pathways helps to reverse intrinsic T cell exclusion in tumors. Inhibition of the WNT/β-catenin pathway by p21-activated kinase 4 inhibitors or the endogenous Dickkopf family binding to lipoprotein receptor-associated proteins may increase tumor invasion of cytotoxic T lymphocytes[52,53]. Inhibition of well-established biochemical pathways, CDK4/6, phosphoinositide 3-kinase (PI3K)/AKT, or MAPK, involved in tumor growth and differentiation can lead to a significant upregulation of tumor-infiltrating T lymphocytes with the regulation of granzyme B and CC chemokine ligand 4/5[49,57,58]. Angiogenesis inhibitors play a role in the normalization of the unregulated balance between angiogenesis-promoting and antiangiogenic signals by upregulation of the leukocyte adhesion molecules in tumor endothelial cells resulting in amelioration of tumor vascular abnormalities, improved tissue perfusion, and increased infiltration of immune effector cells[54,59]. CXCR4 is a receptor for CXC ligand (CXCL) 12 and is overexpressed in tumors, and it can reduce the infiltration of cytotoxic T lymphocytes into the TME and mediate the invasion of immunosuppressive cells, such as regulatory T-cells (Treg), into the tumor. Regulation of CXCL12 by inhibiting CXCR4 can promote the infiltration of T lymphocytes into the tumor and reverse immune resistance[60,61]. Finally, since immune cytokines mediate the influx and expansion of leukocytes at tumor sites, cognate receptor expression on tumor and immune cells may induce an antitumor effect. IL-2, IFN, tumor necrosis factor, IL-12, granulocyte-macrophage colony-stimulating factor, promotion of MHC-I expression, and T-cell activation and infiltration enhance antitumor immunity[56,62].

Table 2 A list of completed clinical trials to improve response to immunotherapies targeting colorectal cancer

Regimen	NCT number	Outcome	Completion
T-cell bispecific antibody and CEA combined with atezolizumab	NCT02650713	20% PR and 50% SD	January 2020
Copanlisib plus nivolumab	NCT03711058	No results available	January 2022
Fruquintinib plus geptanolimab	NCT03977090	26.7% ORR, 80% DCR, and 7.33 mo median PFS	December 2021
Regorafenib plus toripalimab	NCT03946917	15.2% ORR and the 36.4% DCR	November 2021
Durvalumab plus tremelimumab	NCT02870920	2% DCR, 1.8 mo PFS, and 6.6 mo OS	December 2021
Anti-TGF-β antibody plus spartalizumab	NCT02947165	Clinical proof of concept with 2 PR cases	June 2021
Pembrolizumab plus celebrex	NCT03638297	83.3% ORR, 12.5% SD, and 4.2% PD	August 2021
Durvalumab and tremelimumab plus FOLFOX	NCT03202758	31.2% PR and CR, 25% SD, and 6 mo PFS	August 2020

CR: Colorectal; CEA: Carcinoembryonic antigen; PR: Partial response; SD: Stable disease; ORR: Objective response rate; DCR: Disease control rate; PFS: Progression-free survival; OS: Overall survival; PD: Progression disease; FOLFOX: Folinic acid, fluorouracil, and oxaliplatin; TGF: Transforming growth factor.

CLINICAL STRATEGIES TO TARGET COLD CRC

Over the past two decades, a multidisciplinary approach to graft novel therapeutic modalities onto the backbone of fluoropyrimidine-based chemotherapy in local and advanced CRC has achieved significant improvements in the therapeutic efficiency of immunotherapy[63]. However, the expected overall survival of patients with microsatellite stable (MSS) CRC is only about 30 mo, indicating an unmet medical need[64]. Therefore, several clinical trials evaluating immune checkpoint inhibitors have focused on designs that can overcome resistance and achieve clinically meaningful responses, but mono and combination therapies utilizing immune checkpoint inhibitors as the mainstay have not yet shown significant clinical success[65-68]. For example, studies using the single agent of pembrolizumab and nivolumab did not find any objective response rates (ORR)[65-67]. In a study of a combination of ipilimumab, nivolumab, and anti-CTLA-4 antibody in CRC patients with high MSI and MSS, the median progression-free survival (PFS) was only 1.4 mo, and no ORR was observed[68]. These results represent the limitations of approaches that do not target multiple molecular pathways involved in immune exclusion. Strategies for converting the cold CRC into hot CRC, which can enhance the responses to immune checkpoint inhibitors by promoting activation or recruitment of cytotoxic T lymphocytes in TME, should have been included in clinical trials. Recently, several trials have been conducted in favor of strategies to enhance immune activity and T lymphocyte infiltration into the TME to achieve substantial anti-tumor immune responses targeting CRC (Table 2). The list of completed clinical trials reflecting the non-clinical strategies includes the following: A phase I study (NCT02650713) in which a T-cell bispecific antibody and CEA combined with atezolizumab (targeting PD-L1) in CEA-positive solid tumors, indicating 20% partial response (PR) and 50% stable disease (SD)[69]; a phase I/II study (NCT03711058) with a combination of copanlisib (PI3K inhibitor) and nivolumab (anti-PD-1 antibody) targeting relapsed/refractory MSS CRC, with a decreasing trend of CD4+ T-lymphocytes mainly comprised of Treg and helper subsets[70]; a phase Ib study (NCT03977090) evaluating the safety and preliminary efficacy of fruquintinib (VEGF inhibitor) with geptanolimab (anti-PD-1 antibody) targeting metastatic CRC, indicating 26.7% ORR, 80% disease control rate (DCR), and 7.33 mo median PFS[71]; a phase Ib/II study (NCT03946917) of regorafenib plus toripalimab (anti-PD-1 antibody) targeting CRC, with 15.2% ORR and the 36.4% DCR[72]; a phase II randomized study (NCT02870920) of durvalumab (anti-PD-L1 antibody) plus tremelimumab (anti-CTLA-4 antibody) in patients with refractory CRC, resulting in 22% DCR, 1.8 mo PFA, and 6.6 mo overall survival[73]; a phase Ib study (NCT02947165) of the anti-TGF-β monoclonal antibody combined with spartalizumab (anti-PD-1 antibody) in patients with MSS CRC, providing a clinical proof of concept with 2 PR cases[74]; a phase II study (NCT03638297) to assess the efficacy of pembrolizumab (anti-PD-1 antibody) combined with celebrex (COX inhibitor) in patients with high MSI metastatic CRC, with 83.3% ORR, 12.5% SD, and 4.2% progressive disease[75]; and a phase I/II study (NCT03202758) to determine the safety and efficacy of durvalumab (anti-PD-L1 antibody) and tremelimumab (anti-CTLA-4 antibody) in combination with folinic acid, fluorouracil, and oxaliplatin in patients with metastatic CRC, with 31.2% PR and CR, 25% SD, and 6 mo PFS[76]. In summary, positive results were obtained targeting cold CRC through a variety of strategies for increasing immune responses, therefore, follow-up studies continue to be performed for treatment found to show significant results. Further, many clinical trials with

Table 3 Ongoing clinical trials to improve response to immunotherapies targeting colorectal cancer

Strategy	NCT number	Intervention
Targeting tyrosine kinase	NCT04764006	Surufatinib (VEGFR1, VEGFR2, VEGFR3, FGFR1, and CSF-1R inhibitor)
	NCT04819516	High-intensity focused ultrasound therapy; toripalimab (anti-PD-1 antibody)
	NCT04963283	Cabozantinib (anti-VEGFR2) plus nivolumab (anti-PD-1 antibody)
Targeting TGF-β	NCT03724851	TEW-7197 (TGF-β receptor ALK4/ALK5 inhibitor)
Targeting Wnt signaling	NCT02521844	ETC-159 (Porcupine inhibitor) plus Pembrolizumab (anti-PD-1 antibody)
Combination with chemotherapy	NCT04301557	Pembrolizumab plus binimetinib (MEK 1/2 inhibitor) plus FOLFOX plus irinotecan
	NCT04895137	FOLFOX6 plus bevacizumab (anti-VEGF A) plus anti-PD-1 antibody
	NCT03374254	Anti-PD-1 antibody plus oxaliplatin plus capecitabine plus radiotherapy then mesorectal excision
Cancer vaccine	NCT04046445	ATP128 (chimeric recombinant protein vaccine) plus BI754091 (IgG4Pro antibody inhibitor) plus VSV-GP128 (recombinant vesicular stomatitis virus)
	NCT04117087	KRAS peptide vaccine plus nivolumab (anti-PD-1 antibody) plus ipilimumab (anti-CTLA4 inhibitor)
	NCT04912765	Neoantigen dendritic cell vaccine plus nivolumab

VEGFR: Vascular endothelial growth factor receptor; FGFR1: Fibroblast growth factor receptor 1; CSF-1R: Colony stimulating factor 1 receptor; PD-1: Programmed cell death 1; TGF-β: Transforming growth factor beta; MEK 1/2: Mitogen-activated protein kinase 1/2; FOLFOX: Folinic acid, fluorouracil, and oxaliplatin; VEGF A: Vascular endothelial growth factor A; CTLA4: Cytotoxic T lymphocyte antigen-4.

various combinatory strategies by tyrosine kinase inhibitors, TGF-β inhibitors, Wnt signaling inhibitors, chemotherapies, and cancer vaccines to enhance immunotherapeutic efficacy are also ongoing (Table 3).

CONCLUSION

Recently, several attempts have been made to conquer CRC with immunotherapies, but poor clinical outcomes were obtained due to the non-immunogenic characteristics of cold CRC. However, a variety of methods of converting cold into hot tumors were obtained through trial and error, and positive results have been drawn based on this background. We will have to carry our journey to a higher level to target cold CRC by discovering useful biomarkers through various efforts that span non-clinical and clinical studies in the future.

FOOTNOTES

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REVIEW

Role of ferroptosis in colorectal cancer

Ya-Qi Song, Xiao-Dan Yan, Yi Wang, Zhen-Zhen Wang, Xin-Li Mao, Li-Ping Ye, Shao-Wei Li

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Abstract

Colorectal cancer (CRC) is the second deadliest cancer and the third-most common malignancy in the world. Surgery, chemotherapy, and targeted therapy have been widely used to treat CRC, but some patients still develop resistance to these treatments. Ferroptosis is a novel non-apoptotic form of cell death. It is an iron-dependent non-apoptotic cell death characterized by the accumulation of lipid reactive oxygen species and has been suggested to play a role in reversing resistance to anticancer drugs. This review summarizes recent advances in the prognostic role of ferroptosis in CRC and the mechanism of action in CRC.

Key Words: Ferroptosis; Colorectal cancer; System X_c^-; Glutathione; Glutathione peroxidase 4

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Core Tip: The prognosis of patients with advanced colorectal cancer is still poor, largely due to resistance to anticancer drugs. Ferroptosis is a novel form of non-apoptotic cell death, mainly characterized by abnormal iron metabolism and the excessive accumulation of lipid peroxidation. Studies have shown that ferroptosis can participate in the process of colorectal cancer (CRC) through the accumulation of lipid peroxides, inhibition of the System X_c^-, disruption of the glutathione/glutathione peroxidase 4 balance, imbalance of iron homeostasis, and mediation of the P53 pathway. Induction of ferroptosis can reverse the resistance of anticancer drugs and improve the prognosis of CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) is a common gastrointestinal malignancy and the second-most common cause of cancer death. During the last decade (2010-2019), CRC mortality declined by about 2% *per* year but increased among those under 50 years old[1]. The oncogenesis and development of CRC involve multiple genes and steps, which is an extremely complex process.

Studies have shown that CRC cells have characteristics of strong proliferation, easy recurrence and easy metastasis[2], but there are few effective therapeutic targets for CRC patients[3]. At present, the comprehensive treatment of CRC includes surgical resection, neoadjuvant chemoradiotherapy, postoperative chemoradiotherapy, targeted therapy, immunotherapy and other methods, but the prognosis of patients with advanced CRC is still poor.

Cell death is a basic life process and can be divided into accidental cell death and regulated cell death (RCD). RCD can be further divided into the category of apoptosis, pyroptosis, necroptosis and ferroptosis[4]. Ferroptosis, first reported in 2012, is a newly defined form of RCD involving iron-dependent, non-apoptotic cell death. The characterization methods included free iron and lipid reactive oxygen species (ROS), particularly lipid hydroperoxides[5], and by cytoplasmic and organelle swelling, chromatin condensation and mitochondrial disorder[6,7]. Studies have shown that the tumor cell survival is highly dependent on an abnormally activated antioxidant system.

Several therapeutic targets associated with ferroptosis have been identified in CRC (Figure 1). The induction of ferroptosis is also considered a promising research direction in cancer resistance.

FERROPTOSIS-RELATED INDICATORS CAN PREDICT THE PROGNOSIS OF CRC

Recently, a growing number of studies have shown that genes involved in ferroptosis are associated with the prognosis of CRC patients. CRC has a high recurrence rate and individual heterogeneity, so it is desirable to have good prognostic biomarkers that can be used to predict high-risk patients in order to help patients obtain appropriate treatment.

In an analytical study combining samples from eight CRC patients with the TCGA public database, changes in ferroptosis in CRC patients at various molecular levels, ranging from DNA, RNA and proteins to epigenetic modifications, were described, and four ferroptosis-related genes associated with the prognosis were identified: Cyclin-dependent kinase inhibitor 2A (CDKN2A), glutathione peroxidase 4 (GPX4), arachidonic acid lipoxygenase 3 (ALOXE3) and LINC00336[8]. Another study constructed a clinical prediction model including GPX4, NOX1 and Acyl-CoA synthetase long-chain family member 4 (ACSL4) that effectively reflected the prognosis, tumor progression and asthma control test responsiveness of CRC patients. It is also worth noting that tumors with low ferroptosis scores may infiltrate more CD4+ and CD8+ T cells and fewer M1 macrophages[9]. ALOX5 is considered a key ferroptosis-related gene associated with a poor prognosis in CRC patients, and it regulates ferroptosis in cancer cells through lipid peroxidation[10,11]. CRC patients with an increased NOX1 expression and decreased BRAF status have a higher survival rate than others, and genes positively correlated with NOX1 are also significantly correlated with the CRC survival rate. The mechanism underlying NOX1 and BRAF mutations needs to be further explored[12]. A prognostic model combining genes related to oxidative stress and ferroptosis can distinguish CRC as hot and cold tumors. Patients in the low-risk group responded better to fluorouracil chemotherapy and immune checkpoint blocking therapy than those in the high-risk group[13].

Long non-coding RNA (lncRNA) is non-coding RNA longer than 200 nucleotides and refers to the major class of transcripts encoded by the genome but mostly not translated into proteins[14]. LncRNA plays a key role in regulating cancer proliferation, the cell cycle, metastasis and programmed death[15,

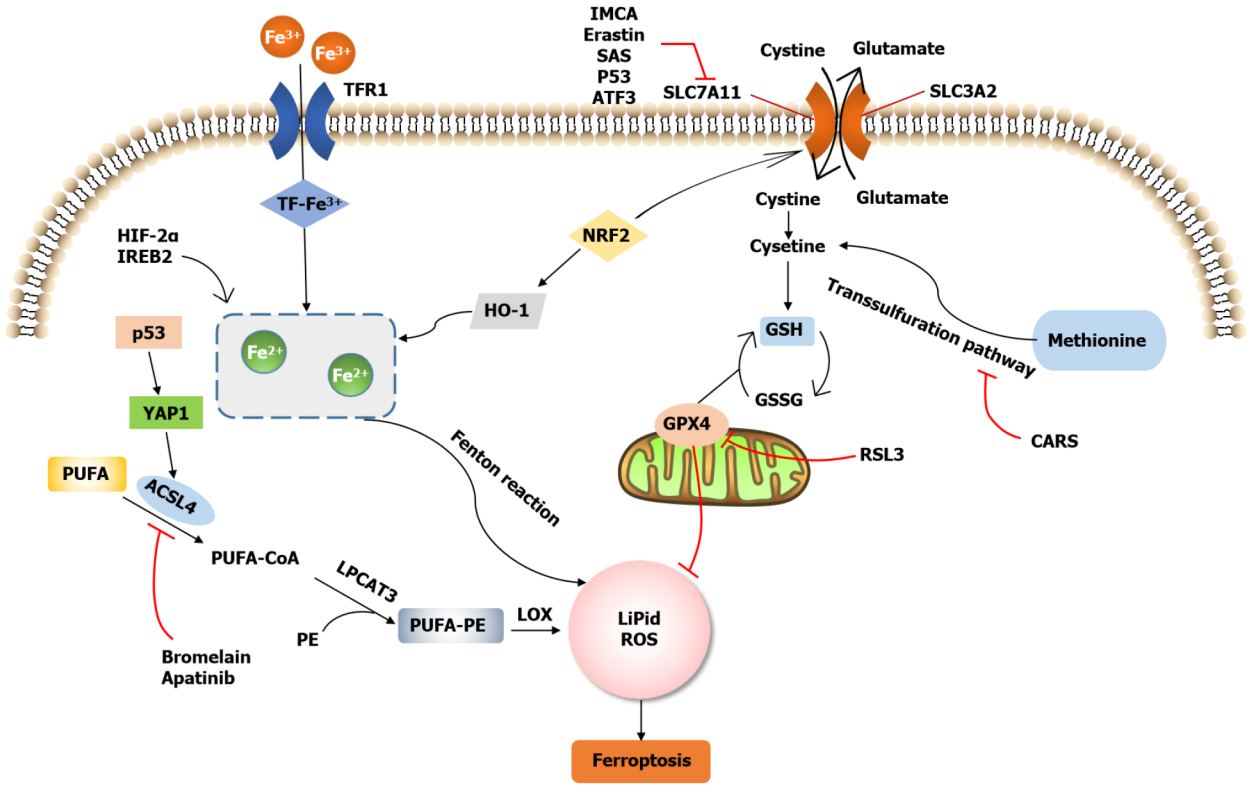

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Figure 1 Mechanisms of ferroptosis in colorectal cancer. The diagram shows several potential regulatory pathways for ferroptosis. Including Xc-system, lipid peroxide accumulation, sulfur transfer pathway, glutathione/glutathione peroxidase 4 lipid repair system, Nrf2-H0-1, cellular iron homeostasis, p53, *etc*. TFR1: Transferrin receptor 1; TF: Transferrin; IREB2: Iron response element-binding protein 2; PUFA: Polyunsaturated fatty acid; ACSL4: Acyl-CoA synthetase long-chain family member 4; LPCAT3: Lysophosphatidylcholine acyltransferase 3; PE: Phosphatidylethanolamine; LOX: Lipoxygenase; NRF2: Nuclear factor erythroid 2-related factor; HO-1: Haem oxygenase 1; SLC7A11: Solute carrier family 7 member 11; IMCA: Benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide; SAS: Sulphasalazine; GSH: Glutathione; GSSG: Oxidized glutathione; GPX4: Glutathione peroxidase 4; RSL3: Ras-selective lethal 3; CARS: Cysteinyl-tRNA synthetase; ATF3: Activating transcription factor 3; YAP1: Yes-associated protein 1.

16]. The abnormal expression of lncRNA is associated with the risk of CRC, imbuing it with clinical potential as a stratification marker, diagnostic index and therapeutic target of CRC[17-20]. A model containing only four lncRNAs was able to well predict the prognosis, vein invasion and lymphatic metastasis in CRC patients, and it was proven that AP003555.1 and AC005841.1 induced ferroptosis by regulating Erastin[21]. The lncRNA model including AC016027.1, AC099850.3, ELFN1-AS1 and VPS9D1-AS1 was able to accurately predict the prognosis of CRC patients and showed great potential to guide individualized treatment[22]. Cai *et al*[23] summarized the details of seven ferroptosis-related lncRNAs to predict the prognosis of CRC patients and found that these lncRNAs were mainly enriched in the mitogen-activated protein kinase (MAPK) signaling pathway, mammalian target of rapamycin (mTOR) signaling pathway and glutathione (GSH) metabolism pathway[23]. LINC00239 increased Nrf2 protein stability by inhibiting Nrf2 ubiquitination and decreased the antitumor activity of erastin and Ras-selective lethal 3 (RSL3)[24]. Circular RNA also plays an important role in ferroptosis. CircABCB10 serves as a sponge of Mir-326 and eventually regulates ferroptosis of CRC by regulating CCL5[25].

In addition to being an independent clinical prognostic factor for CRC patients, genes associated with ferroptosis can also accurately predict the clinical status, including tumor occurrence and progression, drug resistance, somatic mutations and the immune function[26,27], which provides a new research direction for targeted therapy or immunotherapy.

MECHANISM OF ACTION OF FERROPTOSIS IN CRC

The accumulation of lipid peroxides is the core process of ferroptosis

The process of lipid peroxide accumulation in cells is the crucial section of ferroptosis. Polyunsaturated fatty acids (PUFAs) containing diallyl matrigel are prone to hydrogen deprivation, causing the formation of lipid peroxides and inducing ferroptosis[28]. When PUFAs are replaced by monounsaturated fatty acids (MUFAs) in the plasma membrane, lipid ROS accumulation is hindered, and ferroptosis is prevented[29]. With the process of esterification into membrane phospholipids and

oxidation into ferroptosis signals, free PUFAs then can be joined into the lipid signaling mediator syntheses[30]. Several studies have shown that phosphatidylethanolamines, a key phospholipid containing arachidonic acid (AA) or its derivative epinephrine (AdA), are oxidized to oxophosphatidylethanolamines to induce ferroptosis[31]. Elongation of very-long-chain fatty acid protein 5 (ELOVL5) and fatty acid desaturase 1 are can participate in AA and AdA synthesis, which can effectively inhibit ferroptosis[32]. Furthermore, regulatory enzymes involved in membrane phospholipid biosynthesis of PUFAs, such as ACSL4[33] and lysophosphatidylcholine acyltransferase 3 (LPCAT3)[34], can also trigger or prevent ferroptosis. However, the effect of LPCAT3 on ferroptosis was mild compared to that of ASCL4[34].

Bromelain, a pineapple stem extract, potently induces cell ferroptosis and inhibits the proliferation of Kras mutant CRC in Kras mutant cell lines by regulating ACSL-4 Levels compared to Kras wild-type cells[35]. The behavers of Emodin, inhibiting ACSL4 expression, which can inhibit the proliferation and invasion of CRC, bring new research directions for CRC[36].

Apatinib, also known as YN968D1, as a third-line therapy can effectively improve the prognosis of patients with metastatic CRC[37]. ELOVL6 is a target of apatinib. By orienting the ELOVL6, Apatinib can promote ferroptosis with result of ACSL4 regulation, which has been verified by a co-IP assay. This suggests that apatinib inhibits CRC cell viability, at least in part, by targeting ELOVL6/ACSL4 signaling, thus providing novel mechanistic support for the use of apatinib in the clinical treatment of CRC patients[38].

Inhibition of System X_c^- induces ferroptosis

GSH has been known as a crucial antioxidant. It can bind toxic molecules, such as free radicals and heavy metals, and convert them into harmless substances that are excreted[39]. GSH is also the first line of defense in the body to scavenge free radicals, which can effectively inhibit ferroptosis, and has a strong protective effect on the body[40]. System X_c^- is a heterodimer, which was constructed by a heavy-chain subunit and a light-chain subunit (SLC7A11) that assists in the transmembrane transport of cystine and glutamate. Upon entry into the cell, cystine is reduced to cysteine[41], and together with cysteine and glycine, GSH is synthesized intracellularly. Therefore, System X_c^- plays an important role in maintaining GSH homeostasis.

Studies have suggested that System X_c^- may mediate ferroptosis by affecting the glutamate uptake and GSH synthesis[42,43]. Erastin and sulfasalazine are inhibitors of System Xc^- that can lead to intracellular GSH deficiency and ferroptosis by affecting intracellular GSH homeostasis[44,45]. By regulating the expression of SLC7A11, a functional subunit of X_c^-, it can affect the activity of System X_c^- and the susceptibility to ferroptosis in cancer cells[46-48].

Knockdown of SLC7A11 attenuates the viability of CRC stem cells by increasing ROS levels and decreasing cysteine and GSH levels[49]. Talaroconvolutin A is a natural product, and studies have shown that, in addition to inducing ferroptosis by increasing ROS levels in cancer cells, this compound can also promote ferroptosis by down-regulating the SLC7A11 expression and up-regulating the ALOXE3 expression, becoming a new potentially powerful drug candidate for CRC therapy[50]. Copper overload mediated by the copper chelator elesclomol inhibits CRC both *in vitro* and *in vivo*, and one of its pathways may induce ferroptosis by promoting the degradation of SLC7A11[51].

The benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide (IMCA) is considered to significantly inhibit the viability of CRC cells. IMCA can downregulate the expression of SLC7A11 and reduce cysteine and GSH glycine content, which leads to the accumulation of ROS and ferroptosis. In contrast, the overexpression of SLC7A11 was shown to attenuate ferroptosis induced by IMCA, which was confirmed to be involved in the activated protein kinase/mTOR/p70S6k signaling pathway [52].

Petunidin 3-O-[rhamnopyranosyl-(trans-p-coumaroyl)]-5-O-(β-D-glucopyranoside) is a flavonoid compound. CRC cell proliferation can be inhibited by down-regulating SLC7A11 to reduce ferroptosis [53].

Resveratrol (RSV) has been shown to promote ferroptosis by down-regulating the expression of SLC7A11 and GPX4. Combined with bionic nanocarriers, RSV's therapeutic potential as ferroptosis inducing anticancer agent has been developed. The bionic nanomaterial coated the RSV-supported poly (ε-caprolactone) poly (ethylene glycol) nanoparticles on the erythrocyte membrane to improve the transmission efficiency of RSV[54].

Several ferroptosis-related genes are concentrated on System X_c^-. Activating transcription factor 3 (ATF3) promotes ferroptosis by inhibiting System X_c^-[55]. Deficiency of CDKN2A and growth differentiation factor 15 downregulates SLC7A11 expression, thereby sensitizing cells to ferroptosis[56,57].

Radiotherapy and poly-ADP-ribose polymerase inhibitors have been used in clinical trials in the treatment of CRC, the mechanism of which may be ionizing radiation activation of dsDNA that modulates ferroptosis through activation of the ATF3-SLC7A11 pathway. Triggers cGAS signaling mediated tumor control in cancer cell lines and mouse xenograft models[58].

The transsulfuration pathway is a regulator of ferroptosis resistance

More than 40% of the source of cysteine came from diet, and transfer to GSH *via* a biochemical process in body, which can combat the excessive deposition of peroxide. In addition to being transported into

cells by System X_c^-, cysteine can also be converted intracellularly by methionine *via* the transsulfuration pathway[59].

Cystathionine-β-synthase (CBS), an enzymatic component of the transsulfuration pathway, is significantly increased in cells resistant to Erastin-induced ferroptosis[60]. CBS has also been shown to be an independent regulator of ferroptosis[61,62]. Endogenous H2S, a by-product of the transsulfuration pathway, is closely related to tumor cell physiology and is finely regulated in a variety of cancers[63, 64]. Xc(-) transporter-related protein (xCT), a functional subunit of system X_c^-, was shown to interfere with xCT in colon cancer cells, resulting in an increased expression of cystathionine-γ-lyase and CBS, which are majority of the transsulfuration pathway. Additionally, the endogenous H2S levels can be significantly decreased by interfering with xCT. The correlation of xCT and transsulfuration pathway has been investigated that is a makeable metabolic vulnerability.

Cysteinyl-tRNA synthetase, a genetic suppressor of ferroptosis, upturns the sulfur pathway and re-sensitizes cells to erastin, demonstrating a new mechanism for resistance to ferroptosis[65]. This implies that the transsulfuration pathway is a regulator of ferroptosis resistance.

Zinc oxide has outstanding desulfurization ability, and VZnO can effectively reduce the content of H_2S in CRC, effectively deplete GSH in tumor cells and ultimately lead to ferroptosis in CRC cells, providing an effective strategy for CRC treatment[66].

Disruption of the GSH/GPX4 lipid repair system can promote the accumulation of lipid ROS

ROS levels in the body are regulated by the antioxidant defense system, and oxidant/antioxidant imbalance may also contribute to ferroptosis[67]. GPX4 is an important selenoprotein that belongs to the GPX antioxidant defense system and is a considerable enzyme to balance the concentration of GSH and GS-SG. GPX4 protects membrane lipid bilayers by transferring toxic lipid hydroperoxides to nontoxic lipid alcohols[68]. GPX4 has been literately proved as a factor of ferroptosis promotor and can be trigger by ferroptosis inducers, such as Erastin and RSL3. Likewise, direct knockdown of GPX4 Leading to the excessive accumulation of intracellular lipid peroxidation and cell death[69]. Thus, GPX4 is consider as a crucial target to trigger ferroptosis[70,71].

In experiments with three different CRC cells (HCT116, LoVo and HT29), RSL3 was found to trigger cellular ferroptosis in a dose- and time-dependent manner due to increased ROS levels and destabilization of the intracellular iron pool. In a further analysis, GPX4 inhibition was proven to be a key determinant of RSL3-induced ferroptosis, and overexpression of GPX4 rescued RSL3-induced ferroptosis[72]. Aspirin has been reported to have therapeutic benefits for CRC carrying carcinogenic PIK3CA. The mechanism may be that aspirin inhibits protein kinase B/mTOR signaling. The expression of downstream sterol regulatory elm-binding protein 1 was inhibited, and the production of MUFA fat by stearoyl-CoA desaturase-1 was reduced. Thus promoting RSL3-induced ferroptosis in CRC cells[73].

Serine- and arginine-rich splicing factor 9 (SRSF9) is frequently overexpressed in multiple tumor types and manifests as a proto-oncogene[74-76]. SFRS9 upregulates GPX4 protein, which is an obstacle to ferroptosis[77]. Knockdown of SFRS9 may be an effective treatment for CRC. In CRC tissues, the expression of SFRS9 mRNA and protein was significantly higher than that in adjacent tissues. Experiments in mice demonstrate that regulation of GPX4 by SRSF9 is an important mechanism driving CRC tumorigenesis and resistance to Erastin-induced ferroptosis. This molecular mechanism may provide a novel approach to improving the sensitivity of CRC to Erastin[78].

ACADSB is a member of the Acyl-CoA dehydrogenase family, and its overexpression inhibits the migration, invasion and proliferation of CRC cells. Studies have shown that ACADSB negatively regulates the expression of GSH reductase and GPX4 while increasing the concentrations of malondialdehyde, Fe ions and superoxide dismutase. This suggests that ACADSB may affect CRC cell migration, invasion and proliferation by regulating CRC cell ferroptosis[79].

ROS causes GSH accumulation through nuclear factor erythroid 2-related factor

Antioxidant proteins, such as Nrf2, are major antioxidant transcription factors that help prevent the accumulation of excess ROS and maintain redox homeostasis. Downregulation of Nrf2 enhances the sensitivity of cancer cells to ferroptosis promoters[80].

The Warburg effect is thought to be a characteristic of cancer cells, that is, cancer cells will undergo glycolysis beyond very high levels under aerobic conditions[81]. Inhibition of the Warburg effect reduces the ability of cells to proliferate. Therefore, inhibiting the Warburg effect may be a therapeutic strategy for cancer. *In vivo* and *in vitro* experiments in CRC showed that iron-induced ROS activated the expression of Nrf2 in the nucleus has the positive correlation with Warbury enzymes expression and CRC cell proliferation by enhancing the Warburg effect.

Heme oxygenase 1 (HO-1) is a downstream gene of Nrf2, and NGF2 protects against lipid peroxidation[80] and ferroptosis through the transcription of enzymes such as HO-1[82]. Ferroptosis can be effectively alleviated by the elimination of lipid oxidation through the Nrf2/HO-1 axis activation[83, 84].

Tagitin C, a sesquiterpene lactone[85], can induce ferroptosis in HCT116 cells and inhibit the growth of CRC cells. Mechanistically, Tagitinin C induces endoplasmic reticulum stress and oxidative stress as well as nuclear translocation of Nrf2. As a downstream gene of Nrf2, HO-1 was significantly increased with Tagitinin C treatment[86].

Tagitinin C-induced ferroptosis was accompanied by a decrease in GSH levels and an increase in lipid peroxidation. Cetuximab combined with chemotherapy has made great progress in the treatment of metastatic CRC[87], but cetuximab is not effective in CRC patients with KRAS mutations[88,89].

Lysionotin (Lys), a flavonoid, has been demonstrated to successfully inhibit cell proliferation, migration and invasion of HCT116 and SW480 CRC cells *in vitro*. Lys treatment worked by increasing Nrf2 cells' degradation rate to reduce the concentration of Nrf2 protein, inducing ferroptosis and ROS accumulation in CRC cells[90].

P38 MAPK has been investigated that participate in the regulation of Nrf2/HO-1[91,92]. It has been shown that cetuximab can significantly inhibit Nrf2/HO-1 signaling through p38 MAPK activation in KRAS-mutant CRC cell lines, thereby promoting RSL3-induced ferroptosis. This provides a research direction for cetuximab in the treatment of KRAS-mutant CRC[93].

Oxaliplatin is the first-line chemotherapy drug for CRC. By inhibiting the Nrf2 signaling pathway, the sensitivity of CRC cells to oxaliplatin can be enhanced[94,95]. Furthermore, the study found that oxaliplatin significantly inhibited the protein expression of Nrf2, HO-1 and NQ in the Nrf2 signaling pathway in a dose-dependent manner. Therefore, the anticancer effect of oxaliplatin may be enhanced by inhibiting the Nrf2 signaling pathway, leading to ferroptosis and oxidative stress in CRC cells[96].

Cellular iron homeostasis is a key factor in ferroptosis

The primary condition for the initiation of ferroptosis pathway is the need of iron ion. Dietary iron is absorbed primarily in the gut as ferric ion and delivered to the blood by transferrin (TF). In general, extracellular iron transport into the cell through the sequence of complexing with circulating TF, then binds to membrane TF receptor proten-1 (TFR1), finally to the cytoplasmic unstable iron pool. Excess cellular iron is stored as ferritin or transported extracellularly by ferritin[97,98]. Maintenance of cellular iron homeostasis prevents oxidative damage, cytotoxicity and death.

Lipid reactions can be divided into enzymatic and non-enzymatic reactions. Iron can promote the production of ROS through the Fenton reaction, leading to enzymatic lipid peroxidation[99,100], and also acts in a non-enzymatic manner as a cofactor for lipid-oxidizing lipoxygenase. Supplementation of exogenous iron ions can accelerate erastin-induced ferroptosis[5]. Knockout the gene encoding the TFR or upregulate the expression of iron storage proteins can inhibit iron overload and ferroptosis. Iron metabolism can be regulated by inhibition of the major transcription factors, like iron regulatory protein 2, significantly upregulates the expression of iron metabolism-related genes, such as *FTH1* and *FTL*, thereby inhibiting erastin-induced ferroptosis[101]. Iron chelators can prevent the transfer of electrons from iron to oxides, thus inhibiting the production of oxygen free radicals and inhibiting lipid peroxidation to prevent ferroptosis. Therefore, the regulation of iron metabolism and ferritin phagocytosis may become new targets and new pathways for regulating ferroptosis.

Hypoxia-inducible factor-2α (HIF-2α) is a master transcriptional regulator of cellular iron levels[102]. Activation of HIF-2α increases cellular iron in CRC, leading to an increase in lipid ROS and a decrease in GSH production, thus enhancing cellular sensitivity to ferroptosis[103].

There is a conserved miR-545 binding site in the 3' untranslated region of TF, and the overexpression of TF in CRC cells was found to induce increased levels of ROS, MDA and Fe^{2+}, thereby promoting CRC cell death. This suggests that miR-545 may play an oncogenic role in CRC by regulating the iron accumulation in cells[104].

Dichloroacetate attenuates the stemness of CRC cells by chelating iron in lysosomes, leading to the up-regulation of iron concentrations and lipid peroxide levels, thus triggering ferroptosis[105].

OTUD1 is a deubiquitinase of iron-responsive element-binding protein 2 (IREB2), which is mainly expressed in intestinal epithelial cells. OTUD1 promotes TFR1-mediated iron transport through deubiquitination and stabilization of IREB2, leading to increased ROS production and ferroptosis, which is highly associated with a poor prognosis in CRC[106].

Dual role of p53 in mediating tumor ferroptosis

The tumor suppressor gene p53 plays a dual role in mediating ferroptosis in a variety of cancers[99]. Studies have found that p53 can enhance ROS-mediated ferroptosis by inhibiting SLC7A11 protein expression, resulting in decreased cystine import and thus reduced GSH production[46,107]. However, unlike in CRC, p53 can protect CRC cells from ferroptosis by stirring SLC7A11[108]. In addition, p53 can also inhibit CRC cell ferroptosis by blocking dipeptidyl peptidase 4 activity[97,109].

Cytoglobin (CYGB) is a regulator of ROS that plays an important role in oxygen homeostasis and acts as a tumor suppressor[110]. Yes-associated protein 1 (YAP1) is a key downstream target of CYGB. CYGB modulates cellular ferroptosis through p53-YAP1 signaling in CRC cells[111]. A novel antitumor compound optimized from the natural saponin biocide A has also been shown to trigger ferroptosis by activating p53[112].

Ginsenoside Rh4 can increase ROS accumulation, lead to the activation of ROS/p53 signaling pathway, and induce ferroptosis to inhibit the proliferation of cancer cells[113]. Cullin-9 can bind p53 to ubiquitinized heteroribo nucleoprotein C for degradation through whole genome sequencing and external differential expression analysis. Cullin-9 overexpression increases resistance to erastin-induced ferroptosis and is a novel and important regulator of CRC ferroptosis[114].

INDUCTION OF FERROPTOSIS REVERSES RESISTANCE TO ANTICANCER DRUGS

Oxaliplatin prolongs the median disease-free survival and overall survival in patients with advanced CRC, but clinical data suggest that < 40% of patients with advanced CRC benefit from it[115,116]. This may be related to oxaliplatin resistance, and recent studies have shown that induction of ferroptosis can significantly reverse oxaliplatin resistance in CRC cells.

Ferroptosis in CRC cells may be inhibited through the KIF20A/NUAK1/PP1β/GPX4 pathway, which may underlie CRC resistance to oxaliplatin[117]. Deletion of cysteine desulfurase (NFS1) can significantly enhance the sensitivity of CRC cells to oxaliplatin. The mechanism may involve NFS1 deficiency synergizing with oxaliplatin to induce PANoptosis (apoptosis, necroptosis, pyroptosis and ferroptosis), thus increasing the intracellular ROS levels. This also demonstrated that ferroptosis is involved in the oxaliplatin resistance pathway[118]. Obesity is strongly associated with a poor prognosis in patients with advanced CRC, and adipose-derived exosomes reduce susceptibility to ferroptosis in CRC, thereby promoting chemoresistance to oxaliplatin[119].

Combination with chemotherapy with monoclonal antibodies against anti-epidermal growth factor receptor or vascular endothelial growth factor has advanced in the treatment of metastatic CRC. However, inherent resistance to downstream KRAS mutations, so the effect of combination chemotherapy is often less optimistic. β-elemene has broad-spectrum anticancer effects, and it has been demonstrated that combined treatment of β-elemene and cetuximab can induce ferroptosis and inhibit epithelial-mesenchymal transition, thereby improving resistance to KRAS-mutated CRC cells[120].

FAM98A is a microtubule-associated protein involved in cell proliferation and migration. Increased expression of FAM98A can inhibit ferroptosis and promote CRC resistance to 5-fluorouracil (5-FU)[121]. Similarly, PYCR is an oncogene that desensitizes CRC cells to 5-FU cytotoxicity by promoting ferroptosis in CRC cells[122].

In addition, Andrographis Paniculata may also exert a sensitizing effect on CRC treatment by activating ferroptosis[123,124]. Andrographis Paniculata-mediated sensitivity to 5-FU-based chemotherapy in CRC is primarily mediated through activation of ferroptosis and inhibition of the β-catenin/Wnt signaling pathway[123].

NANOTECHNOLOGY PROMOTES FERROPTOSIS IN CRC CELLS

Although increasing iron concentration promotes ferroptosis in tumor cells has been demonstrated experimentally, direct administration of Fe^{2+} is not feasible in the clinic due to the protective effect of cell membranes and the defense mechanism of the tumor immune microenvironment (TME). Nano-drug delivery system (nano-DDSs) has unique physical and chemical properties of nanomaterials, which can not only enhance drug solubility and improve drug circulation time in the body, but also achieve targeted delivery and controlled release of drugs[125]. Therefore, the use of novel nanodelivery systems to improve the efficiency of iron release has great prospects in CRC targeted therapy.

Nano DDS can directly drive the death of iron in tumor cells, and iron-based nanoparticles can be catabolized by acid lysosomes of tumor cells to release Fe^{2+} and iron 3+[126]. Iron-based nanoparticles induce ferroptosis by catalyzing the Fenton reaction, but because of their low reactive oxygen production, they are often used in conjunction with other treatments. Liang *et al*[127] synthesized ultra-small single crystal Fe nanoparticles (bcc-USINPs) that are highly active in the tumor microenvironment and can effectively induce tumor cell ferroptosis and immunogenetic cell death at very low concentrations[127].

In addition to acting directly on cancer cells, nanotechnology also works by acting on key components of TME. Due to TME's weak acidity, abundant angiogenesis and hypoxia conditions, the effectiveness of conventional cytotoxic therapy delivery is limited, while active targeting of nanoparticles may be more useful[128].

Sodium persulfate (NaSO) is a novel chemodynamic therapy (CDT) that can produce •SO and Na, which can cause ferroptosis in cells. Ir780-iodide (IR780) is a phototherapy agent that produces ROS in conjunction with NaSO's CDT to overcome CRC chemotherapeutic resistance. Co-assembly of NaSO and IR780 on the nano platform improved the stability of NaSO and solubility of IR780, significantly enhancing the anti-tumor effect on CRC cell lines[129]. A novel composite nanomaterial PPy@Fe_3O_4 has been demonstrated to regulate the nuclear factor-kappaB signaling pathway and then then inhibit the proliferation, migration and invasion of CRC cells *in vitro*. Interestingly, Fer-1, an ferroptosis inhibitor, reversed changes in transfer-related proteins induced by nanoparticle therapy[130].

CONCLUSION

Ferroptosis, a newly discovered type of RCD mediated by iron-dependent lipid ROS accumulation, plays a role in a variety of diseases of the gastrointestinal tract. Many ferroptosis-related genes have been confirmed to be associated with the prognosis of CRC, and various models have been confirmed to

Table 1 Key molecular targets and inducers involved in the regulation of colorectal cancer ferroptosis

Target	Ferroptosis inducers	Ref.
ACSL4	Bromelain	[35]
	Apatinib	[37]
	Emodin	[36]
SLC7A11	Erastin	[42]
	SAS	[44,45]
	Talaroconvolutin A	[50]
	Copper chelator elesclomol	[51]
	IMCA	[52]
	Pt3R5G	[53]
	RSV	[54]
Transsulfuration pathway	CARS	[65]
	VZnO	[66]
GPX4	Erastin	[67]
	RSL3	[67]
	ACADSB	[79]
Nrf2/HO-1	Cetuximab	[87]
	Lys	[90]
	Oxaliplatin	[94,95]
Cellular iron homeostasis	HIF-2α	[102]
	miR-545	[104]
	Dichloroacetate	[105]
	IREB2	[106]
P53	Ginsenoside Rh4	[113]
	Cullin-9	[114]

ACSL4: Acyl-CoA synthetase long-chain family member 4; SLC7A11: Solute carrier family 7 member 11; SAS: Sulphasalazine; IMCA: Benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide; Pt3R5G: Petunidin 3-O-[rhamnopyranosyl-(trans-p-coumaroyl)]-5-O-(β-D-glucopyranoside); RSV: Resveratrol; CARS: Cysteinyl-tRNA synthetase; RSL3: Ras-selective lethal 3; Nrf2: Nuclear factor erythroid 2-related factor; HO-1: Haem oxygenase 1; Lys: Lysionotin; IREB2: Iron response element-binding protein 2; GPX4: Glutathione peroxidase 4; HIF-2α: Hypoxia-inducible factor-2α.

be able to stratify CRC patients well and facilitate the early identification of high-risk patients. Iron metabolism and the accumulation of lipid peroxides are the core processes in ferroptosis. As mentioned above, GPX4, SLC7A11, ACSL4 and p53 act as key regulators in ferroptosis-related CRC mediation (Table 1). Induction of ferroptosis can reverse the resistance of anticancer drugs or exert a synergistic effect with other anticancer drugs to enhance the sensitivity of antitumor drugs and improve the prognosis of CRC patients. Furthermore, in colon cancer, copper chelators have also been shown to facilitate CRC cell death by promoting the degradation of SLC7A11. The mechanism underlying ferroptosis and the relationship between key regulators and other RCDs, such as autophagy and apoptosis, should be explored in future research.

In animal studies, the underlying mechanism of ferroptosis is complex, involving multiple targeted enzyme systems and metabolic networks. However, the actual clinical situation is more complicated, which requires the combination of gene mutation, epigenetic modification, metabolomics, *etc*. The absorption, transport, storage and metabolism of iron in the body is a very complex process, and how to define the therapeutic iron concentration needs to be carefully considered. In practice, increasing iron concentration to promote ferroptosis in tumor cells seems difficult to achieve due to the presence of defense mechanisms in the TME. At present, many studies have mentioned the promotion of ferroptosis in CRC cells through nanotechnology, and nano-DDSs seems to be able to effectively help solve this problem due to the advantages of targeted delivery and controlled release. In fact, ferroptosis seems to be a double-edged sword in the treatment of gastrointestinal diseases. The role of ferroptosis in different

gastrointestinal diseases is different, and the pros and cons of ferroptosis treatment need to be evaluated more carefully.

At present, research on the mechanism underlying ferroptosis in the colorectum is still in its infancy, and other ferroptosis pathways or related targets, such as the ferroptosis suppressor protein 1/CoQ/nicotinamide adenine dinucleotide phosphate pathway, still need to be further explored. In the future, ferroptosis genes related to the prognosis of CRC also need to be verified. The mechanism underlying ferroptosis and tumor escape in CRC is also worth further in-depth study in order to promote the development of new and effective therapeutic strategies.

FOOTNOTES

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REVIEW

"Cold" colorectal cancer faces a bottleneck in immunotherapy

Jia-Liang Liu, Ming Yang, Jun-Ge Bai, Zheng Liu, Xi-Shan Wang

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Abstract

The advent of immunotherapy and the development of immune checkpoint inhibitors (ICIs) are changing the way we think about cancer treatment. ICIs have shown clinical benefits in a variety of tumor types, and ICI-based immunotherapy has shown effective clinical outcomes in immunologically "hot" tumors. However, for immunologically "cold" tumors such as colorectal cancer (CRC), only a limited number of patients are currently benefiting from ICIs due to limitations such as individual differences and low response rates. In this review, we discuss the classification and differences between hot and cold CRC and the current status of research on cold CRC, and summarize the treatment strategies and challenges of immunotherapy for cold CRC. We also explain the mechanism, biology, and role of immunotherapy for cold CRC, which will help clarify the future development of immunotherapy for cold CRC and discovery of more emerging strategies for the treatment of cold CRC.

Key Words: Immunotherapy; "Cold" colorectal cancer; Immune checkpoint inhibitors; Cancer treatment; Review

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Core Tip: Immune checkpoint inhibitors (ICIs) are usually produced by antibodies, and their effectiveness relies on the antitumor effects of immune cells (especially T cells). Colorectal cancer (CRC) is one of the most common forms of cancer worldwide. Only a limited number of patients are currently benefiting from ICIs due to limitations such as individual differences and low response rates. In this review, we discuss the classification and differences between hot and cold CRC and the current status of research on cold CRC, and summarize the treatment strategies and challenges of immunotherapy for cold CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common forms of cancer worldwide[1]. Globally, CRC is the second most common cancer in women and the third most common in men[2]. More than half of the development of CRC can be attributed to modifiable risk factors such as smoking, unhealthy diet, heavy alcohol consumption, lack of physical activity, and overweight; therefore, the disease is preventable[3]. Despite some progress in the diagnosis and treatment of CRC, it remains a significant cause of cancer-related deaths[2]. The global burden of CRC is expected to increase by 60% by 2030[4]. Therefore, there is an urgent need to develop new preventive and treatment strategies for this disease[1].

In contrast to traditional cancer therapies that affect the proliferation, survival, and metabolic activities of tumor cells[5], immunotherapy mainly works by modulating the tumor microenvironment (TME), restoring anticancer immunity, and stimulating or suppressing the immune system to play an antitumor role[6]. Immune checkpoint inhibitors (ICIs) are usually produced by antibodies, and their effectiveness relies on the antitumor effects of immune cells (especially T cells)[7].

However, most solid tumors have little T-cell infiltration and are defined as non-T-cell inflammatory or "cold" tumors[8]. In CRC, it has been shown that only patients with mismatch repair deficiency (dMMR) or microsatellite instability (MSI) high (dMMR/MSI-H) tumor subpopulations respond to treatment with ICIs[9-11]. Clinical trials related to ICIs have been conducted for the treatment of CRC (Table 1). In these patients, there is an urgent need to improve the efficacy of tumor immunotherapy by improving intratumoral T-cell infiltration and converting cold tumors into "hot" or T-cell inflammatory tumors.

In this review, we discuss the classification and differences between hot and cold CRC and the current state of research on cold CRC, the therapeutic strategies and challenges of immunotherapy, and the pathological mechanisms of cold CRC.

CLASSIFICATION AND DIFFERENCE BETWEEN COLD AND HOT CRC

Tumor-immune system interactions provide a basis for patient stratification and treatment strategies for various cancers, which can more accurately predict survival in CRC[12]. An immune scoring system for tumor classification was developed based on the quantification of two lymphocyte populations (cluster of differentiation 3 [CD3] and CD8)[13,14] at the tumor center and aggressive margins[15-19]. The scoring system has four levels (immune score 0 [i0], i1, i2, i3, and i4). The concepts of hot (highly invasive immune score i4) and cold (noninvasive immune score i0) tumors were introduced[15]. In colon cancer, the consensus immune scoring system has a greater relative prognostic value than pathological T staging, pathological N staging, lymphovascular infiltration, tumor differentiation, and MSI status[20].

Currently, hot and cold tumors are typically referred to as T-cell infiltrated, inflammatory but noninfiltrating, and noninflammatory tumors[15]. This immune classification has been validated in melanoma and breast cancer[21,22]. In addition to the presence of tumor-infiltrating lymphocytes (TILs), other features are the consensus molecular subtype (CMS) classification developed through a comprehensive reassessment and comparison of CRC molecular gene expression profiles: CMS1 and CMS4 are hot tumors (Figure 1); they are considered immunoreactive and highly infiltrated by immune cells. These tumors are immunoreactive and highly infiltrated by immune cells, as opposed to CMS2 and CMS3, which are cold tumors[23]. A small group of CRCs with dMMR/MSI-H benefits from immunotherapy. dMMR/MSI-H in solid tumors, including CRC, suggests a good tumor response to immunotherapy[7]; however, the majority of patients with skilled MMR (pMMR) or microsatellite stable (MSS) CRC do not respond well to this treatment[24]. However, immune scoring is a better predictor of prognosis in CRC patients than MSI testing alone[25], and MSI has been used to predict the response to

Table 1 Clinical trials for immune checkpoint inhibitors in colorectal cancer patients

Name	Targets	Phase	Settings	Trial identifier
Nivolumab and ipilimumab	PD-1 and CTLA4	II	dMMR and/or MSI mCRC	NCT04730544
Camrelizumab and apatinib	PD-L1 and VEGF	II	Locally advanced dMMR/MSI-H CRC	NCT04715633
Toripalimab with or without celecoxib	PD-1 and COX	I and II	Resectable non-metastatic dMMR/MSI-H CRC	NCT03926338
Cetuximab-avelumab	PD-1 and EGFR	II	mCRC	NCT04561336
Nivolumab + relatlimab	PD-1 and LAG3	II	MSS colorectal adenocarcinomas	NCT03642067
Obinutuzumab + atezolizumab + cibisatamab + tocilizumab	CD20, PD-L1, CEA + CD3 and IL-6R	Ib	MSS mCRC	NCT03866239

CEA: Carcinoembryonic antigen; COX: Cyclooxygenase; CTLA4: Cytotoxic T-lymphocyte-associated protein 4; dMMR: Deficient DNA mismatch repair; EGFR: Epidermal growth factor receptor; IL-6R: Interleukin 6 receptor; LAG3: Lymphocyte activation gene 3; PD-1: Programmed cell death protein 1; PD-L1: Programmed cell death-ligand 1; mCRC: Metastatic colorectal cancer; MSI: Microsatellite instability; MSS: Microsatellite stability; VEGF: Vascular endothelial growth factor.

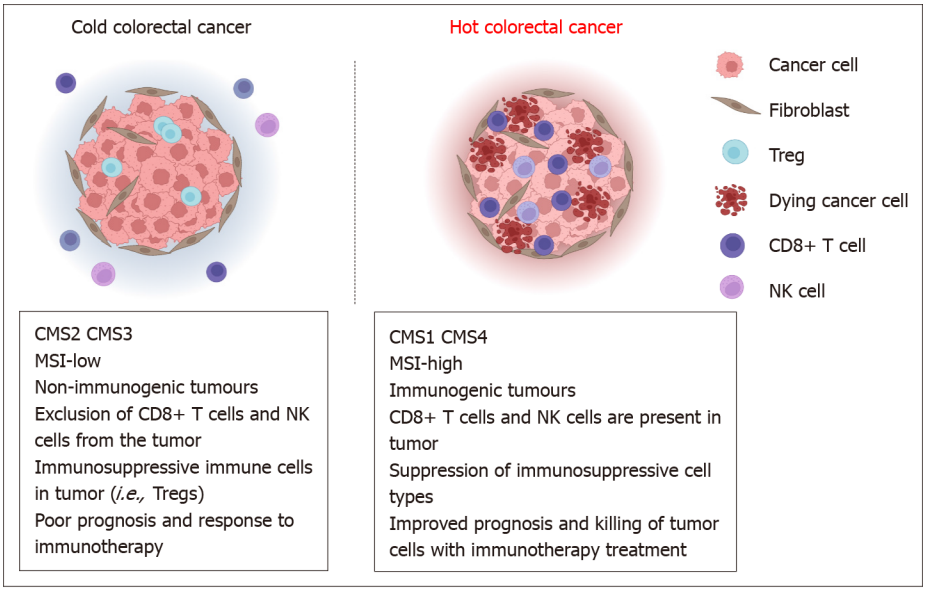

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Figure 1 Difference between cold and hot colorectal cancer. Colorectal cancer (CRC) is divided into hot and cold subtypes. Hot CRC mainly includes the deficient DNA mismatch repair (dMMR)/high microsatellite instability (MSI-H), consensus molecular subtype (CMS)1, and CMS4 subtypes, while cold CRC includes the proficient mismatch repair (pMMR)/low microsatellite instability (MSI-L), CMS2, and CMS3 subtypes. NK: Natural killer; Tregs: Regulatory T cells.

anti-programmed cell death protein 1 therapy in cancer[26]. The expression of anti-programmed cell death ligand 1 (PD-L1) on tumor-associated immune cells, possible genomic instability, and the pre-existing antitumor immune response are characteristics of hot tumors[27].

Currently, the most comprehensive approach to define hot and cold tumors remains the immune scoring system, but there are still some tumors with characteristics intermediate between hot and cold tumors, and the four main categories of tumor classification, namely hot, altered exclusion, altered immunosuppression, and cold, provide classification of the four major tumor categories[28]. This system provides a more comprehensive approach to classification and helps to suggest new ideas for immunotherapy strategies.

With the development of immunotherapy and its achievements, it is important to determine how to use immune scoring to classify tumors and help and guide the choice of treatment. A blanket use of parameters to score may produce bias, which reinforces the need to incorporate the details of each individual case and to adequately integrate clinical practice to develop a rational, standardized, and coordinated scoring approach to guide treatment decisions. For immunotherapy to overcome the bottleneck of cold CRC, a general consensus is still required.

Immunotherapy has made significant progress in cancer treatment[29]. In particular, hot tumors with an immune microenvironment of highly TILs are highly responsive to most immunotherapies, a property that plays a key role in obtaining good antitumor responses to immunotherapy[30-33]. The discovery and development of ICs and IC-related drugs are of importance in cancer immunotherapy. This immunotherapy approach has excellent long-term regression efficacy in hot tumors; however, hot tumors have a low response rate to immunocooled tumors lacking predominant infiltration of tumor immune cells[34-40]. Therefore, the absence or low number of lymphocytes in the TME also serves as a biomarker for cold tumor unresponsiveness to ICIs[41].

Therefore, it is important to consider a proper treatment plan for cold CRC. Classifying tumors according to their immunophenotypes is too homogeneous; an emphasis on tumor heterogeneity can enable us to have a better understanding of individualized cancer treatment[6]. Most solid tumors are non-T-cell inflammatory or cold tumors[8,42], and CRC is no exception. Therefore, there is an urgent need to improve the TME to convert cold tumors into hot or T-cell inflammatory tumors to improve the efficacy of tumor immunotherapy.

It is important to elucidate the mechanisms involved in cold CRC that do not respond to immunotherapy to provide additional insights into the therapeutic strategies that can be developed. In this section, we outline the mechanisms and approaches related to the possible modulation of non-immune-responsive cold CRC to improve the efficacy of treatment approaches against non-immune-responsive tumors.

Increasing tumor inflammation

Establishing an inflammatory response in the TME is a key goal of immunomodulatory approaches for all cold tumors, including CRC. Infection by pathogens can activate the immune system, thus stimulating a series of immune attacks[43]. The involvement of such pattern recognition receptors can activate immune cells and lead to an immune system-mediated antitumor response[44]. Interventional radiology has enabled the precision treatment of local tumors, and a variety of therapeutic substances, including pattern recognition receptor agonists, such as tumor lysing peptides or lysing viruses, cytokines, encoded nucleic acid sequences, bispecific T-cell participants, nanoparticles or particles, and immune cells, can be delivered locally[45]. The immunogenic cell death pathway induced by precise radiotherapy and cryoablation or radiofrequency ablation that produces massive tumor antigen release can convert tumors into *in situ* vaccines, which provides us with new insights and options[45,46].

Although a growing number of studies has demonstrated the effectiveness of radiotherapy[47-50], the benefits obtained in these trials cannot be attributed exclusively to radiotherapy. Explicit demonstration of the contribution of radiation to the immunotherapeutic response is challenging but crucial. The optimal integration of radiobiology and tumor immunology may lead to potentially significant clinical benefits.

Precision therapy is limited by several operational, clinical, and biological factors, in addition to the numerous complications that may arise from injecting drugs or biologics directly into the tumor. Therefore, a systemic approach to tumor-specific therapy remains attractive. Chemotherapeutic regimens as systemic treatments can induce immunogenic cell death by releasing damage-associated molecular patterns[51,52] and activating necrotic or apoptotic pathways[53]. Some studies have indicated that drugs such as 5-fluorouracil can induce apoptosis of myeloid-derived suppressor cells (MDSCs) and increase CD8 cell function to enhance inflammatory immunity[54].

Immune microenvironment analysis of patients with liver metastases from CRC has revealed that cytotoxicity and memory T-cell density are significantly higher in patients who received preoperative chemotherapy than in patients with untreated metastasis[55-57], suggesting that the use of chemotherapy can induce tumor inflammation to some extent, providing insight into the transformation of cold to hot CRC. Epidermal growth factor receptor (EGFR), vascular EGFR kinase, and mitogen-activated protein kinase kinase (MEK) inhibitors are widely used in the clinical solid tumor routine, and the clinical effects and related mechanisms of EGFR (cetuximab and panitumumab) and angiogenesis (bevacizumab, afliximab, or ramucizumab) as first- and second-line targeted agents for metastatic CRC are being actively investigated[58-61]. Although there is a lack of knowledge regarding the detailed molecular mechanisms of action between targeted drugs and immunity, *in vivo* studies have shown that the activated mitogen-activated protein kinase (MAPK) signaling pathway can inhibit major histocompatibility class I components and antigen-presentation mechanisms. Use of MAPK inhibitors enhances T-cell-mediated killing of tumor cells[62].

MEK inhibitors may also be involved in the immune effects of tumors[63,64]. This effect promotes antigen presentation on the surface of tumor cells to activate the recognition of CD8 T lymphocytes, which then kills tumor cells. Additionally, it has been found that inhibition of the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathway can prevent the activation of immunosuppressive pathways[65], thereby affecting the immune microenvironment.

There is a large body of literature that strongly supports the idea that drugs targeting oncogenes and non-oncogenes can beneficially affect the TME in a wide range of tumors and thus enhance tumor immune responses. However, many therapeutic methods have not yet been used in the routine management of cancer patients, as their preclinical and clinical development has only recently begun.

For example, toll-like receptors (TLRs) are highly expressed in immune cells in the TME, and in clinical development, the involvement of TLR agonists can activate antitumor immune responses[66]. However, the complexity of the TLR system challenges the selection of agonists. Different agonists may cause different types of inflammatory responses. TLR agonists are currently being used as monotherapy and in combination with ICIs[66,67].

Stimulator of interferon (IFN) genes is an endoplasmic reticulum transmembrane protein, whose mechanism of action is to sense cytosolic DNA, induce type I IFN gene transcription, and promote antigen cross-presentation[68]. IFN gene-stimulating factor agonists increase CD8 T cells in the TME[69] and, when combined with anti-PD-L1, reduce local immunosuppression to mediate a systemic antitumor response[70]. This combination may be used as a method of local immunosuppression, and the study of its agonist is in the clinical development stage.

Cytokines in the TME

Cytokines and chemokines are molecular messengers of the immune system, and many cytokines (*e.g.*, interleukin 2 [IL-2], IL-7, IL-15, IL-21, granulocyte macrophage colony-stimulating factor, and IFN-α) in the immune TME[71-76] regulate the function of T cells, and studies have reported their use as single agents or in combination with other drugs[77]. Accumulation of MDSCs as a group of immature myeloid cells with immunosuppressive functions in tumors attenuates the regulation of immune responses. Regulatory T cells (Tregs) can promote tumor growth by suppressing cytotoxic immune responses. Therefore, therapeutic strategies that specifically eliminate MDSCs or Tregs have been proposed[78]. It has been suggested that monoclonal antibodies against immune checkpoints or agonists of the tumor necrosis factor receptor superfamily may have a modulatory effect on Tregs[79].

Immunosuppressive soluble ligands play an important role in the immune microenvironment. For example, prostaglandin E2 promotes tumor growth and exerts immunosuppressive effects[80]. The TME is also rich in adenosine. The TME is also enriched in adenosine, which is released owing to the death of tumor cells through CD73 and CD39 ectonucleases[81]. The increase in adenosine, a substance enriched in the TME, impairs the implantation and activation of immune cells in the TME. Antagonizing adenosine or its pathway can block CD39 to enhance T-cell proliferation and induce proinflammatory cytokines, which can control tumor growth[82].

Cell therapy

Since 1993, engineered T cells targeting artificial chimeric antigen receptors (CARs) on the surface molecules of tumor cells were first proposed[83], and in 2010, their nature as anticancer drugs was revealed[84]. CAR T-cell-based therapies have shown specific, rapid, high success rates, and long-lasting effects[85]. The principle of action is mainly through T cells with novel properties to induce a tumor rejection response. Patient-derived TILs that can be expanded *in vitro* with recombinant IL-2 have been demonstrated and have made important advances in the treatment of metastatic melanoma and other types of tumors[86-88].

To some extent, the efficacy of T-cell therapy depends on the potency of the T cells themselves; however, T cells can be influenced by the dose (absolute number of T cells injected) as well as the characteristics of the tumor-specific T cells administered. Controlling the dose of tumor-specific T cells is crucial for activation of the endothelial complement by IFN-γ to overcome the vascular endothelial barrier[89]. Therefore, the effect of pericyte therapy alone may be limited, and patients may benefit more from its combination with tumor-targeted interventions aimed at reprogramming the TME. A combination of many factors (intervention of tumor-intrinsic pathways, local inflammatory response, or intercellular messaging) to ensure proper engraftment and function of relayed metastatic T cells may be beneficial for cold tumors. Repeated stimulation of tumor-specific T cell expansion by cell lines established in lesions from patients with resected melanoma has been shown to be effective[90]. Melanoma is the best solid cancer type to respond to adoptive cell therapy[91].

In summary, peritoneal cellular immunotherapy is a promising treatment modality for CRC. The approach is based on the collection T cells from patients, which are expanded *in vitro* and then transfused back into the patient. These T cells are designed to express CARs, which can be designed to recognize not only tumor antigens but also to produce anticancer cytokines or ICIs. However, despite the results of CAR-T therapy in the treatment of hematological tumors such as B-cell malignancies, the effectiveness and applicability of the approach to convert cold tumors into hot tumors, and whether it can be successful in CRC and other solid tumors remains to be elucidated[7,92].

Other possible methods that can be used to make the tumor hotter

As a branch of hyperthermia, modulated electro-hyperthermia (mEHT) has been gradually applied in the treatment of various cancers in recent years[93,94]. The principle of mEHT is delivering locoregional clinical hyperthermia generated by 13.56 MHz amplitude-modulated radiofrequency[95]. A series of studies have shown the effect of mEHT in the treatment of CRC[96-99], and related mechanism studies have also shown its relevance in immunity[100,101]. This treatment may be a good candidate for transforming cold CRC.

In addition, some experiments are being conducted to make tumors hotter, such as combining oncolytic bacteria or viruses or peptides, tumor, virus or dendritic cell antigens with various adjuvants, with the goal of improving CRC immunogenicity. Tumor-associated macrophages, as a key driver of inflammation that facilitates tumor progression, are attractive targets to complement current immunotherapy[102].

As a key target of tumor-associated macrophages, colony-stimulating factor 1 receptor (CSF1R) can bind to CSF1 or IL-34 to activate macrophage proliferation and function[103,104]. CSF1R-specific inhibitors and other macrophage modulators are currently being studied in clinical trials in solid tumors [105].

THE CHALLENGES FOR IMMUNOTHERAPY TARGETING COLD CRC

Despite the widespread use of immunotherapy, poor clinical response to cold tumors is a current challenge[22]. Prior to immunotherapy, the resected tumor (primary or metastatic) should be classified as hot, altered, or cold. Although the tumor sample is valuable in providing information about the disease, it is limited in that it is not representative of the entire tumor landscape[28]. Recently, it was noted that, in addition to the CMS of CRC, the underlying epithelial cell diversity of CRC was summarized in a large transcriptome into two intrinsic subtypes, iCMS2 and iCMS3. This finding refines the CMS[106]. Because of genomic and immune heterogeneity, each sample can be considered an individual tumor[56]. Moreover, immune parameters change over the course of the disease[107].

The concept of personalized cancer immunotherapy is being increasingly promoted. A major challenge for immunotherapy of cold tumors is the need to identify key immune- or tumor-related features at the time of diagnosis to establish a reliable classification strategy to support immunotherapy for maximum efficiency[108].

CONCLUSION

With an increasing number of studies conducted on cold tumors, personalized cancer immunotherapy for individual patients is gaining ground. A major challenge hindering the development of therapeutic strategies may be the lack of mastery of the relationship between cancer and immunity. Even though a great deal of technological innovation and related research has been conducted to achieve some progress, the variability of cancer among individual patients cannot be generalized[108]. Identifying key phenotypic features is of interest when developing treatment strategies, and considerable progress has been made with ICIs approved by the United States Food and Drug Administration for the treatment of patients with dMMR/MSI-HmCRC. Notably, subtype dMMR/MSI-H CRC represents only a small fraction of all CRCs, and most pMMR/MSS mCRC patients do not benefit from ICI treatment alone. Therefore, further tumor states need to be identified, which has led to the continued reporting of new biomarkers, such as comprehensive immune scoring and complete CMS classification, and these results have led to a better understanding of the immune mechanisms of CRC and their relationship to tumor treatment strategies.

FOOTNOTES

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REVIEW

Is the combination of immunotherapy with conventional chemotherapy the key to increase the efficacy of colorectal cancer treatment?

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Abstract

Colorectal cancer (CRC) is among the most prevalent and deadly neoplasms worldwide. According to GLOBOCAN predictions, its incidence will increase from 1.15 million CRC cases in 2020 to 1.92 million cases in 2040. Therefore, a better understanding of the mechanisms involved in CRC development is necessary to improve strategies focused on reducing the incidence, prevalence, and mortality of this oncological pathology. Surgery, chemotherapy, and radiotherapy are the main strategies for treating CRC. The conventional chemotherapeutic agent utilized throughout the last four decades is 5-fluorouracil, notwithstanding its low efficiency as a single therapy. In contrast, combining 5-fluorouracil therapy with leucovorin and oxaliplatin or irinotecan increases its efficiency. However, these treatments have limited and temporary solutions and aggressive side effects. Additionally, most patients treated with these regimens develop drug resistance, which leads to disease progression. The immune response is considered a hallmark of cancer; thus, the use of new strategies and methodologies involving immune molecules, cells, and transcription factors has been suggested for CRC patients diagnosed in stages III and IV. Despite the critical advances in immunotherapy, the development and impact of immune checkpoint inhibitors on CRC is still under investigation because less than 25% of CRC patients display an increased 5-year survival. The causes of CRC are diverse and include modifiable environmental factors (smoking, diet, obesity, and alcoholism), individual genetic mutations, and inflammation-associated bowel

diseases. Due to these diverse causes, the solutions likely cannot be generalized. Interestingly, new strategies, such as single-cell multiomics, proteomics, genomics, flow cytometry, and massive sequencing for tumor microenvironment analysis, are beginning to clarify the way forward. Thus, the individual mechanisms involved in developing the CRC microenvironment, their causes, and their consequences need to be understood from a genetic and immunological perspective. This review highlighted the importance of altering the immune response in CRC. It focused on drugs that may modulate the immune response and show specific efficacy and contrasted with evidence that immunosuppression or the promotion of the immune response is the answer to generating effective treatments with combined chemotherapeutic drugs.

Key Words: Colorectal cancer; Immunotherapy checkpoint inhibitors; Chemotherapy; Immunotherapy; Immune response

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Core Tip: This review focused on the drugs that may modulate the immune response and show specific efficacy in the treatment of colorectal cancer. We then presented the evidence that immunosuppression or promotion of the immune response is the answer to generating effective treatments with combined chemotherapeutic drugs.

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INTRODUCTION

The origin of colorectal cancer (CRC) is heterogeneous. The general classification of CRC is divided into inherited, sporadic, and intestinal bowel diseases. Inherited CRC, which represents approximately 5% of all CRC cases, includes either the presence or absence of colonic polyps, such as Lynch syndrome and serrated polyposis syndrome[1]. Sporadic CRC (approximately 70% of CRC cases) is sustained by environmental and modifiable risk factors, including stress, diet, and age. Sporadic CRC has a monoclonal origin and is characterized by mutation accumulation in oncogenes and tumor suppressor genes. The second pathway of CRC includes the traditional APC-KRAS pathway and the microsatellite instability group, both having an essential role in clinical studies[2]. The third pathway includes intestinal chronic inflammatory diseases, such as Crohn's disease and ulcerative colitis, which could result in colitis-associated colon cancer[3].

From a biological perspective, this evidence demonstrates that the origin of CRC is diverse. The response to therapies is not always homogeneous in patients. The best treatment should be based on the tumor's unique characteristics. Effective treatments need to be broad and involve chemical and immunological molecules. The context of the broad causes of CRC development is highly involved in the low effectiveness of either single chemotherapeutics or classical immunotherapy by checkpoints inhibitor (ICI) administration during this oncological pathology. An in-depth and more precise description of the CRC origin and development, including a role for both immune response and inflammation, can be found in[4,5].

CONVENTIONAL TREATMENTS FOR COLON CANCER AND MECHANISMS OF ACTION FROM A GENETIC PERSPECTIVE

CRC is one of the deadliest diseases in the world. Despite advances in diagnosis, treatment strategies remain an essential bottleneck affecting survival, in which the pathological stage represents the most important prognostic factor for patients with CRC. The accurate classification of lesions is the primary tool to decide the most appropriate treatment and therapy[6]. The treatment for early-stage CRC (stage I and stage II) currently consists of resecting the tumor area with regional lymph nodes, which has a 5-year disease-free survival rate of 95%[7]. In the advanced stage of the disease (stages III and above), the rate of disease-free survival drops from 90% to 50% for surgery alone, requiring the administration of

chemotherapeutics, and only 17%-20% of these patients ultimately survive[8,9].

5-Fluorouracil (5-FU) has been central in treating advanced CRC since 1957. Unfortunately, the response rate to 5-FU as the first-line chemotherapy in advanced CRC is still only 10%-15%. In contrast, 5-FU combined with other anticancer drugs as adjuvants, such as leucovorin and oxaliplatin (FOLFOX) or leucovorin calcium and irinotecan, increases the effectiveness of 5-FU by 50%[10,11].

5-FU is the third most commonly used chemotherapeutic agent for the treatment of solid malignancies worldwide[12]. Heidelberger synthesized it in the early 1950s as a derivate of fluoropyrimidines. This drug was one of the first chemotherapeutics reported to have anticancer activity. It was tested in diverse tumors in rats and mice, where it significantly reduced tumor burden. Additionally, tumoral tissues incorporated this compound more rapidly than normal tissues, which pointed to its potential use as a chemotherapeutic drug[13,14]. In 1962, the Food and Drug Administration approved the use of 5-FU for treating CRC.

In intravenous administration, 5-FU is incorporated rapidly into the cells through facilitated transport as uracil[15]. Subsequently, its metabolism can be driven in two ways, *i.e.*, *via* anabolic and catabolic routes, which compete with each other. In sensitive cancerous cells, the anabolic pathway leads to the conversion of this drug into several active metabolites, such as fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate[16]. The active metabolites interfere with nucleoside metabolism and can be incorporated into RNA and DNA, leading to cytotoxicity and cell death[17,18]. This mechanism is due to its similar structure to pyrimidine, molecules of DNA and RNA, an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen[19]. Fluorodeoxyuridine monophosphate disrupts the function of thymidylate synthase, a key enzyme responsible for providing deoxynucleotide triphosphates, which are necessary for DNA replication and repair, catalyzing the reaction of deoxyuridine monophosphate to deoxythymidine monophosphate synthesis [14,15]. An insufficiency in deoxythymidine monophosphate leads to the depletion of deoxythymidine triphosphate, which perturbs the levels of the other deoxynucleotide triphosphates[16-20] (Figure 1).

5-FU has primarily been used in the treatment of solid cancers of digestive origin, such as colorectal, anal, pancreatic, esophageal, gastric, and ampullary tumors, and less frequently in breast, cervical, and head and neck cancers[21-23]. CRC treatment includes various chemotherapeutic drugs. As the backbone of treatments, 5-FU has been used for more than five decades, and more recently, it has been combined with other chemotherapeutic drugs to potentiate its anticarcinogenic effect[22,24].

The use of alternative broad-spectrum chemotherapeutics in addition to 5-FU has been proposed for colon cancer treatment. Doxorubicin treatment combined with other drugs, such as metformin and sodium oxamate, reduces the proliferation rate of colon cancer cell lines *in vitro*[25]. However, the use of doxorubicin in patients is limited by the side effects frequently associated with this drug, such as hepatotoxicity, nephrotoxicity, pulmotoxicity, and cardiotoxicity[26,27]. Additionally, doxorubicin can lead to chemoresistance in tumor cells through nuclear factor kappa B translocation to the nucleus and DNA binding because of the damage induced by this drug, triggering the expression of antiapoptotic genes[28].

In CRC, nuclear factor kappa B nuclear translocation is a characteristic in more than 70% of patients, limiting the use of doxorubicin[29]. Another disadvantage of this drug is its anthracycline nature since it is extracted from *Streptomyces spp*. Cancer cells frequently show rapid resistance to naturally occurring cancer drugs, diminishing their effectiveness, whereas they are more sensitive to antimetabolites, such as 5-FU and cisplatin, among others[30]. The use of other chemotherapeutics, such as tamoxifen, which is highly effective and frequently used in breast cancer treatment, has an adverse effect in the treatment of CRC[31]. Due to the molecular characteristics of each type of cancer, the successful use of tamoxifen in breast cancer lies in its mechanism of action. Approximately 80% of all breast cancers are positive for the estrogen receptor, and tamoxifen inhibits the expression of estrogen-regulated genes by the competitive inhibition of this receptor. Different reports indicate that tamoxifen has the opposite effect on CRC, increasing the risk of developing this type of cancer[31,32].

MOLECULAR PERSPECTIVE FOR THE USE OF CHEMOTHERAPEUTIC STRATEGIES IN CRC TREATMENT: WHICH IS THE RIGHT DRUG?

CRC is a molecularly heterogeneous disease in which genetics and cellular events accumulate to endow tumor cells with aggressive characteristics, including chemotherapy resistance. Chromosomal instability, mismatch repair, and methylator phenotype are the three major pathways involved in acquiring tumorigenesis and a malignant phenotype and could be present in sporadic and inherited CRC[33,34]. The choice of better therapy is based on cancer-related features and patient-related factors, such as the number and localization of metastases, tumor progression, presence or absence of biochemical markers, and comorbidity[35-37]. Despite all these characteristics, treatment based on the antineoplastic effects of 5-FU is the cornerstone of therapy in advanced CRC stages.

Treatment with 5-FU in combination with other drugs, such as oxaliplatin (OXA), irinotecan, capecitabine, bevacizumab, cetuximab, panitumumab, ziv-aflibercept, regorafenib, and ramucirumab, increases its effectiveness and has been approved by the Food and Drug Administration for the

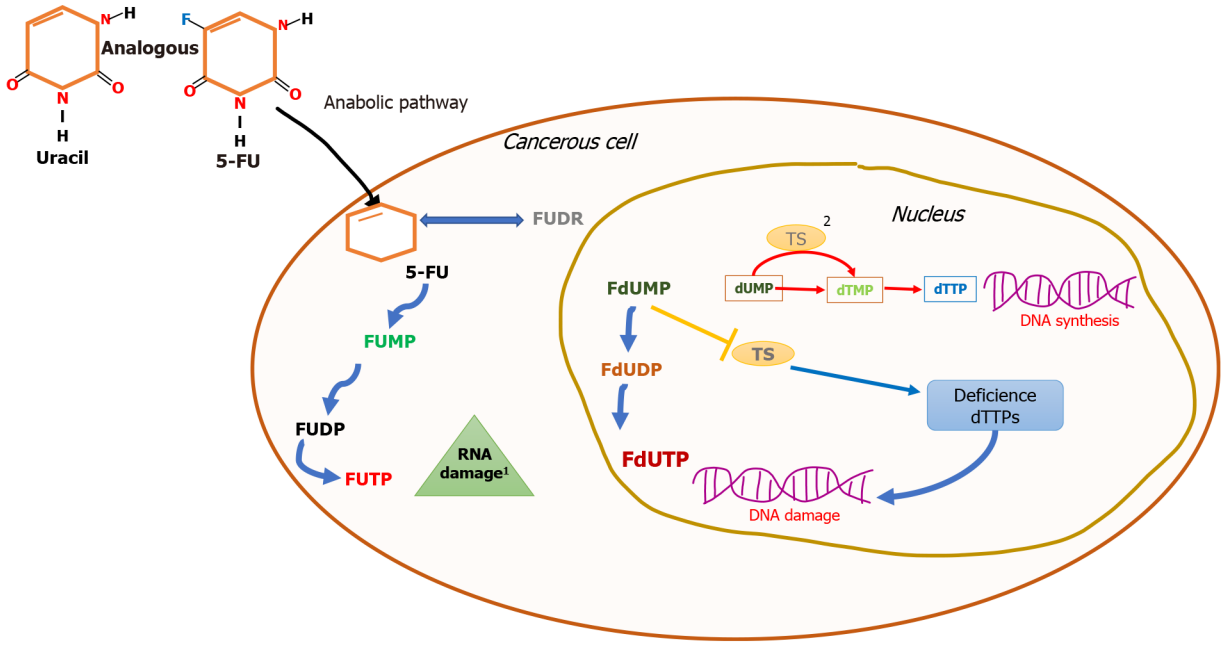

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Figure 1 5-Fluorouracil mechanism of action. The 5-fluorouracil structure is analogous to that of the nucleotide uracil; its ability to disrupt standard RNA processing and function is mediated by three primary metabolites: fluorodeoxyuridine monophosphate, fluorodeoxyuridine diphosphate, and fluorouridine triphosphate. [1]: 5-fluorouracil inhibits thymidylate synthase activity by fluorodeoxyuridine monophosphate metabolite binding, blocking the typical substrate deoxyuridine monophosphate that inhibits deoxythymidine monophosphate synthesis leading to deoxythymidine triphosphate imbalance. The consequent result is DNA damage due to a deficiency in its synthesis and its repair; [2]: DNA replication and repair are regulated by deoxyuridine monophosphate transition to deoxythymidine monophosphate. This step is coordinated by thymidylate synthase; FUMP: Fluorodeoxyuridine monophosphate; FUDP: Fluorodeoxyuridine diphosphate; FUTP: Fluorouridine triphosphate; dUMP: Deoxyuridine monophosphate; dTMP: Deoxythymidine monophosphate; FUDR: Fluorodeoxyuridine; FdUMP: Fluorodeoxyuridine monophosphate; dTTP: Deoxythymidine triphosphate; FdUDP: Fluorodeoxyuridine diphosphate; FdUTP: Fluorodeoxyuridine triphosphate; 5-FU: 5-Fluorouracil; TS: Thymidylate synthase.

management of CRC[38]. Thus, during stages III or IV in resected CRC patients, the use of combination treatment, such as FOLFOX or 5-FU, leucovorin calcium, and irinotecan, is common as a first-line treatment. This strategy significantly increases the survival rate of these patients[39]. The initial chemotherapy scheme and the decision on better combinatory drugs depends on multiple conditions in the patients. In metastatic CRC limited to the liver or lung, surgery and the rapid initiation of chemotherapy appears to be the best option. When CRC cure is not possible, three additional scenarios can arise: (1) Patients with advanced tumors and symptoms require rapid tumor shrinkage to provide palliation, which begins with chemotherapy; (2) Asymptomatic patients with bulky tumor and possible rapid progression are likely to become symptomatic in a short period; and (3) Patients without symptoms but disseminated disease who never had resectable disease but whose tumors remain non-bulky are likely to remain asymptomatic for an extended period. In the last two scenarios, the initiation of chemotherapy can be discussed[22,40]. Previous work in the Nordic population demonstrated that early treatment with 5-FU plus leucovorin in asymptomatic patients with advanced CRC prolonged survival and delayed both disease progression and the onset of symptoms[41]. In another study in Australasian and Canadian populations of asymptomatic patients using the same chemotherapy regimen, no difference was reported between early or delayed chemotherapy use until symptoms appeared[42]. Thus, clinical treatment requires a medical discussion and the patient´s preference when cure is not possible. The spectrum of molecular alterations that offer alternative management for this disease could be explored.

Alterations in genes related to survival, angiogenesis, proliferation, and apoptosis incorporate additional strategies into CRC treatment. The *RAS*, *KRAS*, and *NRAS* genes play an essential role as prognostic and predictive indicators in CRC treatment[43-45]. Mutations in the DNA at position 12 in the KRAS protein are significantly associated with a poor prognosis: a 5-year survival rate of approximately 3%[46]. Patients with this mutation are not candidates to receive treatment with monoclonal antibodies, such as cetuximab or panitumumab, which target the epidermal growth factor receptor (EGFR)[47-50]. Blocking EGFR represents the second line of treatment in patients with wild-type RAS together with the backbone 5-FU, leucovorin calcium, and irinotecan therapy. Therefore, the patient's genetic and tumor-specific factors need to be considered when choosing chemotherapeutic and combination schemes to avoid resistance and undesired responses to these therapies.

Drug resistance and consequent therapy failure are the main problems clinicians face in treating different neoplasms, which limit the quality of life and long-term remission rates as a consequence of tumor growth and spreading leading to 90% of patients dying[51,52]. Drug resistance is a highly complex process that is commonly classified into two types: intrinsic and acquired. Both types of drug resistance lead to the regulation of molecular mechanisms of chemoresistance, such as the activation of transporter pumps, oncogenes, tumor suppressor genes, mitochondrial alteration, DNA repair, autophagy, epithelial-mesenchymal transition, cancer stemness, and exosomes[53,54].

In the intrinsic phenotype, diverse alterations existing before drug administration in the patient complicate the selection of chemotherapy. The inherent genetic mutations in tumors, such as a *KRAS* mutation in exon 2 in codon 12 or 13, are the most frequent mutations associated with poor prognosis and drug resistance in CRC[55,56]. Therapies based on the first line of treatment using FOLFOX or 5-FU, leucovorin calcium, and irinotecan plus cetuximab or panitumumab (anti-EGFR) are ineffective in patients with *KRAS* mutations[57]. Recent studies indicate that mutations in genes related to the pathways that regulate tumor cell survival and proliferation and inhibit apoptosis in tumor cells, such as *AKT1* and *CTNNB1*, contribute to 5-FU chemotherapeutic resistance in CRC. The *CTNNB1* gene encodes the β-catenin protein, which plays a crucial role in cancer by activating the Wnt/β-catenin signaling pathway. This pathway is associated with tumorigenesis and CRC resistance, and it upregulates genes and proteins, such as multidrug resistance gene (*MDR1*) and inhibitor of apoptosis (*Bcl2*), to induce epithelial-mesenchymal transition and regulate the tumor microenvironment (TME)[58-60].

IS CHEMOTHERAPY AN INDUCER OF IMMUNOSUPPRESSION IN CRC?

The heterogeneity of tumors, including CRC, consists of heterogeneity in cancer and infiltrated resident host cells, extracellular matrix, and immune and inflammatory cells, such as macrophages, dendritic cells, myeloid-derived suppressor cells, T cells, mast cells, and natural killer cells. These components comprise the TME, which has a dynamic composition[61]. It is well known that one of the main functions of the TME is to provide a protective function for tumor cells, inducing crosstalk between immune and nonimmune cells that leads to tumor-mediated immunosuppression, supporting tumor growth and survival[62]. Recent reports indicate that the TME in CRC contributes to cancer progression and drug resistance through high interstitial pressure, fibrosis, and the degradation of the therapeutic agent by enzymatic activity and inducing immunosuppression[61,63,64]. These findings indicate that the regulation of immune cells surrounding the tumor has a critical role in the response to therapies for CRC. Thus, chemotherapy and immunotherapies targeting the recovery activity of immune cells are likely necessary to fight CRC.

The study of the effect of chemotherapeutic drugs on immune cells is controversial. The central concept here is that chemotherapy reduces the capacity of the immune system to function, but how could a drug affect the capacity and efficiency of the immune response to induce an efficient post-treatment response? Perhaps the "original" concept has a flawed approach. Evidence suggests that after 5-FU treatment in a mouse model, bone marrow cellularity decreases, but platelets and thrombopoietin, which are close to the immune response, rebound[64,65]. Similarly, the serum of patients diagnosed with stage III/IV CRC who had received FOLFOX chemotherapy showed increased levels of heat shock protein 70, which belongs to the damage-associated molecular patterns recognized by innate receptors [66]. Later, *in vitro* studies showed that the supernatants of dying CRC cells treated with OXA and 5-FU induced a mature phenotype in dendritic cells coexpressing HLA-DR, CD80, and CD86 and producing interleukin-1β, tumor necrosis factor-α, and MIP-1α in a TLR-4-dependent manner[66]. These results strongly suggested that OXA/5-FU treatment induced the activation of the innate immune response during CRC. Additionally, increased numbers of myeloid-derived suppressor cells have been reported in a mouse model of thymoma, and treatment with 5-FU combined with gemcitabine selectively induced apoptosis in myeloid-derived suppressor cells. Consequently, increased antigen-specific CD8+ T cells produced more interferon-γ, generating a T cell antitumor response (Figure 2)[66].

Conversely, high levels of the chemokine CCL20 recruit regulatory T (Treg) cells in CRC patients resistant to FOLFOX[67]. However, in blood samples from metastatic FOLFOX-sensitive CRC patients, a reduced percentage of Foxp3+ Treg cells was recorded after treatment[68]. Therefore, the increase in Treg cells is associated with 5-FU chemoresistance. Thus, evidence of 5-FU chemotherapy suggests a role in the specific and direct reduction of suppressive immune cells during CRC. Additionally, the increased apoptosis-induced death of tumor cells by 5-FU could increase the ability of immune cells to recognize damage-associated molecular patterns released by these dying tumor cells, inducing protective inflammation. Evidence needs to be accumulated in this field to clarify whether chemotherapy may be an inducer of immune cell activation (Figure 3).

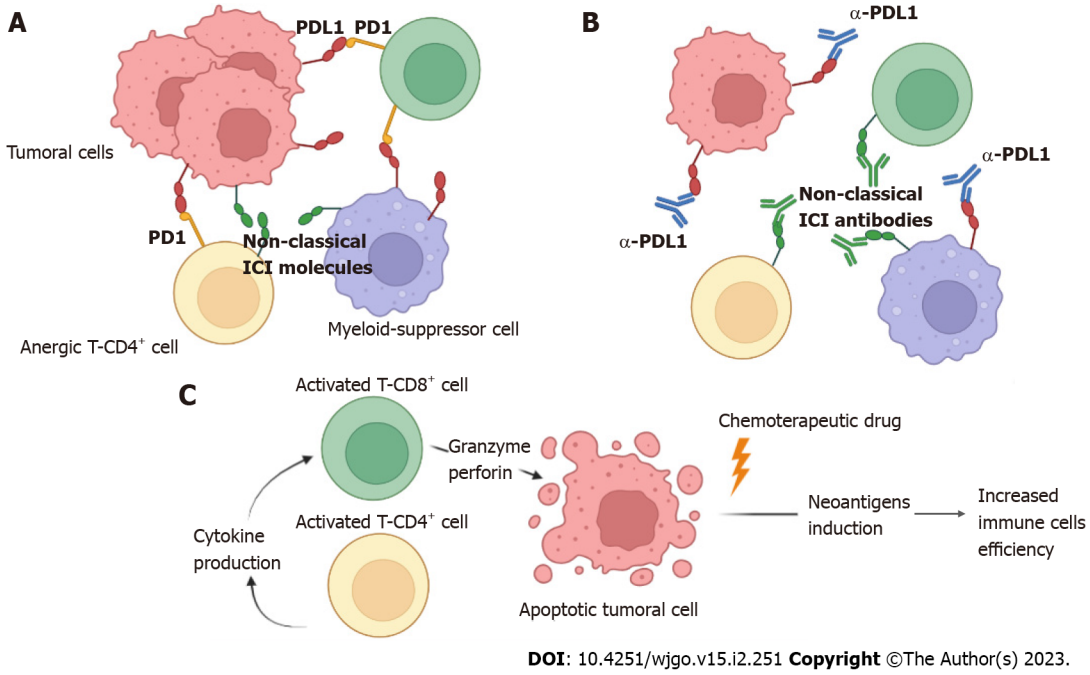

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Figure 2 Mechanism of action using either anti-programmed cell death ligand 1 or anti-nonclassical immune checkpoint inhibitor antibodies to increase the effector immune response in colorectal cancer. A: The programmed cell death ligand 1 molecule expressed on both tumor and myeloid suppressor cells interacts with programmed cell death 1 molecules expressed on exhausted CD8$^+$ and CD4$^+$ T cells, inducing a state of anergy. Additionally, other nonclassical immune checkpoint inhibitor molecules can induce anergy in T cells; B: Anti-programmed cell death ligand 1 antibodies block the interaction of the programmed cell death 1/programmed cell death ligand 1 axis, favoring the return from the state of anergy to exert the effector function of CD4$^+$ and CD8$^+$ T cells, favoring the reduction of the tumor burden. Most likely, nonclassical immune checkpoint inhibitor antibodies may have a similar effect; C: Once CD4$^+$ T cells are activated, they produce cytokines for the efficient activation of CD8$^+$ T cells, which in turn produce granzyme and perforin, inducing apoptosis in tumor cells. The addition of chemotherapeutic drugs increases the induction of neoantigens, favoring immune response activation. PDL1: Programmed cell death ligand 1; PD1: Programmed cell death 1; ICI: Immune checkpoint inhibitor.

CLASSICAL ICIS USED AS MONOTHERAPY DURING CRC

The effectiveness of ICI as an immunotherapy treatment has been evaluated in the last decade. The efficacy of these agents is evident in liquid tumors, such as melanoma, leukemia, and solid non-small cell lung carcinoma. Classical ICIs used to treat these oncological pathologies are anti-programmed cell death 1 (PD1), anti-programmed cell death ligand 1 (PDL1), and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) monoclonal antibodies, where anti-CTLA4 has a lower clinical efficacy[68,69] (Table 1).

Diverse reports suggest single or combined therapy using different antibodies targeted against the same or another molecule. In melanoma therapy, nivolumab, a human immunoglobulin G4 anti-PD1 pathway monoclonal antibody, results in an overall survival (OS) rate of 72.9%, whereas the antineoplastic chemotherapeutic dacarbazine resulted in a survival rate of 42.1%[69]. In metastatic melanoma refractory to chemotherapy, treatment with ipilimumab, an anti-CTLA4 antibody, shows high efficacy when combined with anti-PD1 antibodies (either pembrolizumab or nivolumab)[70]. Additionally, nivolumab and ipilimumab combination treatment prolongs progression-free survival, mainly in patients with tumors testing positive for PDL1 expression[71]. Although *in vitro* studies suggested that atezolizumab, avelumab, and durvalumab, all anti-PDL1 antibodies, more effectively block PD1/PDL1 signaling (Figure 2)[72], evidence of the use of an anti-PDL1 antibody as a single approved treatment without immunotherapy or chemotherapy combination is insufficient to conclude their role in inducing protection in metastatic melanoma[73]. Immunotherapy with the anti-PDL1 antibody atezolizumab has been approved to treat non-small cell lung carcinoma combined with chemotherapy, and anti-PDL1 has been approved to treat triple-negative breast cancer[74,75]. Therefore, increased PDL1 expression in the TME of melanoma patients is an efficient marker to predict response to anti-PD1 treatments, which must be applied in all types of cancer when this immunotherapy is suggested[76].

Notwithstanding the successful use of immunotherapy in the neoplasms mentioned above, few reports show an influential role for classical monoclonal ICI using anti-CTLA4 and anti-PD1/PDL1 axis antibodies in CRC. Most clinical assays were disappointing. For example, tremelimumab, a human immunoglobulin G2 anti-CTLA4 antibody, did not produce clinically meaningful results when it was used as a monotherapy in patients with refractory metastatic CRC[77] (Table 1).

Table 1 Types of immune checkpoint inhibitor antibodies used as monotherapy, combined immune checkpoint inhibitor, and immune checkpoint inhibitor + chemotherapy in colorectal cancer

Antibody name	Isotype	Target molecule	Effectiveness[1] as monotherapy	Effectiveness as combined ICI	Effectiveness as ICI + chemotherapy	Ref.
Ipilimumab	IgG1	CTLA4	Yes	Well tolerated in combination with nivolumab	No	Suzuki *et al*[80], 2021; Lenz *et al*[86], 2022; Cohen *et al*[90], 2020
Tremelimumab	IgG2	CTLA4	No	Yes, durvalumab improved OS and increased lymphoid response	Combined with durvalumab + fluoropyrimidines, oxaliplatin, irinotecan, showed increased OS	Chung *et al*[77], 2010; Kanikarla Marie *et al*[85], 2021; Chen *et al*[102], 2020
Nivolumab	IgG4	PD1	Well tolerated	Well tolerated in combination with low ipilimumab dose, with increased OS	Yes	Overman *et al*[78], 2017; Kawazoe *et al*[79], 2021; Lenz *et al*[86], 2022; Morse *et al*[87], 2019
Pembrolizumab	IgG4	PD1	Well tolerated, increased OS	No	There is no evidence	Haag *et al*[82], 2022
Atezolizumab	IgG1	PDL1	There is no evidence	There is no evidence	Safe when combined with cobimetinib, having no effect on OS. Combined with FOLFOX and bevacizumab showed increased progression-free survival, but adverse events were shown	Eng *et al*[91], 2019; Antoniotti *et al*[94], 2022
Avelumab	IgG1	PDL1	Increased OS but adverse events were shown	There is no evidence	Combined with cetuximab showed increased T cell killing	Haag *et al*[82], 2022; Stein *et al*[97], 2021
Durvalumab	IgG1	PDL1	Increased progression-free survival, but adverse events were shown	There is no evidence	Safe when combined with MEKi, having no effect on OS	Oh *et al*[84], 2022

[1]Effectiveness was considered as increased survival, well tolerated treatment in patients, or simply a lack of side effects associated with the treatment.
IgG: Immunoglobulin G; OS: Overall survival; PDL1: Programmed cell death ligand 1; PD1: Programmed cell death 1; CTLA4: Cytotoxic T-lymphocyte-associated protein 4; ICI: Immune checkpoint inhibitor; FOLFOX: 5-FU therapy with leucovorin and oxaliplatin; MEKi: Inhibitor of MAPK/ERK kinase.

Another monotherapy treatment using nivolumab in CRC patients with microsatellite instability and FOLFOX chemoresistance showed excellent control of the disease, and patients tolerated this treatment well[78]. CRC patients treated with nivolumab combined with an oral heat shock protein 90 inhibitor showed safety profiles and antitumor activity associated with reduced activity of Treg cells and better response of tumor-infiltrating lymphocytes[79]. Interestingly, a report of a woman treated with nivolumab for melanoma with no hereditary CRC background developed colon carcinoma after 7 years of anti-melanoma treatment. The medical service then decided to switch the treatment to ipilimumab, and after four cycles of monotherapy, the colon tumor was in complete remission[80]. Pembrolizumab used as monotherapy in either microsatellite instability-high or mismatch repair-deficient CRC patients produced improvements in health-related quality of life compared to patients treated with leucovorin, 5-FU, and OXA[81]. In one study of refractory mismatch repair, CRC patients treated with pembrolizumab as monotherapy plus maraviroc, an agonist of CCR5 that promotes the activation and recruitment of macrophages inducing immune cell infiltrate in tumors, showed a beneficial toxicity pattern. The OS was higher than expected[82].

ICI with avelumab monotherapy in unresectable metastatic CRC patients who failed FOLFOX chemotherapy showed an OS of 72.2% at 8.1 mo, which was similar to the effect of ICI monotherapy using either pembrolizumab or nivolumab. However, some patients showed treatment-related adverse events[83]. Durvalumab used as monotherapy in microsatellite-instability high/mismatch repair-deficient metastatic CRC patients whose disease had progressed after chemotherapy showed efficiency and a satisfactory progression-free survival of 58.2%; however, side effects were found in 36.4% of patients[84].

Taken together, these results suggest that contrary to anti-CTLA4, ICI monotherapy blocking the PD1/PDL1 axis has a better effect in high microsatellite instability/mismatch repair-deficient metastatic CRC patients who previously displayed chemoresistance. Additionally, combining ICI PD1/PDL1 monotherapy with either antibodies or immune cell stimulators improves treatment efficacy. However, only a small number of clinical trials show increased OS (Table 1). Most likely, the TME reduces the access of ICI antibodies to the target molecules expressed in either immune or epithelial cells (Figure 3).

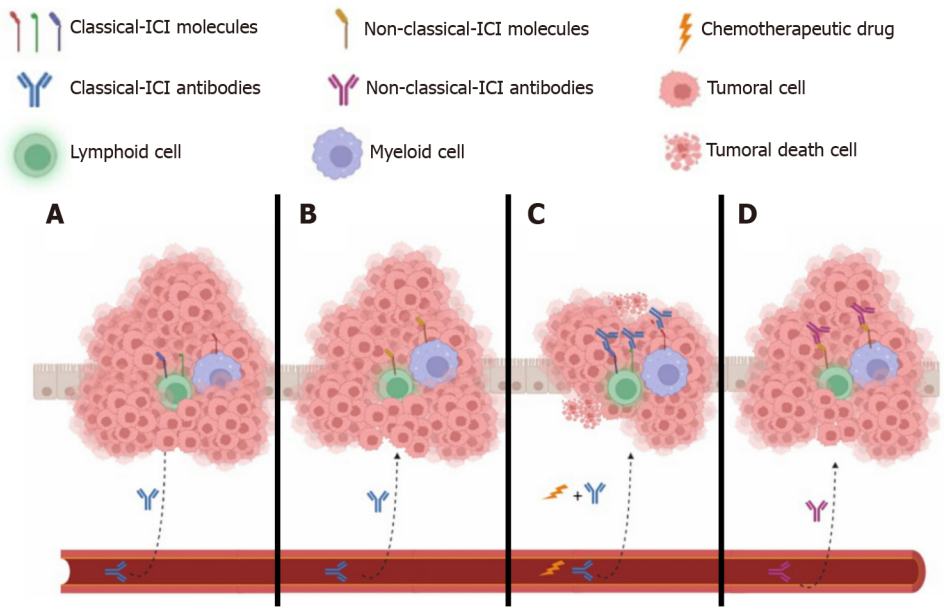

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Figure 3 The tumor microenvironment favors or does not allow the access of both classical and nonclassical immune checkpoint inhibitors to their targets in immune cells. A: The classical immune checkpoint inhibitor (ICI) antibody cannot access its target because immune cells are surrounded by tumor cells, although immune cells may express classical ICIs; B: On the other hand, classical ICIs probably have access to immune cells but may not express the targeting molecules; C: Chemotherapy can induce the death of tumoral cells, favoring the formation of neoantigens that may reactivate immune cells. Additional classical ICI antibodies target individual molecules such as cytotoxic T-lymphocyte-associated protein 4, programmed cell death 1, and programmed cell death ligand 1; D: Finally, immune cells expressing nonclassical ICIs could have effector profiles such as helper T type 1 cells, cytotoxic T cells, M1, or N1 when nonclassical antibodies target them. Additionally, tumor epithelial cells may likely express both classical and nonclassical ICIs. ICI: Immune checkpoint inhibitor.

DOES COMBINED IMMUNOTHERAPY INCREASE THE EFFECTIVENESS OF TREATMENT FOR CRC?

Little evidence of the apparent effect of ICI monotherapy on CRC development is available. Conversely, increasing evidence suggests a better outcome using combined ICI, *i.e.*, the use of two monoclonal antibodies targeting CTLA4 or the PD1/PDL1 axis. To improve the immune response, combination treatment with tremelimumab and durmalumab was used in patients for the preoperative management of resectable CRC and liver metastases. These patients improved their OS to 24.5 mo; interestingly, their $CD4^+$, $CD8^+$, and B cells displayed an activated profile[85]. Additionally, first-line treatment consisting of nivolumab plus low-dose ipilimumab treatment in patients with microsatellite instability and metastatic CRC without previous chemotherapy showed that this combination was well tolerated at the primary endpoint, with robust and durable clinical benefit. However, the study is ongoing, and OS data are not yet available[86]. Previous ICI studies using similar inclusion criteria and antibody doses showed that follow-up over 12 mo of combined treatment resulted in an 85% OS[87]. Recently, the combined use of ipilimumab plus nivolumab before surgery in either mismatch repair-deficient or mismatch repair-proficient CRC patients induced an antitumoral response associated with a lack of signs of cancer after surgery with increased infiltration of $CD8^+PD1^+$ cells; the authors suggested that this combined ICI therapy may be the standard treatment for mismatch repair-deficient CRC patients [21].

Additionally, circulating tumor DNA detection in the blood of patients with durable and ongoing responses to ipilimumab plus nivolumab could be used as a monitoring response and dynamic marker of this combined ICI treatment[88,89]. Finally, a study attempted to analyze whether pseudo progression was observed in mismatch repair-deficient CRC patients treated with nivolumab plus ipilimumab, showing that this treatment rarely induces and confirming a high disease control rate of 86%[90]. Taken together, this evidence strongly suggests that the combined ICI blockade of CTLA4-PD1/PDL1 in mismatch repair-deficient CRC patients is highly successful. However, these patients represent only a tiny fraction of all CRC-diagnosed patients. Therefore, more clinical trials must be developed to obtain sufficient evidence to conclude the positive effects of the classic ICI combination.

USE OF EITHER MONOTHERAPY OR COMBINED IMMUNOTHERAPY PLUS CHEMOTHERAPY TO INCREASE THE EFFECTIVENESS OF TREATMENTS FOR CRC

The combined use of atezolizumab plus aobimetinib, a MAPK/ERK kinase 1 and 2 inhibitor that increases $CD8^+$ cell infiltration in tumors of patients with microsatellite-stable metastatic CRC, showed no improvement in OS and was consistent with the safety of using both drugs[91] (Table 1). A similar effect showing only acceptable tolerance to this treatment was observed with durmalumab plus inhibitor of MAPK/ERK kinase in microsatellite-stable metastatic CRC patients[89]. No improved OS was observed using atezolizumab combined with cobimetinib in metastatic CRC patients[92]. A multicenter phase I/II study in June 2017 aimed to analyze the role of durmalumab plus tremelimumab combined with FOLFOX chemotherapy in patients with metastatic CRC, expecting a 6-mo progression-free survival of over 70.7%; however, the authors do not have the final results to date[93].

Recently, the combination of atezolizumab with FOLFOX chemotherapy plus bevacizumab (monoclonal anti-vascular endothelial growth factor antibody) in mismatch repair metastatic CRC patients induced a median progression-free survival of 13 mo, while FOLFOX plus bevacizumab alone resulted in 11 mo of progression-free survival, suggesting that the addition of atezolizumab improves progression-free survival. However, 42% of patients showed neutropenia, and 27% displayed severe adverse events[94]. The same treatment consisting of atezolizumab combined with FOLFOX plus bevacizumab was used in patients with untreated unresectable metastatic CRC, showing no improvement in OS and safety signals[95]. The use of the anti-EGFR antibody cetuximab plus avelumab for treating wild-type *RAS* metastatic CRC patients was safe; the authors suggested that an analysis of circulating DNA in plasma could be an indicator of the positive effects of this treatment. However, the data are insufficient to show the impact on the OS rate[96].

Treatment with cetuximab plus the ICI avelumab in microsatellite stable metastatic CRC patients showed that subclones of tumors expressing PDL1 mutations mediated the resistance to direct avelumab antitumor effects but also increased T cell killing[97]. An analysis of the neutrophil-to-lymphocyte ratio in the blood of chemorefractory metastatic CRC patients treated with cetuximab plus avelumab showed that a high neutrophil-to-lymphocyte ratio was a poor prognostic factor. Thus, the neutrophil-to-lymphocyte ratio could also be a predictor for the effectiveness of the combined ICI cetuximab plus avelumab[98].

Regorafenib, an inhibitor of protein kinases in tumor angiogenesis used in combination with avelumab in microsatellite stable CRC patients, showed increased infiltration of $CD8^+$ T cells associated with better outcomes, with an OS of 10.8 mo[99]. Metastatic CRC patients who previously received two radiotherapies and who were treated with durvalumab plus tremelimumab before the third round of radiotherapy showed increased circulating, differentiated, and proliferating $CD8^+$ T cells, but the authors concluded that this finding does not meet the prespecified endpoint criteria to consider this combined ICI plus radiotherapy worthwhile for further study; specifically, the authors suggested an objective response rate of at least 25%, but they only obtained a response rate of 8.3%[100].

The addition of FOLFOX-based chemotherapy to avelumab plus an adenovirus vector vaccine capable of inducing a $CD4^+$/$CD8^+$ T cell response in mismatch repair-deficient microsatellite instability-high metastatic CRC patients showed no improvement in progression-free survival[101]. However, The Canadian Cancer Trials Group suggests that combining tremelimumab and durmalumab to treat patients with high microsatellite instability who had previously received chemotherapy (fluoropyrimidines, OXA, irinotecan, and others) may prolong OS. They correlated the increased effectiveness of this immunotherapy combination with the tumor mutation burden elevated in plasma[102].

It is essential to mention that some research about the combination of chemotherapy and ICI is under development[93]. For example, a phase II trial in 2020 will show whether atezolizumab combined with OXA, radiotherapy, and bevacizumab may increase progression-free survival in microsatellite instability CRC patients[103]. Additionally, in microsatellite instability-high metastatic CRC patients with deficient mismatch repair, a study is currently underway to prove the improvement of disease-free survival by ICI with avelumab plus fluoropyrimidine; the authors suggested that this ICI plus chemotherapy treatment would improve the expected 3-year disease-free survival rate by 12%[104]. Evidence showing that combining ICI with chemotherapy improves treatment efficacy continues to accumulate.

Most clinical trials reported here are recent, and perhaps the evidence is insufficient to conclude that a treatment criterion has already been established. Consequently, evidence supports the hypothesis that the use of classical ICIs improves chemotherapy treatment, mainly in CRC patients with high microsatellite instability. It is crucial for patients who do not have a good prognosis with chemotherapy alone to have a better response with the combination of classical ICI antibodies.

MULTIOMICS, INDIVIDUALIZED IMMUNOTHERAPY, ADOPTIVE TRANSFER OF "TRAINED" IMMUNE CELLS, AND NONCLASSICAL ICIS ARE A NEW HOPE FOR CRC

In recent years, mechanisms have begun to be developed to understand and explain why, in some cases, classical immunotherapy is sufficient to generate benefits in some patients. Multiple factors participate in the development of any pathology, such as the patient's clinical history, genetics, and the ability of their immune cells to act during cancer. We emphasize the importance of the recruitment of immune cells to the TME; in the case of melanoma, non-small cell lung cancer, and leukemia, the capacity of the immune cells to access is greater, which together with ICIs increases the bioavailability of monoclonal antibodies to find the antigens expressed in the required enclosures to be detected and removed or blocked[105]. In contrast, other types of oncological pathologies are available when access to the tumor site is more difficult for both immune cells and classical ICI antibodies, such as CRC[106]. In addition, the causes of CRC are multiple, and we cannot attempt to generalize a unique treatment for all varieties of CRC to reduce the statistics of this oncological pathology on the rise.

The single-cell multiomics technique has shed light on the complexity of the individual immune response elicited against any agent. This approach facilitates the individual characterization of groups of cells by identifying the gene transcripts at a specific time[107]. This technique depends on the efficiency of flow cytometry to distinguish and separate individual cells, the equipment used to amplify mRNA transcripts and synthesize complementary strand DNA, and sequencing equipment, allowing for robust data with high precision and certainty[108]. The advancement of these latest-generation technologies allows not only the expression of the classic ICI (CTLA4, PD1/PDL1) to be distinguished but also the characterization of mRNA transcripts in specific immune or epithelial cell populations at a particular time. These transcripts, already expressed as proteins, can individually be proposed as new nonclassical ICIs in patients[109], generating a wide range of therapeutic targets that, as in the case of CRC, increase the efficiency of previous treatments.

Some surface molecules involved in suppressive functions for activated T cells, Treg cells, macrophages, neutrophils, and epithelial cells have been proposed as immunotherapy targets. Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), T cell immunoglobulin and mucin domain 3 (TIM3), LAG3, CD39, CD73, CD47, and SIRP-1α (a do not eat me signal)[110] warrant evaluation as monotherapy or combined therapy in CRC.

TIM3 is an overexpressed inhibitory receptor in active immune cells, including myeloid and lymphoid cells, with suppressive and modulatory characteristics. It is relevant in reducing interferon-γ production by helper T type 1 cells after binding to its ligand, galectin-9[111]. TIM3 is also overexpressed by Treg cells in a colitis-associated colon cancer mouse model[112]. The combination of TIM3 with anti-PD1 ICI antibodies is a good prospect in a murine breast cancer model[113]. Sabatolimab, an anti-TIM3 antibody, has already been used as a treatment in ovarian, CRC, and non-small cell lung cancer combined with an anti-PD1 antibody, being well tolerated and improving antitumor activity [114]. GITR has a role in the immunomodulation of effector T cells and increases tumor resistance[113, 115]. Treatment with an anti-GITR antibody combined with pembrolizumab improves the disease control rate compared with anti-GITR used as monotherapy for treating CRC, melanoma, and adrenal carcinoma[116].

Lymphocyte activation gene-3 (LAG3 or CD223) has a structure similar to that of the CD4 molecule, joining major histocompatibility complex II in antigen-presenting cells. However, two of their immunoglobulin-like domains can bind receptors in tumor cells[81]. Blockage of LAG3 induces increased interleukin-2 production and enhances T cell proliferation[117]. Therefore, LAG3 has been proposed as an ICI; the genes *LAG3* and *IDO1* were shown to be overexpressed in a phase II study of pembrolizumab use as ICI monotherapy in patients with esophageal squamous cell carcinoma. The authors suggested that a combination of ICIs is needed to induce immunity against this tumor[118]. CD73 is an extracellular adenosine receptor expressed in immunosuppressant cells (such as Treg cells), favoring tumor progression[119].

Recently, single-cell RNA sequencing in a colitis-associated CRC murine model showed that an anti-CD73 antibody has a significant role in improving the anticancer functions of Treg cells, and exhausted $CD8^+$ T cells became activated $CD8^+$ T cells. In contrast, anti-PD1 antibodies in the same model depleted Treg cells and M2 macrophages[120], suggesting a synergistic role for the new ICI anti-CD73 that may improve the positive effects of anti-PD1 monotherapy. *Ex vivo* samples of blood and tumors from microsatellite instability CRC patients showed that atezolizumab alone could reactivate T cells.

Furthermore, adding tiragolumab, an anti-TIGIT antibody, restored intraepithelial CD4 T and CD8 T cell function by favoring interferon-γ and tumor necrosis factor-α production[120,121]. TIGIT is a receptor upregulated in natural killer and activated T cells when the modulation of their effector abilities is necessary for the microenvironment, such as cancer. It is also overexpressed in Treg cells [122]. The use of avelumab plus the adoptive transfer of autologous dendritic cell vaccine in chemotherapy-treated mismatch repair-proficient metastatic CRC patients had a successful result because this treatment was well tolerated. Furthermore, treatment was terminated early because 11% of patients were disease free at 6 mo, and progression-free survival was increased by 40%[123]. This evidence also suggests that new ICI research could open other possibilities for specific and beneficial

treatment for CRC patients because either immune or epithelial cells may express nonclassical ICI molecules (Figure 2).

CONCLUSION

Chemotherapy using 5-FU remains the primary treatment for CRC, despite its high toxicity and low efficacy. New strategies targeting ICIs have been useful in some oncological pathologies; however, evidence showing the effectiveness of classical ICI monotherapy in CRC is scarce. A combination of classical ICI antibodies targeting CTLA4 and PD1/PDL1 molecules showed stronger efficacy for CRC treatment. Finally, classical ICI plus conventional chemotherapy is effective, as evidenced by increased OS, but these strategies are not yet well established, and some clinical studies are ongoing. Evidence suggests that chemotherapy produces neoantigens, increasing tumor immunogenicity that may activate immune responses[124]. This increased immunogenicity is likely the reason for a better response when classical ICI plus chemotherapy is used and may represent a pathway to design new therapeutic strategies aimed at improving the response in CRC patients based on immunological reactivation combined with conventional chemotherapy. Knowledge of the TME in CRC is essential to understand immunosuppression. New options for nonclassical ICIs obtained by single-cell sequencing are shedding light on this area and will probably improve the effectiveness of many treatments.

We are possibly on the verge of major findings in the study of CRC, where the immune response will continue playing a leading role and where new proposals with nonclassical ICIs may reduce the current statistics and poor prognoses for this oncological pathology.

FOOTNOTES

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MINIREVIEWS

Serum biomarkers for the differentiation of autoimmune pancreatitis from pancreatic ductal adenocarcinoma

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Abstract

Autoimmune pancreatitis (AIP), a chronic inflammation caused by the immune system attacking the pancreas, usually presents imaging and clinical features that overlap with those of pancreatic ductal adenocarcinoma (PDAC). Serum biomarkers, substances that quantitatively change in sera during disease development, are a promising non-invasive tool with high utility for differentiating between these diseases. In this way, the presence of AIP is currently suspected when serum concentrations of immunoglobulin G4 (IgG4) antibody are elevated. However, this approach has some drawbacks. Notably, IgG4 antibody concentrations are also elevated in sera from some patients with PDAC. This review focuses on the most recent and relevant serum biomarkers proposed to differentiate between AIP and PDAC, evaluating the usefulness of immunoglobulins, autoantibodies, chemokines, and cytokines. The proposed serum biomarkers have proven useful, although most studies had a small sample size, did not examine their presence in patients with PDAC, or did not test them in humans. In addition, current evidence suggests that a single serum biomarker is unlikely to accurately differentiate these diseases and that a set of biomarkers will be needed to achieve adequate specificity and sensitivity, either alone or in

combination with clinical data and/or radiological images.

Key Words: Autoimmune pancreatitis; Pancreatic ductal adenocarcinoma; Serum; Biomarkers; Differentiation

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Core Tip: The imaging and clinical features of autoimmune pancreatitis commonly overlap with those of pancreatic ductal adenocarcinoma. This study reviews the most recent and relevant serum biomarkers proposed to differentiate between these diseases of the pancreas, including serum immunoglobulins, autoantibodies, chemokines, and cytokines, evaluating their usefulness for this purpose. One of the key conclusions is that a panel of various serum biomarkers appears to be necessary for an accurate differentiation between these diseases, either alone or in combination with clinical data and/or radiological images. Importantly, further research is warranted to assess the usefulness of these promising serum biomarkers in clinical practice

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare entity that represents 2%-10% of chronic pancreatitis (CP) cases [1]. Elevated serum concentrations of immunoglobulin (Ig), especially IgG4, have been observed in the majority of AIP patients[2], and Umehara *et al*[3] introduced the concept of IgG4-related disease in 2011, including AIP as one of these disorders. AIP can be classified into types 1 and 2, with more than 90% of cases corresponding to type 1[4]. Type 1 is associated with high serum IgG4 concentrations, unlike type 2[5]. Given the large proportion of cases that are type 1, type 1 AIP is referred to as AIP in this review.

The clinical and radiological characteristics of AIP can mimic those of pancreatic ductal adenocarcinoma (PDAC), leading to misdiagnosis and therapeutic errors that increase the morbidity and mortality of patients. This difficulty in differentiating AIP from PDAC has been well documented, with up to 15% of neoplasms being classified as AIP and up to 36% of AIP cases diagnosed as cancer. It should also be borne in mind that AIP, like other forms of CP, increases the risk of PDAC and therefore requires close follow-up[6]. The similarity between AIP and PDAC means that invasive methods must be applied, when possible, to establish the differential diagnosis using histological criteria.

The current diagnostic criteria for AIP, displayed in Table 1, were established in 2011 by consensus among international experts[7], who recognized that AIP has two different histopathological and clinical subtypes, types 1 and 2, as noted above.

Although serum IgG4 antibodies are used for the diagnosis of AIP, elevated IgG4 concentrations are not AIP-specific and are observed in other diseases, including PDAC[8]. In a study of 510 patients, Ghazale *et al*[9] observed increased serum IgG4 in around 10 % of PDAC cases, yielding false positives. In addition, not all patients with AIP have elevated serum IgG4 Levels, resulting in false negatives and an inadequate diagnostic accuracy[10]. Hence, this serological biomarker alone does not define the disease, and its usefulness is more limited in type 2 AIP.

Radiological criteria should also not be used alone, because they can lead to an erroneous differential diagnosis between AIP and PDAC[11]. Current recommendations require an exhaustive study to establish the diagnosis, including histological and morphological criteria and the response of patients to corticosteroid treatment[8]. Although not included in recommendations, clinical characteristics can also help the differential diagnosis of PDAC and the two AIP subtypes. Weight loss and anorexia are more frequently observed in PDAC, while other organs are more commonly involved in AIP[12]. Ultrasound endoscopy plays a key role in the diagnosis, allowing the morphology to be assessed and a core needle biopsy to be obtained before the proposal of a percutaneous biopsy or videolaparoscopy[13].

Hence, new serological biomarkers other than IgG4 antibodies are needed for the differential diagnosis in order to rule out malignancy and establish the appropriate treatment, avoiding unnecessary surgical resection and the erroneous treatment of patients. Some authors have increased the diagnostic potential of IgG4 by combining it with other serum biomarkers. Chang *et al*[14] increased the diagnostic accuracy to differentiate between AIP and PDAC by combining IgG4 and carbohydrate antigen 19-9 (CA 19-9) levels, with cutoff values of > 140 mg/dL and < 37 U/mL, respectively, obtaining

Table 1 Diagnostic criteria for autoimmune pancreatitis

Radiology	Serology	Histology	Response to steroid
Parenchyma:Diffuse enlargement with enhancement	IgG4 > 2 x upper limit of normal value	At least three	< 2 wk radiologically demonstrable resolution or marked improvement pancreatic/extrapancreatic manifestations
Duct: > 1/3 length of the main pancreatic duct		Periductal lymphoplasmacytic infiltrate without granulocytic infiltration	
Atypical: Segmental/focal narrowing with duct < 5 mm		Storiform fibrosis	
Other organ involvement: Bile duct: Segmental/multiple proximal or distal stricture		Obliterative phlebitis	
Retroperitoneal fibrosis		> 10 IgG4-positive cells/high power field	
Salivary/lachrymal glands: Symmetrically enlarged			
Renal involvement			

IgG4: Immunoglobulin G4.

a sensitivity of 64%, specificity of 92%, and diagnostic accuracy of 82 %. When the authors increased the cutoff to 280 mg/mL for IgG4 and 85 U/mL for CA 19-9, they reported a higher diagnostic accuracy of 86.9%. These results differ from those described by van Heerde *et al*[15], who considered less strict cutoff levels (1 g/L for IgG4 and 74 U/mL for CA 19-9) and obtained a sensitivity of 94 % and specificity of 100 %. These discrepancies highlight the need to study large samples of patients with homogeneous clinical characteristics to ensure the reproducibility of data on diagnostic accuracy. However, the search for new biomarkers is hampered by the fact that AIP is a rare entity, limiting sample sizes. In this review, we summarize and discuss the progress made in the search for new serum biomarkers for the diagnosis of AIP.

CLASSIC SEROLOGICAL MARKERS IN AIP AND PDAC

IgG4

IgG4 is the only serological biomarker currently included in diagnostic criteria for AIP, specifically type 1 AIP[7]. Values above 135-140 mg/dL were previously established as the cut-off point for the diagnosis, varying in sensitivity and specificity according to the population under study[16].

Absolute values are not taken into account in the diagnosis because of the interlaboratory variability in normal values, and patients are considered positive when their IgG4 concentrations are two-fold higher than the upper limit of normality[17]. European guidelines on IgG4 disease published in 2020 describe the IgG4 concentration has having diagnostic value when concentrations are four-fold higher than the upper limit of normality, which is only observed in a minority of patients[18]. Indeed, when jaundice secondary to a pancreatic mass is present, only a value 92-fold higher than the upper limit of normality is considered strongly suggestive of AIP[7].

Besides its elevation in PDAC patients[8,19], the usefulness of this serum biomarker is also reduced by its lack of specificity and sensitivity to differentiate between primary sclerosing cholangitis and cholangiocarcinoma, which can increase the false positive rate by up to 40%[20]. Serum IgG4 is also not useful for the diagnosis of type 2 AIP associated with inflammatory bowel disease[16] and must be accompanied at least by suggestive radiological findings to have diagnostic value in AIP[21]. Finally, normal IgG4 concentrations have been reported in up to 20% of AIP patients, even in the active phase [22].

CA 19-9

CA 19-9 is a tumor marker that is detectable in serum and widely used in the clinical management of PDAC[23]. CA 19-9 is elevated in the majority of PDAC patients and is useful for monitoring purposes; however, this biomarker is not useful for the early diagnosis of PDAC detection because of the substantial number of false positives and negatives[24].

Furthermore, CA 19-9 is commonly elevated in AIP patients, almost 40% of whom have concentrations above 100 U/mL[15]. In this way, individual measurements of either CA 19-9 or IgG4 are unable to distinguish AIP from PDAC[25].

The numerous limitations of CA 19-9 include the influence on its concentrations of the presence of jaundice and cholangitis, among many other factors. Nevertheless, it is widely used because it is accessible and cheap, and the sensitivity and specificity can be improved by its combination with other clinical, serological, histological and/or morphological criteria[26].

Hence, the combination of various serological biomarkers appears necessary to distinguish between AIP and PDAC. In this line, Yan *et al*[27] proposed combining CA 19-9 with globulin, eosinophils, and hemoglobin for the differential diagnosis. Elevated concentrations of eosinophils and globulin together with reduced concentrations of Hb and CA 19-9 were found to identify patients with AIP with a sensitivity of 92% and specificity of 79%, a relatively high diagnostic value.

NOVEL SERUM BIOMARKERS PROPOSED FOR THE DIFFERENTIATION OF AIP FROM PDAC

Igs

IgG1 and IgG2 have been studied in relation to AIP. IgG1 has been proposed as a diagnostic marker for AIP and IgG4-associated disease due to its involvement in the immunogenesis of the disease[28]. However, IgG2 concentrations were lowest in AIP and highest in IgG4-associated disease with orbital involvement[29].

There are different glycoforms of IgG subclasses, and different patterns of glycosylation have been described between patients with AIP and PDAC. Quantitative analysis of the IgG glycosylation profile may therefore allow the differential diagnosis between these entities to be established with high precision[30].

In addition, the proportion of different types of Igs has also shown some promise as a biomarker. An increase in IgG and inversely proportional reduction in IgA and IgM have been reported in AIP and IgG4-associated disease. In addition, elevated IgE has been described as having diagnostic and prognostic value for disease relapse in both entities[31].

Autoantibodies

Anti-annexin A11, anti-laminin 511-E8, and anti-galectin-3 autoantibodies have been implicated in the pathogenesis of AIP over recent years. Hubers *et al*[32], proposed annexin 11, a calcium-dependent phospholipid-binding protein, as an autoantigen in AIP. They showed that annexin A11-specific IgG4 competitively inhibited the pathogenic binding of annexin A11-specific IgG1 to shared epitopes, suggesting that the IgG1-mediated pro-inflammatory response could be downregulated by IgG4. Laminin 511-E8 is a truncated form of laminin 511, which is part of the extracellular matrix of the pancreas. Shiokawa *et al*[33] detected laminin 511-E8 in 51% of AIP patients (n = 51) compared with 1.6% of controls (n = 122) and suggested that it is an autoantigen in this disease. Galectin-3, which has been associated with fibrotic disorders, has also been proposed as a candidate biomarker[34]. In addition, anti-trypsinogen autoantibodies have been observed in sera from AIP patients and related to the loss of acinar cells[35].

Autoantibodies to amylase-2A and heat-shock protein 10 (HSP10) were previously found to be present not only in AIP but also in fulminant type 1 diabetes. Amylase-2A autoantibodies have not been detected in toxic CP or PDAC, while anti-HSP10 antibodies have been reported in a small percentage of patients[36,37].

Anti-plasminogen-binding protein autoantibodies have been observed in almost 95% of AIP patients (n = 35). Interestingly, these antibodies were presented by IgG4-negative patients with AIP but not by IgG4-positive patients with type 2 AIP[38]. Anti-pancreatic secretory trypsin inhibitor has also been suggested as a potential AIP-specific antibody, although it was detected in serum from less than half of AIP patients[39].

Other proposed autoantibodies have been those against carbonic anhydrase II, but they are not AIP-specific and are observed at high levels in other disorders such as Sjögren's syndrome[40]. In the same way, high concentrations of anti-lactoferrin antibodies have been described in immune diseases other than AIP such as ulcerative colitis[41], and anti-prohibitin antibodies are detectable not only in AIP patients (73.5%, n = 34) but also in patients with Mikulicz's disease (53%, n = 15%) or retroperitoneal fibrosis (54%, n = 11)[42].

Felix *et al*[43], studied the spectrum of autoantibodies in patients with AIP (n = 14 with type 1 and 11 with type 2) or PDAC (n = 26) and healthy controls (n = 22), showing elevated titers of both novel and previously reported antibodies against a variety of autoantigens, including carboxypeptidase A1 precursor, procarboxypeptidase A2, trypsin-1-preproprotein, and vimentin, among others. The authors found 68 autoantigens in AIP, 26 in PDAC and 21 in both diseases. The researchers selected 13 autoantibodies with potential to discriminate between the two types of AIP and also proposed antitransaldolase antibody as a biomarker to differentiate between type 2 AIP and PDAC.

Chemokines and cytokines

The Th2 immune response is a prominent feature of AIP, and some Th2 chemokines might therefore be useful as AIP biomarkers. Increased serum concentrations of C-C Motif Chemokine Ligand 17 have been reported in patients with IgG4-related disease, but this biomarker has not been explored in AIP [44].

Increased expressions of C-X-C motif chemokine ligand (CXCL) 9 and CXCL10 were recently demonstrated in an experimental model of AIP, but data on their concentrations in patients are not yet available[45].

Th2 cytokines have also been proposed as AIP biomarkers. Thus, interleukin (IL)-5 was found to be upregulated in patients with IgG4-related sclerosing cholangitis and suggested as a biomarker of AIP [46].

Serum concentrations of interferon (IFN)-alpha and IL-33 were found to be higher in patients with AIP than in those with chronic alcoholic pancreatitis or healthy controls. These concentrations were positively correlated with the serum concentrations of IgG4 antibodies. In addition, the observation of decreased IFN-alpha and IL-33 after treatment with corticosteroids, unlike IgG4 concentrations, suggests that they may be useful for following the response to treatment[47].

In a very interesting study, Ghassem-Zadeh *et al*[48] investigated the serum cytokine profile of patients with AIP (n = 29), CP (n = 17), and PDAC (n = 27) and its capacity to discriminate AIP from the other conditions. The authors found that serum levels of IL-1 beta, IL-7, IL-13, and granulocyte colony-stimulating factor (G-CSF) were higher in patients with AIP *vs* PDAC. The best diagnostic utility to differentiate AIP from PDAC was shown by IL-7 alone [area under the curve (AUC) = 0.780], obtaining a marginal added value when it was combined with G-CSF (AUC = 0.782). In the same line, G-CSF alone evidenced a better capacity to identify patients with CP from those with AIP (AUC = 0.804). In addition, the expression of tumor necrosis factor was found to be higher in PDAC tissue lysates than in either type of AIP.

Other cytokines suggested as potential AIP biomarkers include B cell-activating factor and proliferation-inducing ligand, which were found to be higher in patients with AIP than in healthy controls [49]. A decrease in these cytokines has also been observed after treatment with corticosteroids[45].

CONCLUSION

AIP and PDAC often course with similar symptoms, and biomarkers that can differentiate between them are needed for early initiation of the appropriate clinical action protocol. If this milestone is reached, it will be possible to avoid pancreatic resection in patients with AIP and incorrect steroid treatment in patients with PDAC.

Serum markers may be useful in patients with the presence of compatible symptoms and radiological findings, which have a low positive predictive value. Thus, some symptoms, such as abdominal pain and diabetes, may be present in both entities.

In addition, radiological criteria for suspicion of AIP are frequently not all present to establish a given diagnosis. Given the improved safety and performance of histological sampling of the pancreas by endosonography, this procedure should be added in cases of diagnostic doubt. However, the absence of malignancy does not definitively rule out neoplasia and, in the absence of histological criteria for a definitive AIP diagnosis, active suspicion of neoplasia should be maintained. In this context, the combination of serum biomarkers with all these tests can have a high qualitative and quantitative value to achieve a reliable diagnosis in these patients. This review describes serological biomarkers proposed for this purpose.

Increased serum concentrations of IgG4 antibody are a feature of AIP, but there are two main drawbacks to its usefulness as optimal AIP biomarker: (1) It is elevated in PDAC patients; and (2) It is not increased in a fraction of AIP patients. These problems have been addressed by numerous studies of new biomarkers for AIP diagnostics. These include biomarkers related to AIP immunopathogenesis, such as certain cytokines and chemokines, which have shown usefulness in research involving a small number of patients, although most studies did not examine the presence of these biomarkers in patients with PDAC. Some potential biomarkers have also been identified in experimental models but need to be tested in humans.

Another aspect to highlight is that, given the nature of these diseases, the use of a single serum biomarker is unlikely to accurately differentiate between AIP and PDAC. As observed in this study, almost all authors propose the utilization of a set of biomarkers to achieve high specificity and sensitivity for their reliable differentiation.

Translational application in this field will be achieved over the medium term, but further research is required on the numerous biomarkers proposed to date, recruiting larger samples of patients with AIP and assessing their presence in patients with PDAC. This is needed to verify their true specificity and to analyze their possible application in combination with clinical symptoms and/or radiological tests to achieve accurate differentiation between AIP and PDAC.

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FOOTNOTES

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MINIREVIEWS

Evaluation of polygenic risk score for risk prediction of gastric cancer

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Abstract

Genetic variations are associated with individual susceptibility to gastric cancer. Recently, polygenic risk score (PRS) models have been established based on genetic variants to predict the risk of gastric cancer. To assess the accuracy of current PRS models in the risk prediction, a systematic review was conducted. A total of eight eligible studies consisted of 544842 participants were included for evaluation of the performance of PRS models. The overall accuracy was moderate with Area under the curve values ranging from 0.5600 to 0.7823. Incorporation of epidemiological factors or *Helicobacter pylori* (*H. pylori*) status increased the accuracy for risk prediction, while selection of single nucleotide polymorphism (SNP) and number of SNPs appeared to have little impact on the model performance. To further improve the accuracy of PRS models for risk prediction of gastric cancer, we summarized the association between gastric cancer risk and *H. pylori* genomic variations, cancer associated bacteria members in the gastric microbiome, discussed the potentials for performance improvement of PRS models with these microbial factors. Future studies on comprehensive PRS models established with human SNPs, epidemiological factors and microbial factors are indicated.

Key Words: Polygenic risk scores; Gastric cancer; *Helicobacter pylori*; Gastric microbiome

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Core Tip: A systematic review was conducted to evaluate current polygenic risk score (PRS) models in gastric cancer risk prediction. Our study showed that PRS models had the potential to predict the risk of gastric cancer with a moderate accuracy. The prediction models' performance could be improved after incorporating epidemiological factors or *Helicobacter pylori* (*H. pylori*) status. The potential of *H. pylori* genomic variations and members of the gastric microbiome were discussed as candidates for gastric cancer prediction models.

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INTRODUCTION

Gastric cancer (GC) is the fourth most commonly diagnosed type of cancer worldwide and the second leading cause of cancer-related death[1]. According to the latest cancer statistics, there were approximately 26380 new gastric cancer cases and 11090 deaths in the United States in 2022[2]. The occurrence of gastric cancer results from a combination of risk factors, including host genetic factors and *Helicobacter pylori* (*H. pylori*) infection[3,4]. Genetic variations play an important role in the occurrence and progression of gastric cancer[5,6]. Genome-wide association studies have identified many single nucleotide polymorphisms (SNPs) in the human genome that are involved in the development of gastric cancer. *H. pylori* infection affects approximately half of the world's population. The pathogen is considered a definite carcinogen of gastric cancer[7]. Epidemiological studies have revealed that age, sex, alcohol consumption and smoking are risk factors for gastric cancer[8].

To prevent the development of gastric cancer, it is important to identify individuals at high risk for cancer and apply intervention measures to impede the progression of the disease. Many studies have been conducted to explore the performance of biomarkers or models established with risk factors for predicting gastric cancer risk. Models based on epidemiological factors, including age, sex and *H. pylori* infection, have adequate performance in the prediction of gastric cancer risk[9,10]. Genetic variations of *H. pylori* show great potential for use in the prediction of gastric cancer risk[11]. Cancer-associated SNPs have been reported to be valuable in stratifying gastric cancer risk based on the genetic background[12,13].

Polygenic risk score (PRS) models are established with a number of SNPs or genetic variants to explore the combined effect of multiple genetic variations in the risk prediction of disease[14]. They show improved performance in the prediction of the risk of breast, prostate, and colorectal cancer and diseases involving multiple genetic factors[15-17]. Calculations of PRS vary among different models. The simplest way to calculate the PRS is summing the number of all risk alleles[18]. Considering variations in the cancer risk associated with different SNPs, each risk allele is weighted by its odds ratio (OR) value for cancer. PRS is then calculated as a sum of weighted risk alleles[14]. To date, studies have been conducted employing PRS models to predict gastric cancer risk. Different sets of cancer-associated SNPs have been used in the PRS models. In certain studies, epidemiological factors have been included in the establishment of the models. To assess the performance of PRS models in the prediction of gastric cancer risk, this article aimed to comprehensively analyze the accuracy of PRS models for risk prediction through a systematic review of related studies and discuss potentials in the performance improvement of PRS models with the inclusion of *H. pylori* genetic variations and bacterial members of the gastric microbiome for use in the future.

PERFORMANCE OF CURRENT PRS MODELS

To assess the performance of PRS models for the prediction of gastric cancer risk, a systematic review was conducted. The details of the methods for the systematic review can be found in Supplementary material. The PRISMA flow chart (Figure 1) shows the article retrieval and filtering process. A total of 165 articles were retrieved from PubMed, Web of Science, and Embase electronic database using the search strategies in the Methods (Supplementary material). After removing duplicates, a total of 96 articles were further screened. According to the topics and abstracts, 37 articles were selected and analysed for eligibility. Of them, 8 studies were eligible and included in our systematic review in accordance with the inclusion and exclusion criteria[19-26]. The reasons for exclusion are shown in the PRISMA flow diagram.

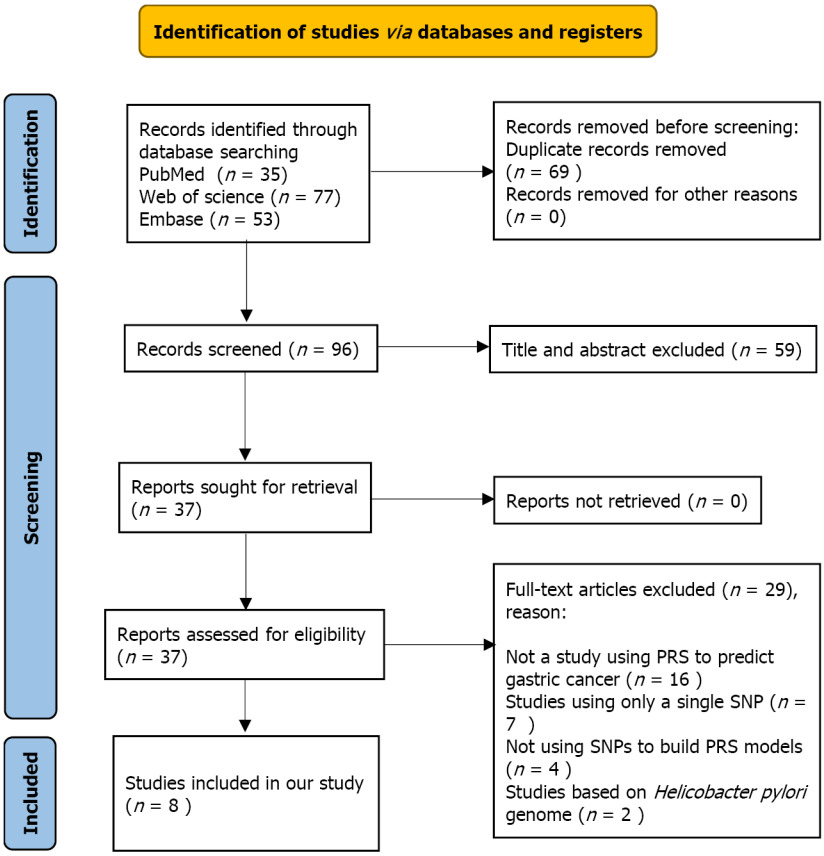

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Figure 1 PRISMA flow diagram. PRISMA flow diagram showed the process of the study selection. SNP: Single nucleotide polymorphism; PRS: Polygenic risk score.

The characteristics of the included studies are shown in Table 1. All of the studies were published in the last five years. Six of them were case-control studies, and two were cohort studies. The study areas included China (5), Korea (1), Japan (1) and Europe (1). The sample sizes in the included studies ranged from 1088 to 400807. A total of 544842 participants were included. All the studies established a PRS model to predict the risk of gastric cancer.

Details regarding the establishment and evaluation of PRS models in these studies are described in Table 2. The accuracy of the PRS models was assessed with the Area under the curve (AUC) in five included studies. The performance of the models was moderate, with AUC values ranging from 0.5600 to 0.7823. The highest performance has been reported by Ishikura *et al*[25] from Japan, with an AUC of 0.7677 in the training set and 0.7823 in the validation set. Two studies from China have used ORs to evaluate the performance of PRS models[19,23]. OR values for the highest quartile with respect to the lowest are 1.14 and 1.19, indicating that the performance of the models was unsatisfactory. The hazard ratio (HR) was used in the remaining study with a value of 2.08 for the highest quantile with respect to the lowest. Overall, the performance of the current PRS models varies considerably among the included studies and appears to have a moderate predictive power for gastric cancer. Factors affecting the performance are indicated.

Pearson correlation analysis of sample sizes and AUC values demonstrated that there was no significant correlation between them (r = -0.51, P = 0.380). This result suggested that the variations in the sample size of the included studies had minimal influence on the predictive power of the PRS model. The number of genetic variants in the models ranged from 3 to 112 SNPs. Pearson correlation analyses were performed to explore whether the number of SNPs was related to the predictive power of the model. The results showed that there was no significant correlation between the number of SNPs and AUC (r = 0.85, P = 0.067). Our results are consistent with previous systematic reviews of breast cancer [27]. This suggests that the inclusion of more SNPs in the PRS model would not improve the performance in the prediction of gastric cancer risk.

All of the included studies used the weighted PRS method instead of the simple counting method. The weight of risk alleles is crucial for the performance of the PRS models[14,28]. Of note, the same SNP, such as rs2294008, has been used in different models, but the weights varied greatly (Table 1). Accordingly, the predictive power varied among studies (Table 2). Generally, the weight of a risk allele derives from the OR of risk alleles for the development of gastric cancer. In the included studies, the ORs mostly came directly from the results of case-control comparisons. They have not been, however,

Table 1 Main characteristics of the included studies

Ref.	Population	Design	Group	Sample size	Sex, (%)	Age, (%)	SNPs (RA, OR)
Mao *et al* [19], 2017	Chinese	Case-control	GC, HC	2631, 4373	Male: 5100 (72.8%); female: 1904 (27.2%)	< 60 yr 3299 (52.9%); ≥ 60 yr 3705 (47.1%)	rs1514175 (A, 1.01), rs2815752 (A, 1.07), rs574367 (T, 1.11), rs12463617 (C, 1.05), rs1861411 (A, 1.02), rs6545814 (G, 1.05), rs10513801 (T, 1.17), rs2535633 (G, 0.98), rs16858082 (T, 0.96), rs261967 (C, 1.02), rs888789 (A, 0.99), rs6890814 (C, 0.99), rs4713766 (A, 1.05), rs9356744 (T, 1.03), rs9473924 (T, 0.98), rs17150703 (G, 1), rs4735692 (A, 1.02), rs11142387 (C, 1.06), rs1211166 (A, 1.02), rs11191580 (C, 0.92), rs10160804 (A, 0.99), rs11030104 (A, 1.02), rs11604680 (G, 0.97), rs2237892 (T, 1), rs671 (G, 1.12), rs897057 (C, 1.04), rs7989336 (A, 1.03), rs9568867 (A, 1.03), rs4776970 (A, 1.05), rs1558902 (A, 1.04), rs2531995 (T, 1.05), rs4788102 (A, 1.08), rs7503807 (A, 1), rs9299 (T, 0.9), rs591166 (A, 1.08), rs11671664 (G, 0.97), rs3810291 (A, 1.02)
Choi *et al*[20], 2020	European	Cohort	GC	272 cases in 400807 individuals	Male: 186372 (46.5%); female: 214435 (53.5%)	NR	rs2990223 (G, 1.27), rs10036575 (T, 1.23), rs2294008 (T, 1.21)
Jin *et al* [21], 2020	Chinese	Cohort	GC	Training set: 10254 cases and 10914 controls; validation set: 692 cases in 100220 individuals	Training set: NR; validation set: Male: 42862 (42.8%); female: 57358 (57.2%)	Training set: NR; validation set: < 60 yr 69805 (69.7%); ≥ 60 yr 30415 (30.3%); mean in case: 60.82 ± 9.33; mean in controls: 53.64 ± 11.00	NR
Qiu *et al* [22], 2020	Chinese	Case-control	GC, HC	1115, 1172	Male: 1615 (70.6%); female: 672 (29.4%)	< 60 yr 1162 (50.8%); ≥ 60 yr 1125 (49.2%)	rs13361707 (C, 1.47), rs2294008 (T, 1.19), rs4072037 (T, 1.38), rs3762272 (T, 1.21), rs2274223 (G, 1.35), rs80142782 (T, 1.36)
Wang *et al*[23], 2020	Chinese	Case-control	GC, HC	2631, 4373	Male: 5100 (72.8%); female: 1904 (27.2%)	< 60 yr 3299 (52.9%); ≥ 60 yr 3705 (47.1%)	rs1801133 (A, 1.02), rs2275565 (G, 1.01), rs4660306 (T, 1), rs1047891 (A, 1), rs9369898 (A, 1), rs548987 (C, 0.98), rs42648 (G, 1.01), rs1801222 (A, 0.99), rs12780845 (A, 1.01), rs7130284 (C, 1.01), rs2251468 (C, 1.03), rs154657 (A, 1.01), rs12921383 (C, 1.01), rs838133 (A, 1.02), rs234709 (C, 0.99)
Duan *et al*[24], 2021	Chinese	Case-control	GC, HC	544, 544	Male: 825 (75.8%); female: 263 (24.2%)	Mean in case: 57.80 ± 12.06; mean in controls: 57.02 ± 11.97	rs1859168 (C, 1.09), rs3815254 (A, 0.98), rs4784659 (C, 0.55), rs579501 (A, 0.71), rs77628730 (A, 1.26), rs6989575 (C, 1.03), rs7816475 (A, 1.19), rs6470502 (T, 0.51), rs1518338 (C, 1.08), rs2867837 (G, 0.95), rs12494960 (A, 2.62), rs74798803 (T, 0.97), rs7818137 (T, 1.2), rs550894 (T, 1.13), rs3825071 (A, 1.48), rs580933 (G, 0.98), rs7943779 (A, 1.54), rs911157 (T, 1.74), rs16981280 (C, 0.76), rs2273534 (C, 0.92), rs957313 (T, 1.04)
Ishikura *et al*[25], 2021	Japanese	Case-control	GC, HC	Training set: 696 cases and 1392 controls; validation set: 795 cases and 795 controls	Training set: Male: 1560 (74.7%); female: 528 (25.3%); validation set: Male: 1180 (74.2%); female: 410 (25.8%)	Training set: < 60 yr 1034 (49.5%); ≥ 60 yr 1054 (50.5%); validation set: < 60 yr 621 (39.1%); ≥ 60 yr 969 (60.9%)	rs4072037 (G, 1.35), rs2294008 (T, 0.62), rs7849280 (G, 0.24)
Park *et al* [26], 2021	Korean	Case-control	GC, HC	450, 1136	Male: 836 (52.7%); female: 750 (47.3%)	Mean in case: 55.4 ± 10.7; mean in control: 52.1 ± 8.5	rs2294008 (T, 1.2), rs6656150 (C, 0.8), rs8280142782 (C, 0.6), rs760077 (A, 0.8), rs140081212 (A, 0.8), rs4460629 (T, 0.8)

SNP: Single nucleotide polymorphism included in studies; GC: Gastric cancer group; HC: Healthy controls; NR: Not reported/Not retrieved; RA: Allele associated with gastric cancer; OR: Odds ratio.

confirmed in a validation set. This might account for the different weights that have been used among studies. It appears that a validation of the OR is required to eliminate the bias of weights among studies, improving the consistency in the performance of PRS models.

To improve the accuracy of the PRS model in the prediction of cancer risk, certain epidemiological factors implicated in cancer development have been considered[29,30]. A number of epidemiological factors are associated with the occurrence of gastric cancer. Individuals of male sex and older age are at increased risk for gastric cancer[9,31]. Previous studies have revealed environmental factors in gastric cancer development. A family history has been considered to be significantly associated with the

Table 2 Development and evaluation of Polygenetic risk scores for predicting gastric cancer

Ref.	No. of SNPs included	SNP selection	PRS and related methods used to calculate it	AUC or OR (95%CI) of model with PRS	AUC or OR (95%CI) of model with PRS and Clinical risk factors	Difference	Clinical risk factors included
Mao *et al* [19], 2017	37	Significance level and linkage disequilibrium	Weighted PRS using weights derived from the same study	OR for the highest quartile respect to the lower quartile: 1.14 (1.01-1.29)	-	-	-
Choi *et al* [20], 2020	3	Significance level	Weighted PRS using weights derived from literature	AUC: 0.56 (0.53- 0.60); HR for the highest quintiles respect to the lower quintiles: 1.75 (1.18-2.59)	-	-	-
Jin *et al* [21], 2020	112	Significance level	Weighted PRS using weights derived from the same study	HR for the highest quintiles respect to the lower quintiles: 2.08 (1.61-2.69)	HR for participants with a high genetic risk and an unfavorable lifestyle respect to those with a low genetic risk and a favorable lifestyle 5.14 (2.04–12.93)	-	Smoking, alcohol consumption, consumption of preserved foods, intake of fresh fruit and vegetables
Qiu *et al* [22], 2020	6	Significance level and validated to be associated with gastric cancer risk	Weighted PRS using weights derived from the same study	AUC 0.653	AUC 0.684	0.031	BMI
Wang *et al*[23], 2020	15	Significance level	Weighted PRS using weights derived from literature	OR for the highest quartile respect to the lower quartile: 1.19 (1.04–1.37)	-	-	-
Duan *et al*[24], 2021	21	Prediction functions through bioinformatics tools	Weighted PRS using weights derived from the same study	AUC 0.737 (0.71-0.76); OR for the highest 10% respect to the 40-60%: 5.75 (3.09-10.70)	AUC for PRS + *Hp* infection: 0.752 (0.690-0.814); AUC for PRS + family history of tumor: 0.773 (0.702-0.843)	PRS + *Hp* infection: 0.014; PRS + family history of tumor: 0.036	*Hp* infection, family history, smoking, alcohol consumption
Ishikura *et al*[25], 2021	3	Significance level	Weighted PRS using weights derived from the same study	AUC for training set: 0.6287 (0.6039–0.6530); AUC for validation set: 0.5673 (0.5391–0.5960)	AUC for training set: 0.7677 (0.7465–0.7890); AUC for validation set: 0.7823 (0.7694–0.8140)	Training set: 0.139; validation set: 0.215	Smoking, alcohol consumption, fruit and vegetable intake, and ABCD classification
Park *et al* [26], 2021	6	Significance level	Weighted PRS using weights derived from literature	AUC: 0.565 (0.535–0.596); OR for the highest tertile respect to the lower tertile: 2.03 (1.51–2.72)	AUC: 0.607 (0.576–0.638); OR for the highest tertile respect to the lower tertile: 2.53 (1.92–3.34)	0.042	A sex-specific prediction model

SNP: Single nucleotide polymorphism; PRS: Polygenic risk score; AUC: Area under the curve; OR: Odds ratio; HR: Hazard ratio; CI: Confidence interval; BMI: Body mass index; *Hp*: *Helicobacter pylori*; ABCD classification: *Helicobacter pylori* infection-related factor.

occurrence of gastric cancer, with OR of more than 2[32]. Studies have revealed a close association between alcohol consumption and the risk of gastric cancer. A meta-analysis showed that heavy alcohol consumption increases the risk of gastric cancer[33,34]. Smokers have been reported to be at high risk of gastric cancer[35]. In this study, epidemiological factors were included in five studies in addition to genetic variations (Table 2). After taking into account the epidemiological factors, the AUC achieved using each model increased by 0.014 to 0.215. To explore whether the predictive performance of the PRS model was improved after epidemiological factors were included, the Mann-Whitney test was performed. The results showed that the AUC values were significantly increased from 0.56-0.74 to 0.61-0.78 after epidemiological factors were considered (P = 0.047).

H. pylori is a major cause of gastric cancer. The risk of non-cardia gastric cancer in *H. pylori*-infected individuals is 6 times higher than that in uninfected individuals[36]. Only one of the included studies

took *H. pylori* into account in the establishment of the PRS model. The performance of the model for the prediction of gastric cancer risk increased with an AUC value increasing from 0.737 to 0.752. *H. pylori* infection serves as a biomarker for gastric cancer and has been combined with other epidemiological factors to predict gastric cancer risk. Tan *et al*[37] found that *H. pylori* infection alone had moderate power for predicting gastric cancer risk with an AUC of 0.66. The accuracy of prediction was improved after other clinical factors were incorporated. A study consisting of 14929 participants demonstrated that *H. pylori* infection combined with seven epidemiological factors has a high predictive power with an AUC value of 0.76[38]. These findings suggest that incorporating *H. pylori* status into PRS models boosts the predictive power for gastric cancer risk.

In many types of cancer, PRS models have shown great power for risk prediction[39,40]. Our analyses demonstrated that the performance of current PRS models is promising in predicting the risk of gastric cancer. Nonetheless, the predictive power is not as satisfying as expected. Inclusion of epidemiological factors and *H. pylori* infections likely enhances the performance of the PRS model for the prediction of gastric cancer risk.

RISK PREDICATION WITH MICROBIAL FACTORS

In the reported PRS models, the risk of gastric cancer has been predicted mainly based on the genetic susceptibility resulting from genetic variations and common cancer risk factors, including age, sex, smoking status, and alcohol consumption. Previous studies have reported serum pepsinogen status could reflect the extent of atrophic change in gastric mucosa[41]. The combination of serum pepsinogen status and *H. pylori* status serves as a valuable marker for stratifying the risk of gastric cancer[42]. Individuals with decrease status of pepsinogen and *H. pylori* infection had a higher risk of gastric cancer compared with healthy control, with a HR value of 6.0[43]. Furthermore, recent studies have demonstrated that many microbial factors play roles in gastric carcinogenesis. Infection with *H. pylori* causes gastric cancer in only a minority of individuals[44]. Genetic differences between strains of *H. pylori* account in part for the differential outcomes of the infection among individuals[45]. The dysbiotic gastric microbiome plays an important role in the development of gastric cancer[46,47]. In addition, studies have shown that other bacteria may play an important role in promoting cancer, following the structural imbalance of the stomach microbiome induced by *H. pylori*[48,49]. As we mentioned, multiple studies have reported other bacteria that are associated with gastric cancer[50,51]. We believe that the gastric microbiome can be used as a valuable candidate to establish a prediction model for the occurrence of gastric cancer.

Gastric cancer-associated SNPs of H. pylori

The genome of *H. pylori* is substantially diverse[45]. There is a high level of differences in the gene contents, deletion/insertion, genetic inversion, sequence variations and SNPs[52]. Genetic variations in virulence genes, including *cagA*, *vacA*, and *babA*, are closely associated with gastric cancer risk[53,54]. Genome-wide association studies have identified a number of gastric cancer-associated SNPs in the *H. pylori* genome[55,56]. These cancer-associated genetic variations of *H. pylori* can be used in the risk prediction of gastric cancer. During the process of screening the studies (Figure 1), we observed that studies used gastric cancer-associated SNPs of the *H. pylori* genome to predict gastric cancer risk. Using a model comprising six validated loci in the *cag* pathogenicity island, a study on 1220 subjects demonstrated a sound predictive power for gastric cancer with an AUC of 0.65[57]. Berthenet *et al*[55] generated a risk score model with 12 gastric cancer-associated SNPs identified by a GWAS study of *H. pylori*. The results of this study have shown that the model is capable of predicting gastric cancer risk. A recent report established a PRS model with gastric cancer-associated SNPs selected from previous studies[11]. The model based on *H. pylori* SNPs achieved good predictive performance. These results convincingly support that the incorporation of *H. pylori* genomic variations into current PRS models would considerably enhance the accuracy in the prediction of gastric cancer risk.

Cancer-associated bacteria in the gastric microbiome

Dysbiosis of the gastric microbiome promotes the development of gastric cancer[46,47]. Many bacteria in the gastric microbiome possess carcinogenic potential[50,51,58].

An observational study of 1043 patients demonstrated a significant enrichment of *Streptococcus anginosus* (*S. anginosus*) and *Streptococcus constellatus* (*S. constellatus*) in gastric cancer[58,59]. The abundances of *S. anginosus* and *S. constellatus* serve as novel faecal signatures of early gastric cancer. Coker *et al* demonstrated an association between *S. anginosus*, *Peptostreptococcus stomatis*, *Parvimonas micra*, *Slackia exigua*, *Dialister pneumosintes* and gastric cancer[60]. These bacteria could form a synergistic network, leading to additional contributions to the disease. They could be used as potential tissue markers for gastric cancer with AUC values of 0.82 and 0.81 in the discovery and validation cohorts, respectively. Png *et al*[61] conducted a cohort study involving 43 participants to identify potential carcinogenic bacteria. The study demonstrates that the *Moryella* genus, *Vibro* genus, *Comamonadaceae* family, *Paludibacter* genus, *Agrobacterium* genus, and *Clostridiales* order in the gastric microbiome are

associated with gastric cancer. The model containing analyses of these bacteria is capable of predicting early gastric cancer with an AUC of 0.82. It has been shown that a random forest model generated with bacterial members of the gastric microbiome has a high performance in risk prediction[62,63]. These findings collectively support that bacterial members of the gastric microbiome have potential in the risk stratification of gastric cancer. Despite the requirement of further validation, the inclusion of the analysis of these bacteria in PRS models most likely enhances the accuracy in the prediction of gastric cancer.

CONCLUSION

Our systematic review showed that PRS models have great potential in the prediction of gastric cancer. Incorporation of other risk factors for gastric cancer could increase the accuracy of the models. To further increase the predictive performance of PRS models for gastric cancer, a comprehensive PRS model generated with the analysis of epidemiological risk factors, genetic variations of *H. pylori*, and bacterial members of the gastric microbiome in addition to human genetic variations requires further evaluation. PRS models with high accuracy would benefit the development of individual risk scores, facilitating the prevention of gastric cancer.

FOOTNOTES

Author contributions: Wang XY and Liang SZ collected sequencing data; Wang LL and Xu L analyzed the data; Wang XY, Yu MC, and Zhang QY wrote the manuscript; Dong QJ designed the research and supervised the manuscript; all authors reviewed the manuscript and approved the final version of the manuscript.

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ORIGINAL ARTICLE

Basic Study

Cancerous inhibitor of protein phosphatase 2A enhances chemoresistance of gastric cancer cells to oxaliplatin

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Abstract

BACKGROUND

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a newly discovered oncogene. It is an active cell proliferation regulatory factor that inhibits tumor apoptosis in gastric cancer (GC) cells. CIP2A is functionally related to chemoresistance in various types of tumors according to recent studies. The underlying mechanism, however, is unknown. Further, the primary treatment regimen for GC is oxaliplatin-based chemotherapy. Nonetheless, it often fails due to chemoresistance of GC cells to oxaliplatin.

AIM

The goal of this study was to examine CIP2A expression and its association with oxaliplatin resistance in human GC cells.

METHODS

Immunohistochemistry was used to examine CIP2A expression in GC tissues and adjacent normal tissues. CIP2A expression in GC cell lines was reduced using small interfering RNA. After confirming the silencing efficiency, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium and flow cytometry assays were used to evaluate cell proliferation and apoptosis caused by oxaliplatin treatment. Further, the key genes and protein changes were verified using real-time quantitative reverse transcription PCR and Western blotting, respectively, before and after intervention. For bioinformatics analysis, we used the R software and Bioconductor project. For statistical analysis, we used GraphPad Prism 6.0 and the Statistical Package for the Social Sciences software version 20.0 (IBM,

Armonk, United States).

RESULTS

A high level of CIP2A expression was associated with tumor size, T stage, lymph node metastasis, Tumor Node Metastasis stage, and a poor prognosis. Further, CIP2A expression was higher in GC cells than in normal human gastric epithelial cells. Using small interfering RNA against CIP2A, we discovered that CIP2A knockdown inhibited cell proliferation and significantly increased GC cell sensitivity to oxaliplatin. Moreover, CIP2A knockdown enhanced oxaliplatin-induced apoptosis in GC cells. Hence, high CIP2A levels in GC may be a factor in chemoresistance to oxaliplatin. In human GC cells, CIP2A regulated protein kinase B phosphorylation, and chemical inhibition of the protein kinase B signaling pathway was significantly associated with increased sensitivity to oxaliplatin. Therefore, the protein kinase B signaling pathway was correlated with CIP2A-enhanced chemoresistance of human GC cells to oxaliplatin.

CONCLUSION

CIP2A expression could be a novel therapeutic strategy for chemoresistance in GC.

Key Words: Cancerous inhibitor of protein phosphatase 2A; Gastric cancer; Oxaliplatin; Chemoresistance; Akt

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Core Tip: Gastric cancer (GC) is primarily treated with oxaliplatin-based chemotherapy. Patients who receive chemotherapy often develop resistance. Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a novel oncogene. Recent studies suggested that CIP2A is linked to chemoresistance in various cancers. The purpose of this study was to look into the relationship between CIP2A expression and oxaliplatin resistance in GC. The findings revealed that GC tissues have higher CIP2A expression than matched adjacent normal gastric tissues, and CIP2A expression plays an important role in the chemoresistance of GC, suggesting a new treatment strategy for GC.

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INTRODUCTION

Gastric cancer (GC) is the most common solid tumor originating from the digestive system and is one of the most severe and fatal malignancies worldwide[1]. GC has a high mortality rate of 75.0% and accounts for 8.8% of all cancer-related deaths[2]. Advanced-stage GC is associated with rapid metastatic growth, relapse, and a poor prognosis with a 5-year survival rate of 30%-50%. Clinically, GC is treated *via* surgical resection and chemotherapy, which is a viable option[3]. The primary treatment for GC is neoadjuvant or adjuvant therapy. Chemoresistance is a major challenge with few benefits. Further, the aggressiveness of GC is attributed in part to intrinsic and extrinsic chemoresistance[4]. As a result, identifying the molecular mechanism of chemoresistance in GC is critical.

S-1 (tegafur, gimeracil, and oteracil potassium capsules) or capecitabine in combination with oxaliplatin is currently used as adjuvant therapy for GC in various East Asian institutions[5,6]. Oxaliplatin is a third-generation platinum analog commonly used to treat GC, resulting in a large amount of platinum-DNA adducts that are poorly identified by the mismatch repair system[7]. Although oxaliplatin initially has a high responsiveness rate, patients eventually develop resistance[8]. Protein kinase B (Akt), also known as protein kinase B, is involved in a variety of critical cellular processes such as cell proliferation and migration, metastasis, and cancer progression[9]. Drug resistance in various types of human cancers is influenced by changes in Akt expression or activity[10-12]. Moreover, trastuzumab resistance is primarily determined by Akt signaling activation in breast cancer[12,13]. The aberrant Akt signaling pathway activation-mediated epithelial-mesenchymal transition is important in the development of doxorubicin resistance in GC cells[14]. Akt signaling has been linked to oxaliplatin resistance in GC cells in several studies[15-17].

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is an oncogene that inhibits c-Myc degradation. CIP2A has been shown to facilitate the proliferation of various cancer cells[18]**.** Moreover, CIP2A is overexpressed in a variety of cancers, including breast, head and neck, prostate, lung, and

digestive system cancers[18-20]. In human GC, cancer tissues had higher CIP2A levels than noncancerous tissues. As a result, CIP2A may play an oncogenic role in human GC progression and is correlated with a poor prognosis[21-23]. Recent studies have linked increased CIP2A expression to doxorubicin resistance in human breast and colon cancer cells[24]. Moreover, CIP2A overexpression is associated with cisplatin chemoresistance in human non–small cell lung carcinoma, and Akt phosphorylation may play a role in this process[25]. The effect of CIP2A on oxaliplatin resistance in human GC is unknown.

The current study sought to investigate CIP2A expression in tumor tissue and its relationship to clinicopathological features and prognosis in GC patients. We investigated the expression of CIP2A and its relationship with oxaliplatin resistance in human GC cells as well as the possible mechanisms involved.

MATERIALS AND METHODS

Patients and their clinicopathological characteristics

Between January 2012 and December 2015, 108 paired primary gastric carcinoma tissue and adjacent normal tissue (> 5 cm from the tumor margin and noncancerous tissues determined by the pathologist) specimens were collected from patients undergoing D2 radical resection at the Department of Gastroenterological and Oncological Surgery of the First Hospital of Lanzhou University. Prior to surgery, none of the patients received chemotherapy, radiotherapy, targeted therapy, or immunotherapy. Patients with GC ranged in age from 26-years-old to 78-years-old (mean: 57.3 ± 6.8 years). Table 1 shows the clinicopathological characteristics of patients. The Tumor Node Metastasis stage of the tumor was determined using the 8th edition of the American Joint Committee on Cancer staging manual[26]. All patients were followed up for at least 5 years after surgery. Further, following surgery, all patients received six cycles of S-1 (tegafur, gimeracil, and oteracil potassium capsules) combined with oxaliplatin. To detect the mRNA and protein expression of CIP2A, 18 frozen GC tissue and paired normal tissue specimens were selected. All pathological results were evaluated independently by two specialized pathologists who were blinded. The ethics review board of the First Hospital of Lanzhou University approved this study, and each participant provided written informed consent. All experiments were performed in accordance with the principles of the Declaration of Helsinki.

Immunohistochemistry analysis

The expression of CIP2A in 108 pairs of GC tissue and matched adjacent normal tissue samples was evaluated *via* immunohistochemistry (IHC) using the SP method. Formaldehyde-fixed and paraffin-embedded 4-μm-thick samples were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling in a pressure cooker. The endogenous peroxidase activity was blocked with H_2O_2. Primary antibodies (CIP2A antibody 1:500, Santa Cruz Biotechnology, United States) were added to the sections and incubated for 1 h at 37 °C in the dark. The secondary antibodies were then added at 37 °C for 30 min. Next, DAB (3,3′-Diaminobenzidine) chromogenic reagent was added to develop, and hematoxylin was added for staining. In the negative control group, phosphate-buffered solution (PBS) was used instead of the primary antibody. The IHC score of each slide was calculated by multiplying the intensity of staining by the average percentage of positive cells[27]. The staining intensity scores were classified as follows: colorless (no staining), 0; light yellow (weak staining), 1; yellow-brown (moderate staining), 2; and brown (strong staining), 3. The average percentages of positive cells were as follows: 1 = 1%-25%, 2 = 26%-50%, 3 = 51%-75%, and 4 = 76%-100%. Based on the Statistical Package for Social Sciences software version 20.0, the optimal cutoff IHC score was set as 6. In the final analysis, samples with a score of ≥ 6 were classified as $CIP2A^{high}$ expression, whereas those with a score of < 6 were classified as $CIP2A^{low}$ expression.

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) and The National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) were used to obtain data on *CIP2A* mRNA expression. Moreover, R software and the Bioconductor project were used to analyze and process data on *CIP2A* mRNA expression. Data on the mRNA expression of *CIP2A* were processed with log2 and standardized with R software. For the survival analysis of *CIP2A*, the online tool Kaplan–Meier plotter (http://www.kmplot.com/gastric) was used to assess the prognostic value of *CIP2A* in GC patients. The Kaplan–Meier plotter database contains 875 patients with clinicopathologic information about GC from the National Center for Biotechnology Information Gene Expression Omnibus 208853 dataset. Moreover, the survival data were analyzed online. The Kaplan–Meier survival curves were drawn using GraphPad (GraphPad Prism 6.0, La Jolla, CA, United States).

Cell culture, reagents, and small interference RNA

Human GC cell lines MKN-45 and AGS, as well as normal human gastric epithelial cells (GES-1)

Table 1 Association between the expression of cancerous inhibitor of protein phosphatase 2A and clinicopathological features in patients with gastric cancer

Prognostic variables	Number	Expression of CIP2A		χ^2	*P* value
		Low	High		
Sex				1.766	0.184
Male	71	27	44		
Female	37	19	18		
Age, yr				0.175	0.676
< 60	68	30	38		
≥ 60	40	16	24		
Tumor location				0.097	0.756
Proximal + middle	37	15	22		
Distal	71	31	40		
Histological grade				0.001	0.973
G1 + G2	26	11	15		
G3	82	35	47		
Tumor size in cm				5.975	0.015[a]
< 5	84	41	43		
≥ 5	24	5	19		
T stage				5.472	0.019[a]
T1–T2	36	21	15		
T3–T4	72	25	47		
N stage				12.428	0.000[a]
N0	47	29	18		
N1–N3	61	17	44		
TNM stage				5.168	0.023[a]
I + II	69	35	34		
III + IV	39	11	28		

[a]$P < 0.05$.

CIP2A: Cancerous inhibitor of protein phosphatase 2A; G: Grade; N: Node; T: Tumor; TNM: Tumor Node Metastasis.

(Chinese Academy of Sciences, China), were cultured in RPMI-1640 (Hyclone Laboratories Inc., United States) supplemented with 1% penicillin and streptomycin (North China Pharmaceutical Company, Inc., China) and 10% fetal bovine serum (Hyclone Laboratories Inc., United States). Oxaliplatin was purchased from the Hengrui Medicine Co., Ltd. (Jiangsu, China). Further, Invitrogen Inc. (Carlsbad, CA, United States) provided the unique *CIP2A* small interfering RNA (siRNA) and negative control. The *CIP2A* siRNA sequence is 5′-GACAACUGUCAAGUGUACCACUCUU-3′[28]. To deliver the siRNA into the MKN-45 and AGS cells, Lipofectamine™ 2000 (Invitrogen Inc., Carlsbad, CA, United States) was used based on the manufacturer's instructions. In addition, MK-2206 was acquired from Cell Signaling Inc. (InvivoGen, San Diego, CA, United States).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium assay

Cell proliferation was detected using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) assay. Cultured cells were separated in 96-well plates for 12 h (5000 cells/well). Moreover, 20 μL/well of the MTT reagent was added to each sample after treatment. The sample was then incubated at 37 °C for 4 h before being washed with PBS. Following that, 200 μL of dimethyl sulfoxide was added. The 490-nm optical density was evaluated. The rate of cell proliferation was calculated as the score of surviving cells. Cell viability was measured as a percentage of survival (control group: 100%).

Real-time quantitative reverse transcription PCR

Total RNA was extracted in an RNase-free environment using TRIzol reagent (Invitrogen, United States), and cDNA was obtained using PrimeScript™ RT Master Mix (Takara Biotechnology Co., China) according to the manufacturer's instructions. Real-time quantitative reverse transcription (RT-q) PCR was performed using the 7500 Fast PCR System (Applied Biosystems, CA, United States) with SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., China). The reactions were carried out using the 20-μL reaction system *per* the manufacturer's instructions. Moreover, glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene. The primer sequences were as follows: *CIP2A* (accession no. NM020890) sense 5′-GGCACTTGGAGGTAATTTCT-3′, anti-sense 5′-CTGGTTTCAATGTCTACTGCTAG-3′, glyceraldehyde-3-phosphate dehydrogenase (accession no. NM002046) sense 5′-AAGGCTGGGGCTCATTTG-3′, and anti-sense 5′-AGGAGGCATTGCTGATGATC-3′. All primers were provided by Takara Biotechnology Co. The expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase was used to normalize the mRNA expression.

Immunoblotting assay

PBS and radio immunoprecipitation assay lysis buffer (Beyotime Biotechnology, China) was used to treat the cells, which were supplemented with 1 mmol/L phenylmethanesulfonyl fluoride. They were centrifuged for 15 min at 12000 × g at 4 °C. The supernatant was then collected, and the protein concentration was determined using the BCA protein assay (Beyotime Biotechnology, China). Using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, an equal amount of sample (50 μg) was isolated and moved into the polyvinylidene fluoride membrane. The samples were then blocked with 5% nonfat milk and incubated with the following primary antibodies: CIP2A (2G10-3B5, Santa Cruz Biotechnology, United States), phospho-Akt (Ser473, Santa Cruz Biotechnology, United States), Akt (Cell Signaling Technology, Inc., United States), and β-actin (Zhongshan Golden Bridge Biotech, China). β-actin was used as an internal control. After that, samples were incubated with secondary antibodies (Zhongshan Golden Bridge Biotech, China) (1:5000). The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., United States) was used to obtain the results. Moreover, data were analyzed using Quantity One (Bio-Rad Inc.).

Annexin V assay of apoptosis

After 48 h of siRNA treatment, the GC cells were treated with a specific dose of oxaliplatin (2 μg/mL) for 24 h. They were then collected, washed with PBS, and suspended with propidium iodide annexin V-fluorescein 5-isothiocyanate (BD Pharmingen, United States) in the binding buffer of annexin V. Fluorescence was detected using a flow cytometer (BD Biosciences, San Jose, CA, United States) after 20 min in the dark at room temperature. The cells were then counted using Flow Cytometry Cell Quest, and data were analyzed using Magnetic Cell Sorting Quant.

Statistical analysis

All examinations were performed in triplicate, and the results were presented as mean ± standard deviation. To compare absorbance values and percentages of apoptotic and viable cells, the two-tailed Student's *t* test was used. The Pearson's χ^2 test was used to examine the relationship between CIP2A and clinicopathological features. Moreover, survival analysis was conducted using the Kaplan–Meier method, and the difference in survival curves was examined using the log-rank test. We also ran univariate and multivariate Cox proportional hazards regression analyses. All statistical analyses were performed using GraphPad Prism 6.0 and the Statistical Package for the Social Sciences software version 20.0 (IBM, Armonk, United States). SigmaPlot 10.0 (Systat Software Inc., United States) was utilized to display the results. Further, P values of < 0.05 ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$) were considered significant.

RESULTS

High CIP2A expression in GC tissues and its correlation with clinicopathological features

To investigate the clinical value of CIP2A in GC, we assessed the expression of CIP2A in 108 pairs of GC tissue and matched normal tissue samples using IHC and hematoxylin and eosin staining (Figure 1A). CIP2A was found in the nucleus and, more specifically, the cytoplasm of GC cells (Figure 1A). CIP2A expression was found to be significantly higher in tumor tissues. Meanwhile, CIP2A expression was absent or significantly reduced in adjacent normal gastric tissues (Figure 1A). CIP2A expression in GC tissues was significantly higher than that in adjacent normal gastric tissues (Figure 1B). RT-qPCR and Western blot analysis were performed to detect the expression of CIP2A in 18 pairs of fresh GC tissue and adjacent normal gastric tissue samples. Results showed that the expression of CIP2A in GC tissues was significantly higher than that in adjacent normal gastric tissues (Figure 1C and D). This finding supported the IHC analysis results of paraffin-embedded tissues (Figure 1A). The mRNA expression of *CIP2A* was then assessed using The Cancer Genome Atlas data from paired ($n = 27$, $P < 0.05$, Figure 1E)

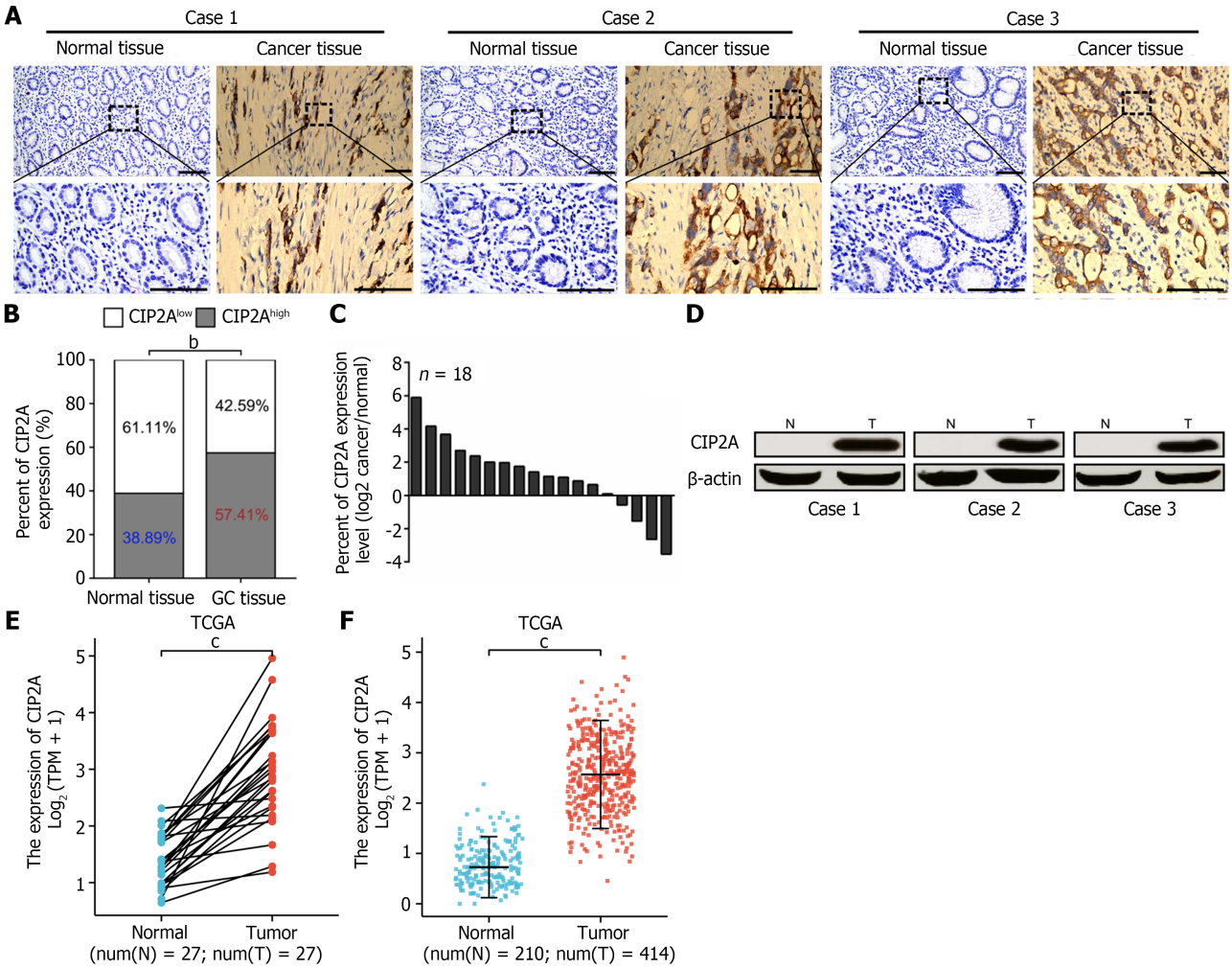

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Figure 1 Cancerous inhibitor of protein phosphatase 2A was highly expressed in gastric cancer tissues. A: Representative immunohistochemistry images of cancerous inhibitor of protein phosphatase 2A (CIP2A) in the adjacent normal gastric tissues and gastric cancer (GC) tissues (scale bar = 50 μm); B: The percentage of CIP2A^{high} expression was significantly higher in GC tissues than in adjacent normal gastric tissues; C: The mRNA expression levels of *CIP2A* were higher in 18 fresh frozen GC tissues than in matched adjacent normal gastric tissues; D: Western blot analysis showed that CIP2A expression level in tumor tissues (T) was significantly higher than that in adjacent normal tissues (N). Representative images were displayed in paired fresh surgical GC tissues; E and F: Based on data in The Cancer Genome Atlas (TCGA), including paired sample data and unpaired sample data (F), GC tissues had a higher *CIP2A* expression than adjacent normal gastric tissues. $^{b}P < 0.01$. $^{c}P < 0.001$.

and unpaired databases (n = 210, normal samples; n = 414, tumor samples, $P < 0.05$, Figure 1F). CIP2A expression was significantly higher in GC tissues than in adjacent normal gastric tissues (Figure 1).

We further investigated the relationship between CIP2A expression and clinicopathological features in 108 patients with GC. As shown in Table 1, high CIP2A expression was associated with tumor size (P = 0.015), T stage (P = 0.019), N stage (P = 0.000), and Tumor Node Metastasis stage (P = 0.023) but not with sex (P = 0.184), age (P = 0.676), tumor location (P = 0.756), and histological grade (P = 0.973).

According to these findings, CIP2A expression was significantly higher in GC tissues. CIP2A could thus be an oncogene that promotes tumor development in GC.

Association between the overexpression of CIP2A and poor prognosis in patients with GC

We investigated the prognostic value of CIP2A expression in GC patients. In 108 GC patients followed up for a median of 58 mo (range: 3–74 mo), 61 (56.48%) died and 47 (43.52%) survived. According to the Kaplan–Meier survival analysis, patients with high CIP2A expression had a shorter overall survival (hazard ratio: = 1.814, 95% confidence interval = 1.038–3.032, P = 0.0375, Figure 2A) and progression-free survival (hazard ratio = 1.805, 95% confidence interval = 1.039–3.043, P = 0.0383, Figure 2B) than those with low CIP2A expression. Based on the National Center for Biotechnology Information Gene Expression Omnibus 208853 dataset, a similar trend was discovered, confirming the prognostic significance of *CIP2A* expression in patients with GC (Figure 2C and D).

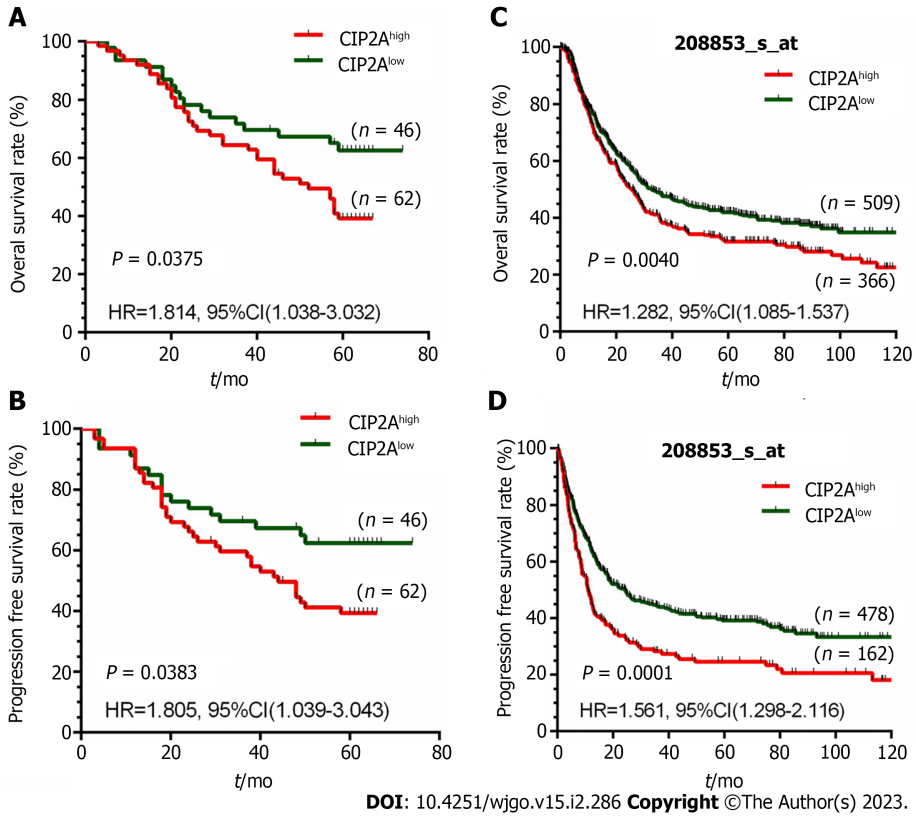

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Figure 2 High expression of cancerous inhibitor of protein phosphatase 2A was associated with poor overall survival rate and progression-free survival rate in patients with gastric cancer. A: Based on the Kaplan–Meier analysis, a high cancerous inhibitor of protein phosphatase 2A (CIP2A) expression was associated with overall survival (P = 0.0375) in 108 patients with gastric cancer; B: Based on the Kaplan–Meier analysis, a high CIP2A expression was associated with progression-free survival (P = 0.0383) in 108 patients with gastric cancer; C and D: According to the Kaplan–Meier analysis of data on gastric cancer from the 208853_s_at dataset, patients with CIP2A^{high} expression tumors had a significantly lower overall survival rate (P = 0.0040) (C) and progression-free survival rate (P < 0.0001) (D) than those with CIP2A^{low} expression tumors. CI: Confidence interval; HR: Hazard ratio.

The Cox proportional risk regression model was used to evaluate CIP2A expression and the prognostic factors in GC patients. Univariate analysis showed that tumor size, T stage, N stage, Tumor Node Metastasis stage, and CIP2A expression (Tables 2 and 3) were significantly correlated with prognosis in patients with GC. According to the multivariate analysis, a significant increase in CIP2A expression was associated with lower overall survival and progression-free survival rates in patients with GC. Further, the expression of CIP2A was an independent prognostic factor in patients with GC (P = 0.046 and 0.042, Tables 2 and 3). As a result, CIP2A could be an important oncoprotein in the development of GC.

High expression of CIP2A in human GC cell lines

CIP2A has been shown to promote the proliferation of the human GC cell lines AGS and MKN-45. However, its molecular mechanism remains unknown. To determine the expression of CIP2A in AGS, MKN-45, and GES-1, RT-qPCR and immunoblotting were performed. The mRNA expression of *CIP2A* in human GC cell lines AGS and MKN-45 was higher than in the human GC cell line GES-1 (Figure 3A). According to the protein expression analysis, CIP2A expression was higher in the human GC cell lines AGS and MKN-45 (Figure 3B and C). Due to their high expression of CIP2A, AGS and MKN-45 were selected.

Proliferation of human GC cells decreased after knockdown of CIP2A

To assess the role of CIP2A in the growth of human GC cell lines, siRNA targeting CIP2A was transfected into AGS and MKN-45. RT-qPCR revealed that the mRNA expression of *CIP2A* knockdown in MKN-45 cells was 90% lower than that of scrambled siRNA and mock cells (P < 0.01). Meanwhile, the mRNA expression of *CIP2A* knockdown in AGS cells was 88% lower than that of scrambled siRNA and mock cells (P < 0.01). There was no statistically significant difference between the last two samples (Figure 4A and B). These findings were confirmed by immunoblotting (Figure 4C-F). Based on the MTT assay, the downregulation of CIP2A expression significantly reduced the proliferation of AGS and MKN-45 cells. When compared to scrambled siRNA and mock cells, CIP2A siRNA significantly reduced the rate of AGS and MKN-45 cell proliferation (P < 0.05) (Figure 4G and H).

Table 2 Univariate and multivariate analyses of overall survival in patients with gastric cancer

Prognostic variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	*P* value	Hazard ratio	95% confidence interval	*P* value
Sex	0.99	0.584–1.679	0.97	-	-	-
Age	1.372	0.820–2.296	0.229	-	-	-
Tumor location	0.925	0.545–1.569	0.772	-	-	-
Histological grade	0.736	0.420–1.288	0.283	-	-	-
Tumor size	2.417	1.399–4.176	0.004[a]	0.87	0.504–1.504	0.619
T stage	3.184	1.687–6.007	0.000[a]	2.651	1.295–5.423	0.008[a]
N stage	2.034	1.196–3.457	0.009[a]	1.414	0.709–2.818	0.325
TNM stage	1.946	1.168–3.243	0.011[a]	0.912	0.469–1.774	0.786
CIP2A expression	2.319	1.288–3.927	0.003[a]	1. 802	1.012–3.210	0.046[a]

[a]$P < 0.05$.

T: Tumor; N: Node; TNM: Tumor Node Metastasis; CIP2A: Cancerous inhibitor of protein phosphatase 2A.

Table 3 Univariate and multivariate analyses of progression-free survival in patients with gastric cancer

Prognostic variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% confidence interval	*P* value	Hazard ratio	95% confidence interval	*P* value
Sex	0.973	0.574–1.650	0.919	-	-	-
Age	1.363	0.814–2.281	0.239	-	-	-
Tumor location	0.913	0.538–1.548	0.735	-	-	-
Histological grade	0.73	0.417–1.278	0.27	-	-	-
Tumor size	2.43	1.407–4.197	0.004[a]	0.869	0.504–1.499	0.614
T stage	3.15	1.669–5.943	0.000[a]	2.644	1.294–5.420	0.009[a]
N stage	2.044	1.202–3.476	0.008[a]	1.451	0.728–2.891	0.29
TNM stage	1.917	1.150–3.194	0.013[a]	0.88	0.452–1.713	0.88
CIP2A expression	2.309	1.282–3.911	0.003[a]	1.821	1.021–3.247	0.042[a]

[a]$P < 0.05$.

T: Tumor; N: Node; TNM: Tumor Node Metastasis; CIP2A: Cancerous inhibitor of protein phosphatase 2A.

Increased sensitivity to oxaliplatin in human GC cells induced by the knockdown of CIP2A

CIP2A may be overexpressed in human GC cells. Thus, there could be a correlation between CIP2A expression and oxaliplatin sensitivity. To test this theory, CIP2A knockdown cells were treated with oxaliplatin at various concentrations. CIP2A knockdown increased susceptibility to oxaliplatin significantly (Figure 5A and B). The half maximal inhibitory concentrations of oxaliplatin in CIP2A-downregulated MKN-45 and AGS cells were 2.9 μg/mL and 3.6 μg/mL, respectively. In the control sample, the concentrations were 5.3 μg/mL and 6.2 μg/mL, respectively ($P < 0.05$). The susceptibility of MKN-45 and AGS cells to oxaliplatin were strengthened by 45% and 42%, respectively. Thus, cells with downregulated CIP2A expression were more sensitive to oxaliplatin treatment.

Oxaliplatin-induced apoptosis in human GC cells induced by the knockdown of CIP2A

Flow cytometric analysis was used to determine whether CIP2A knockdown promoted cell apoptosis. Following oxaliplatin treatment, propidium iodide and annexin V staining were performed. Interestingly, CIP2A knockdown significantly enhanced apoptosis caused by oxaliplatin (2 μg/mL). The apoptosis rates of CIP2A-downregulated MKN-45 and AGS cells added to oxaliplatin exhibited 33.5% and 23.6%, respectively. Control rates in oxaliplatin-treated cells were 16.3% and 11.6%, respectively ($P < 0.05$). In addition, siRNA transfection of CIP2A did not increase the apoptosis rate (Figure 6A-C). As a result, CIP2A knockdown caused cell apoptosis. Hence, high CIP2A expression could be a factor of

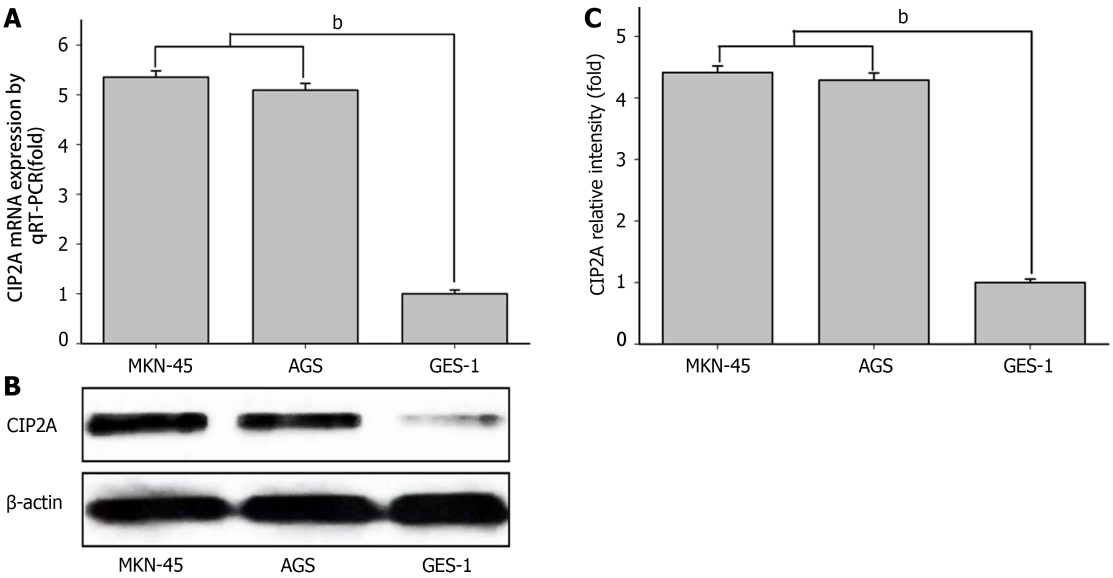

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Figure 3 Expression of cancerous inhibitor of protein phosphatase 2A in human gastric cancer cells. A: The mRNA expression of cancerous inhibitor of protein phosphatase 2A (CIP2A) in human gastric cancer cell lines (MKN-45 and AGS) and normal gastric epithelial cells (GES-1); B: The expression of cancerous inhibitor of protein phosphatase 2A was examined *via* immunoblotting; C: Immunoblotting was quantified using a spectrophotometer. The results consisted of three independent tests. Gastric cancer cell lines *vs* GES-1. $^{b}P < 0.01$. RT: Reverse transcription.

oxaliplatin resistance in GC cells.

Akt phosphorylation of human GC cells regulated by CIP2A

To validate the potential mechanisms by which CIP2A promoted the chemoresistance of human GC cells, the protein expression and phosphorylation level of Akt signaling in CIP2A knockdown AGS and MKN-45 cells were evaluated. According to the findings, CIP2A knockdown significantly reduced Akt phosphorylation levels (Ser473). However, it had no effect on the level of Akt protein expression (Figure 7A-C). Based on these findings, CIP2A influenced Akt activity in human GC cells, and CIP2A knockdown decreased cell proliferation and increased sensitivity to oxaliplatin in human GC cells by decreasing the Akt activity.

Increased sensitivity to oxaliplatin in human GC cells caused by chemical inhibition of Akt signaling

Moreover, to validate the effects of Akt signaling in CIP2A with respect to sensitivity to oxaliplatin, MK-2206, an allosteric Akt inhibitor, was used to pretreat the high expression CIP2A human GC cell line. The expression of the indicated proteins and sensitivity to oxaliplatin were assessed using immunoblotting and the MTT assay, respectively. Pretreatment with MK-2206 reduced p-Akt levels (Figure 8A and B) while increasing sensitivity to oxaliplatin (Figure 8C and D) in MKN-45 and AGS cells. Thus, Akt signaling may play a role in sensitizing CIP2A overexpression in human GC cells exposed to oxaliplatin.

DISCUSSION

When first diagnosed, most patients have middle- and late-stage GC. The only curative treatment option for gastric tumors is surgical resection. However, patients frequently relapse after resection. Therefore, after 1B resection, combination therapy has become the standard treatment for advanced-stage disease [29]. As the first-line chemotherapy regimen, a platinum–fluoropyrimidine-based treatment is often used[30]. Oxaliplatin, as the third-generation platinum derivative, has been used successfully to treat GC[31,32]. According to the CLASSIC trial, XELOX (capecitabine and oxaliplatin) is superior to observation alone after D2 radical gastrectomy. Hence, chemotherapy is effective[33]. Although patients initially respond well to oxaliplatin, they eventually develop resistance[34,35]. Tumor cells can gain resistance to the cytotoxic effects of oxaliplatin, similar to other anticancer drugs[36]. However, its specific molecular mechanism remains unknown, and it must be investigated further.

CIP2A promotes cell proliferation and tumorigenesis in numerous types of tumors by maintaining c-Myc[18,22,37]. In addition, CIP2A overexpression is correlated with a poor prognosis[21,37]. Interestingly, recent studies on breast, colon, and lung cancer cells have revealed that overexpression of CIP2A may induce chemoresistance in cancer cells[24,25]. The current study sought to determine the

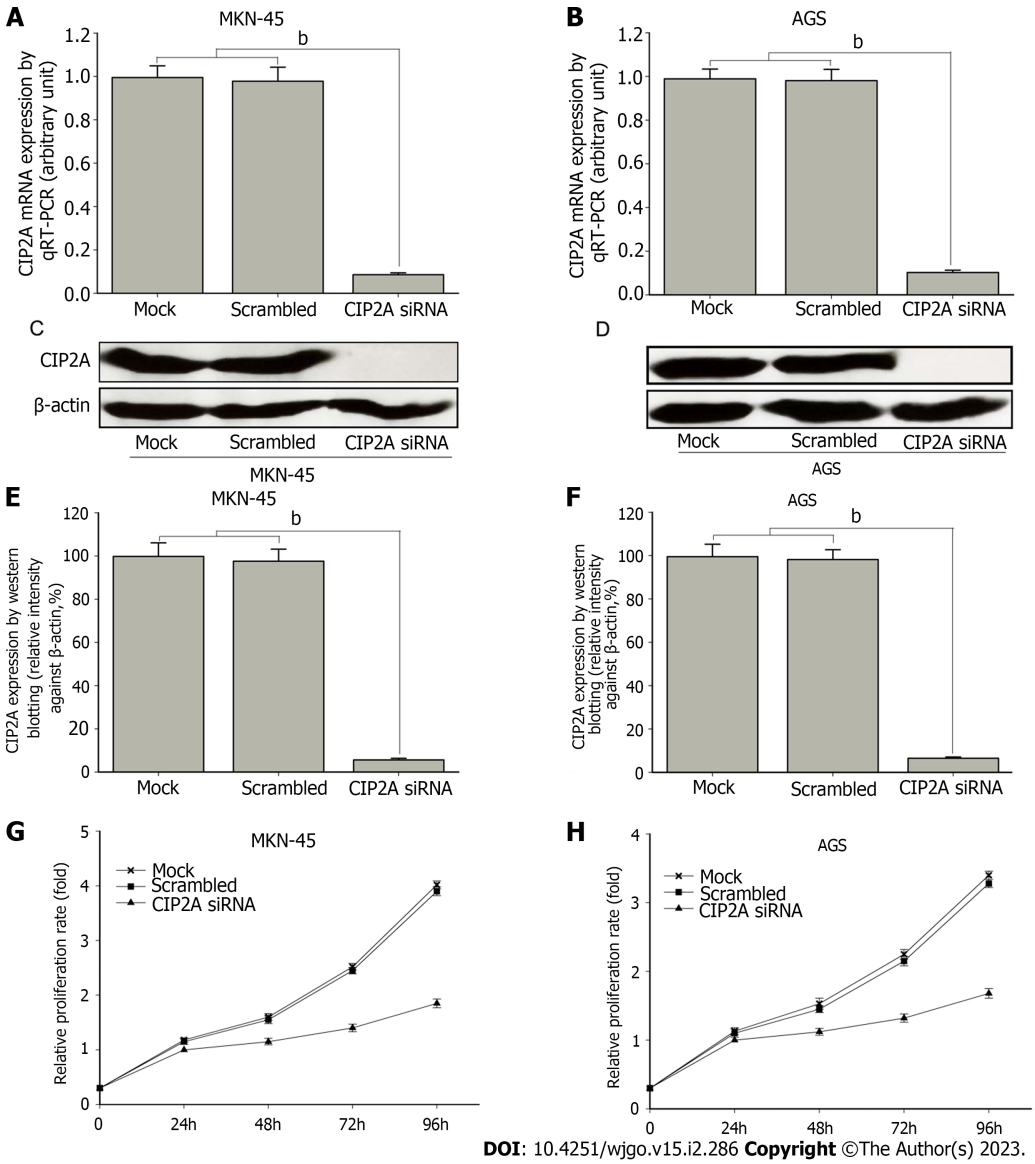

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Figure 4 Small interference RNA targeting cancerous inhibitor of protein phosphatase 2A effectively downregulated expression and decreased cell proliferation in human gastric cancer cells. MKN-45 and AGS cells were transfected with negative control small interference RNA (siRNA) or cancerous inhibitor of protein phosphatase 2A (*CIP2A*) siRNA for 48 h. A: *CIP2A* expression in MKN-45 cells was determined *via* real-time quantitative reverse transcription (RT) PCR; B: *CIP2A* expression in AGS cells was determined *via* real-time quantitative RT PCR; C and D: CIP2A expression was determined *via* immunoblotting; E and F: Data were quantified using a spectrophotometer. The CIP2A siRNA group *vs* the control siRNA group. G and H: The downregulation of CIP2A expression could decrease cell proliferation in MKN-45 and AGS cells based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium assay. Data showed a significant reduction in the proliferation of CIP2A siRNA-treated cells. The results were three independent tests. $^{b}P < 0.01$.

association between CIP2A expression and oxaliplatin sensitivity.

Using The Cancer Genome Atlas data analysis, we discovered that the expression of *CIP2A* in GC tissues was significantly higher than that in adjacent normal gastric tissues. We also confirmed consistent results in GC tissue specimens. Survival analysis revealed that *CIP2A* expression was significantly correlated with overall survival and progression-free survival in patients with GC. Moreover, CIP2A expression in GC cells was significantly higher compared to GES-1 cells. CIP2A overexpression has previously been observed in several GC cells[38], and this finding is similar to the current study. As a result, MKN-45 and AGS were studied further because they have high CIP2A expression[18,39-41].

We performed siRNA knockdown of CIP2A expression to investigate its biological function in GC cells. Results showed that CIP2A silencing reduced the growth rate of MKN-45 and AGS cells, indicating that CIP2A plays an important role in GC cell proliferation. A similar finding has been reported in our previous studies[42]. To investigate the association between CIP2A expression and drug sensitivity, we knocked down CIP2A in GC cells and tested their sensitivity to oxaliplatin treatment. Previous studies have shown that the knockdown of CIP2A significantly increased the susceptibility of GC cells to oxaliplatin. Based on some reports, CIP2A can promote the proliferation of colon cancer

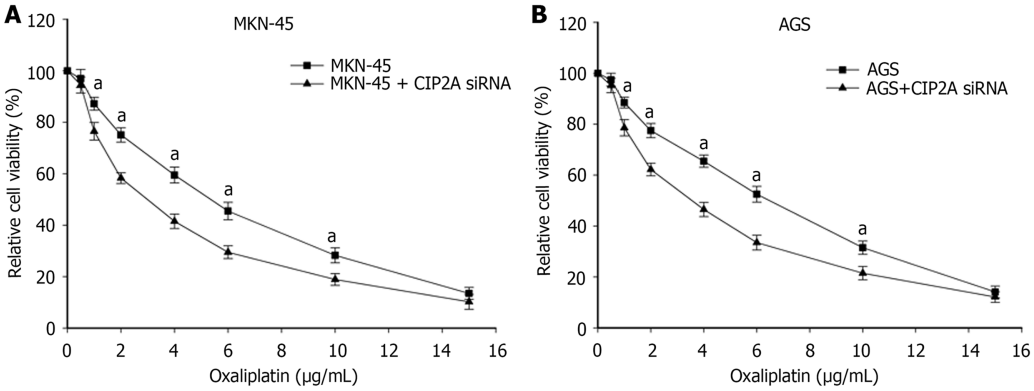

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Figure 5 Downregulation of cancerous inhibitor of protein phosphatase 2A significantly increased sensitivity to oxaliplatin in human gastric cancer cells. A: MKN-45 cells were transfected with cancerous inhibitor of protein phosphatase 2A (CIP2A) small interfering RNA (siRNA) for 48 h and were added with oxaliplatin at different concentrations for 24 h; B: AGS cells were transfected with cancerous inhibitor of protein phosphatase 2A siRNA for 48 h and were added with oxaliplatin at different concentrations for 24 h. The cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium assay. The results consisted of three independent tests. The siRNA group *vs* the control siRNA group. $^{b}P < 0.01$.

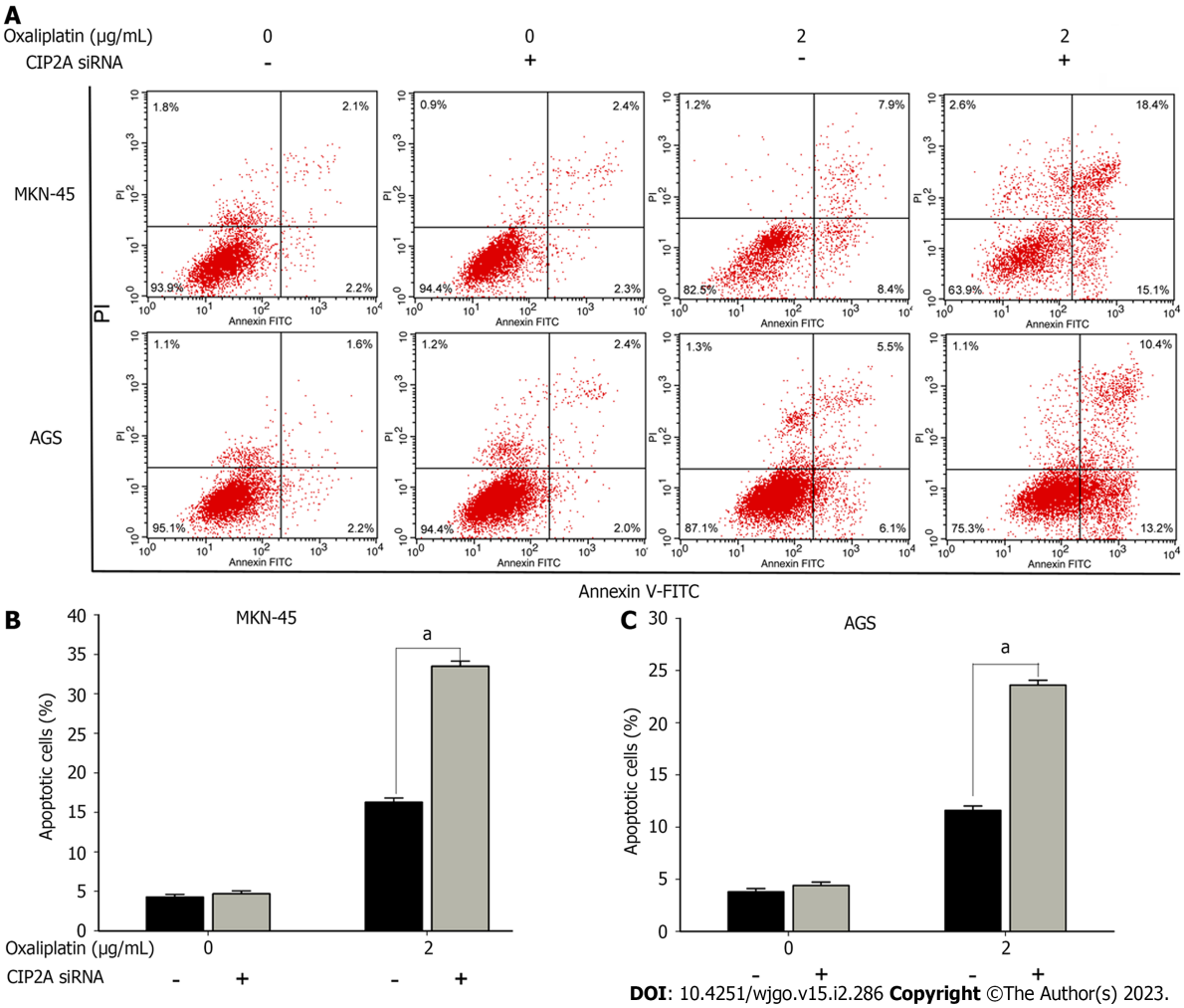

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Figure 6 Downregulation of cancerous inhibitor of protein phosphatase 2A promoted oxaliplatin-related apoptosis in human gastric cancer cells. Control small interference RNA (siRNA) and cancerous inhibitor of protein phosphatase 2A (CIP2A) siRNA-transfected human gastric cancer cells were exposed to oxaliplatin (2 μg/mL). A: At 24 h after treatment, apoptosis was examined *via* annexin V/propidium iodide staining and flow cytometry; B and C: The percentage of apoptotic cells was quantitatively presented in MKN-45 (B) and AGS (C) cells. The results consisted of three independent tests. The cancerous inhibitor of protein phosphatase 2A siRNA group *vs* the control siRNA group. $^{a}P < 0.05$. V-FITC: V-fluorescein 5-isothiocyanate.

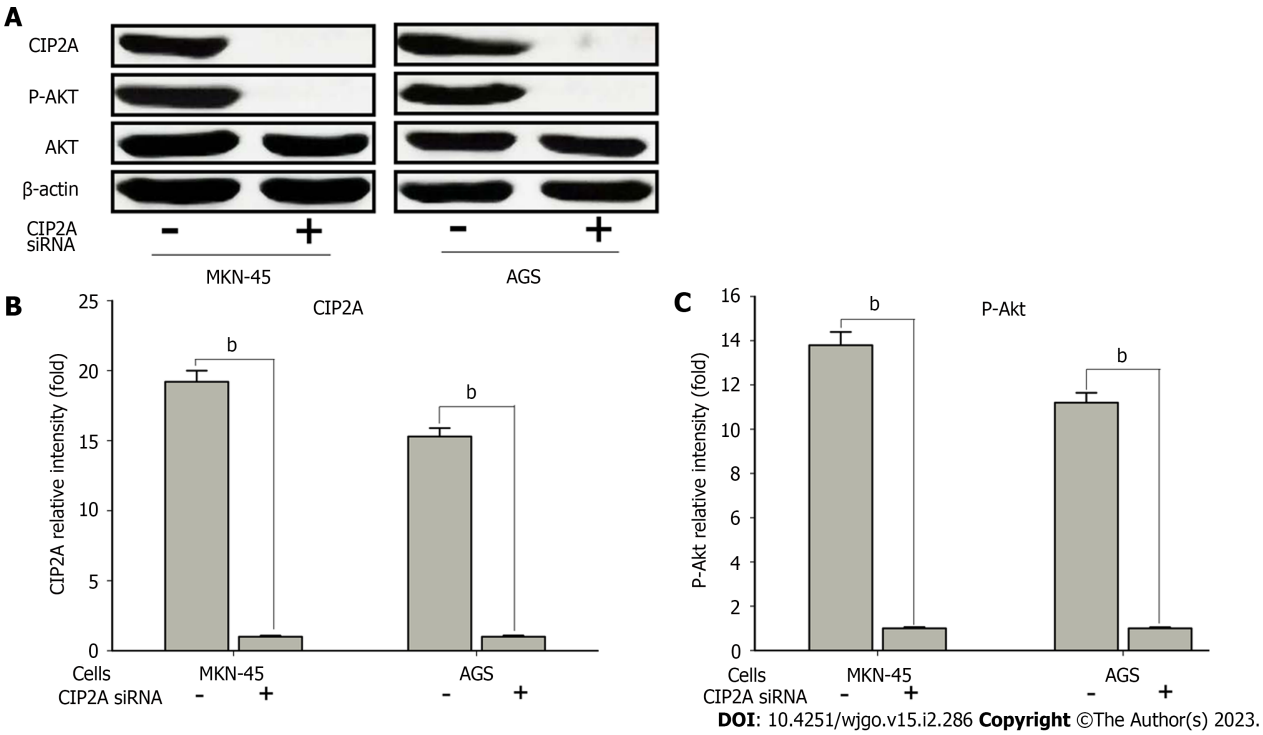

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Figure 7 Cancerous inhibitor of protein phosphatase 2A affected protein kinase B phosphorylation in human gastric cancer cells. MKN-45 and AGS cells were transfected with the control small interfering RNA (siRNA) or cancerous inhibitor of protein phosphatase 2A (CIP2A) siRNA for 48 h. A: Immunoblotting was used to analyze the indicated protein expression; B and C: The CIP2A (B) and phosphorylated protein kinase B (p-Akt) (C) protein levels were quantified using a spectrophotometer. The results consisted of three independent tests. $^{b}P < 0.01$. Akt: Protein kinase B.

cells, and CIP2A knockdown significantly increased sensitivity to oxaliplatin in colon cancer cells[43,44]. As a result, GC cells with CIP2A expression downregulation were more sensitive to oxaliplatin treatment.

Oxaliplatin has the ability to cause apoptosis in GC cells[45,46]. Nevertheless, the underlying mechanism is unknown. Previous studies have shown that CIP2A plays an important role in lung cancer cell apoptosis when treated with cisplatin[47]. The biological impact of CIP2A is a common phenomenon in tumor cells. According to a recent study, the downregulation of CIP2A expression in MKN-45 and AGS cells increased apoptosis and oxaliplatin sensitivity, which could be a cause of oxaliplatin resistance in GC. Therefore, CIP2A knockdown made GC cells more susceptible to oxaliplatin-induced apoptosis, enhancing the cytotoxic effect of oxaliplatin.

According to a previous study, inhibiting the Akt pathway sensitizes GC cells to apoptosis caused by cisplatin[48]. Therefore, we discovered that signaling was correlated with the biological behaviors of CIP2A. Since the phosphorylation of Akt is a critical step in phosphoinositide 3-kinase/Akt signaling activation[49], we confirmed its association with CIP2A expression. CIP2A knockdown caused the phosphorylation of Akt in both MKN-45 and AGS cells, according to the findings. Moreover, the chemical inhibition of the Akt signaling pathway increased oxaliplatin sensitivity in human GC cells. Several studies have found that the downregulation of CIP2A expression can improve the efficacy of chemotherapeutic drugs and inhibit Akt signaling in colorectal and lung cancers[26,38]. As a result, the association between CIP2A and the Akt signaling pathway might be involved in the biological functions of GC cells. This interaction could be correlated with oxaliplatin resistance in GC.

CONCLUSION

A high expression of CIP2A can promote chemoresistance to oxaliplatin in GC, and Akt signaling may play a role in this mechanism. The inhibition of CIP2A significantly improved sensitivity to oxaliplatin in human GC cells. As a result, suppressing CIP2A expression may be an indirect strategy for more effectively treating patients with GC. Nevertheless, further clinical trials on the role of this signaling pathway should be conducted.

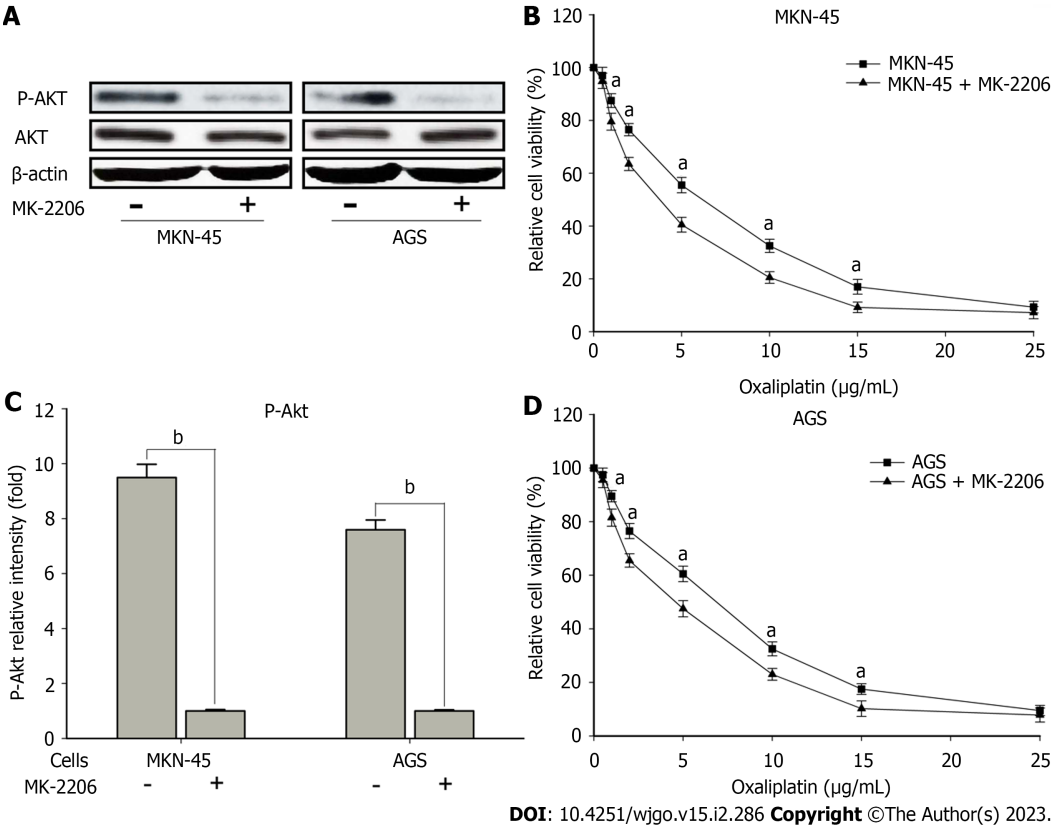

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Figure 8 Chemical inhibition of protein kinase B signaling significantly increased sensitivity to oxaliplatin in human gastric cancer cells. MKN-45 and AGS cells were added to inhibitor of protein kinase B (MK-2206; 20 μM) for 2 h. A: Immunoblotting was performed to evaluate the corresponding protein expression; B: The phosphorylated protein kinase B (P-Akt) protein levels were quantified *via* densitometry; C and D: The pretreated cells were exposed to oxaliplatin at different concentrations for 24 h, and the viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium assay. The results consisted of three independent tests. The MK-2206-treated group *vs* the control group. [a]*P* < 0.05. [b]*P* < 0.01. Akt: Protein kinase B.

ARTICLE HIGHLIGHTS

Research background

Cancerous inhibitor of protein phosphatase 2A (CIP2A) plays a key role in various types of tumors, which may be related to the resistance of gastric cancer (GC) cells to oxaliplatin.

Research motivation

The mechanism of drug resistance in gastric cancer needs to be further studied, and CIP2A expression in GC cells and the mechanism of oxaliplatin resistance may be a breakthrough.

Research objectives

To explore the expression of the CIP2A in human GC cells and its correlation with oxaliplatin resistance.

Research methods

Immunohistochemistry was used to examine CIP2A expression in GC tissues and adjacent normal tissues. *CIP2A* gene expression in GC cell lines was reduced using small interfering RNA. After confirming the silencing efficiency, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium and flow cytometry assays were used to evaluate cell proliferation and apoptosis, respectively, caused by oxaliplatin treatment. Further, the key genes and protein changes were verified using real-time quantitative reverse transcription PCR and Western blotting, respectively, before and after intervention. For bioinformatics analysis, we used the R software and Bioconductor project. For statistical analysis, we used GraphPad Prism 6.0 and the Statistical Package for the Social Sciences software version 20.0.

Research results

High CIP2A expression was associated with tumor size, T stage, lymph node metastasis, Tumor Node Metastasis stage, and poor prognosis. CIP2A knockdown inhibited cell proliferation and significantly increased the susceptibility of GC cells to oxaliplatin. CIP2A regulated the phosphorylation of protein

kinase B, and chemical inhibition of the protein kinase B signaling pathway was significantly associated with increased sensitivity to oxaliplatin.

Research conclusions

CIP2A expression was closely related to chemotherapy resistance of GC cells. The protein kinase B signaling pathway was correlated with CIP2A-enhanced chemoresistance of human GC cells to oxaliplatin.

Research perspectives

Regulation of CIP2A expression may be one of the key points in the treatment of GC chemotherapy resistance.

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FOOTNOTES

Author contributions: Zhao YX, Yang Z, Dang JY, and Wang F performed the experiments and image acquisition; Zhao YX, Ze Yang, Ma LB, and Wang HY designed the study and wrote the manuscript; Zhao YX, Yang Z, and Ma LB edited the manuscript.

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ORIGINAL ARTICLE

Basic Study

Increased CD4/CD8 Lymphocyte ratio predicts favourable neoadjuvant treatment response in gastric cancer: A prospective pilot study

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Abstract

BACKGROUND

Despite optimal neoadjuvant chemotherapy only 40% of gastric cancer tumours achieve complete or partial treatment response. In the absence of treatment response, neoadjuvant chemotherapy in gastric cancer contributes to adverse events without additional survival benefit compared to adjuvant treatment or surgery alone. Additional strategies and methods are required to optimize the allocation of existing treatment regimens such as FLOT chemotherapy (5-Fluorouracil, Leucovorin, Oxaliplatin and Docetaxel). Predictive biomarkers detected using immunohistochemistry (IHC) methods may provide useful data regarding treatment response.

AIM

To investigate the utility of CD4, CD8, Galectin-3 and E-cadherin in predicting neoadjuvant FLOT chemotherapy tumour response in gastric adenocarcinoma.

METHODS

Forty-three adult patients with gastric adenocarcinoma, of which 18 underwent neoadjuvant chemotherapy, were included in a prospective clinical cohort. Endoscopic biopsies were obtained from gastric cancer and normal adjacent gastric mucosa. Differences in expression of Galectin-3, E-cadherin, $CD4^+$ and $CD8^+$ molecules between tumours with and without treatment response to neoadjuvant chemotherapy were assessed with IHC. Treatment response was graded by clinical pathologists using the Tumour Regression Score according to the College of American Pathologists criteria. Treatment response was defined as complete or near complete tumour response, whereas partial or poor/no response was defined as incomplete. Digital IHC images were annotated and quantitatively assessed using QuPath 0.3.1. Biomarker expression between responsive and incomplete response tumours was assessed using a two-sided Wilcoxon test. Biomarker expression was also compared between normal and cancer tissue and between 15 paired tumour samples before and after chemotherapy. We performed a preliminary multivariate analysis and power analysis to guide future study. Statistical analyses were completed using R 4.1.2.

RESULTS

The ratio between $CD4^+$ and $CD8^+$ lymphocytes was significantly greater in treatment responsive tumours (Wilcoxon, $P = 0.03$). In univariate models, $CD4^+/CD8^+$ ratio was the only biomarker that significantly predicted favourable treatment response (Accuracy 86%, $P < 0.001$). Using a glmnet multivariate model, high $CD4^+/CD8^+$ ratio and low Galectin-3 expression were the most influential variables in predicting a favourable treatment response. Analyses of paired samples found that FLOT chemotherapy also results in increased expression of $CD4^+$ and $CD8^+$ tumour infiltrating lymphocytes (Paired Wilcoxon, $P = 0.002$ and $P = 0.008$, respectively). Our power analysis suggests future study requires at least 35 patients in each treatment response group for CD8 and Galectin-3 molecules, whereas 80 patients in each treatment response group are required to assess CD4 and E-cadherin biomarkers.

CONCLUSION

We demonstrate that an elevated $CD4^+/CD8^+$ Ratio is a promising IHC-based biomarker to predict favourable treatment response to FLOT neoadjuvant chemotherapy in locally advanced gastric cancer.

Key Words: CD4; CD8; Galectin-3; Neoadjuvant chemotherapy; Treatment response; Gastric cancer

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Core Tip: In the absence of treatment response, neoadjuvant chemotherapy for gastric cancer may contribute to adverse events without additional survival benefit compared to adjuvant treatment or surgery alone. Identifying patients that are likely to achieve favourable tumour response following neoadjuvant chemotherapy is of critical importance. In this pilot study, we investigate the utility of CD4, CD8, Galectin-3 and E-cadherin molecules in predicting which patients will benefit from neoadjuvant therapy using immunohistochemistry in pre-treatment biopsies. We demonstrate that an elevated ratio between $CD4^+$ and $CD8^+$ lymphocytes is a promising biomarker to predict treatment response to neoadjuvant chemotherapy in locally advanced gastric cancer.

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INTRODUCTION

Gastric cancer is the fifth most common cancer and the third most common cause of cancer death worldwide[1-3]. The poor prognosis associated with gastric cancer is in part related to significant tumour molecular heterogeneity[4-6]. Despite insight gained from extensive genomic and transcriptomic profiling, molecular classification systems such as those proposed by The Cancer Genome Atlas and Asian Cancer Research Group have yet to manifest improvement in the clinical management of gastric cancer.

In North America, the standard of care for locally advanced gastric cancer is neoadjuvant chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and docetaxel (FLOT4)[7]. Advantages of neoadjuvant chemotherapy include improved survival compared to surgery alone, greater R0 resection and reduction in nodal stage[8-11]. Previous research has demonstrated that pathologic complete response (pCR) or treatment response defined as Tumour Regression Grade 1-3 is significantly associated with improved prognosis[12,13]. However, pCR occurs in only 3-15% of cases and complete or partial response in approximately 40% of patients[9,12,14]. Irrespective of efficacy, cytotoxicity of chemotherapy is associated with adverse events including peripheral neuropathy, neutropenia, infection or death[7]. Prior evidence suggests that, in the absence of treatment response, neoadjuvant chemotherapy in gastric cancer contributes to adverse events without additional benefit compared to adjuvant treatment or surgery alone[9,15]. Specific treatment response may be related to underlying tumour biology as exposure to neoadjuvant chemotherapy in microsatellite instability in gastric cancer has been demonstrated to relate to worse survival outcomes[16-18]. Thus, in order to improve outcomes, it is of paramount importance to identify clinicopathologic or molecular biomarkers to identify treatment responders.

Immunohistochemistry (IHC) is a proven molecular pathology technique with a record of providing prognostic and therapeutic biomarkers in oncology. In gastric cancer, prominent IHC-based biomarkers may be prognostic or therapeutic as in the case of E-cadherin and human epidermal growth factor receptor 2, respectively[19,20]. However, there is a lack of predictive biomarkers to inform treatment response to more common regimens such as neoadjuvant chemotherapy.

Here we investigate a panel of biomarkers that we hypothesize may provide value in predicting tumour response. Galectin-3 is a lectin protein that facilitates cancer tumorigenesis and prognosis[21-24]. Pre-clinical models suggest that increased Galectin-3 expression is associated with chemotherapy resistance[25,26]. Recent work has implicated cell-surface expression of Galectin-3 with chemoresistance in gastrointestinal cancer stem cells[27]. E-cadherin is a cell-cell adhesion molecule that plays an important role in gastric cancer development, classification and prognosis[4,5,28]. In-vitro study has previously suggested that germline mutations in E-cadherin related to Hereditary Diffuse Gastric Cancer increases chemoresistance to taxol based agents[29]. However, study of breast cancer cell lines have identified heterogenous effects of E-cadherin expression on chemotherapy response[30,31]. We also assess whether $CD4^+$ and $CD8^+$ tumour infiltrating lymphocytes (TILs) and the relative proportion of these cells influence neoadjuvant chemotherapy response. The CD4/CD8 ratio is a marker of immune effector function and is associated with multiple disease states. A normal circulating CD4/CD8 ratio ranges from 1.5-2.5, and lower ratios in resident tissues or circulation are related to worse HIV related outcomes, cardiovascular disease and cancer[32]. Both $CD4^+$ and $CD8^+$ T cells are essential components to the tumour microenvironment and their composition in relationship to other immune cells such as macrophages, antigen presenting cells and natural killer cells influence the effectiveness of the host response to cancer[33]. Increasing evidence recognises the association of greater TILs to favourable cancer prognosis and chemotherapy response in colon and gastric cancer[34-39]. To date, no studies have investigated the role $CD4^+$ or $CD8^+$ TILs in neoadjuvant chemotherapy response for gastric cancer.

To guide future studies, we performed a prospective pilot study to evaluate if these selected biomarkers provide predictive value in evaluating treatment response following neoadjuvant FLOT chemotherapy.

MATERIALS AND METHODS

Study design

We performed this single-center, prospective pilot study at the University of Alberta in Edmonton, Alberta, Canada from January 2018 to January 2022. All human clinical participants consented according to the approved ethics protocol granted by the Health Research Ethics Board of Alberta (Study ID: HREBA.CC-17-0228_REN5). Treatment naïve Stage I-IV sporadic gastric adenocarcinoma patients aged greater than 18 years were included. A subset of patients enrolled was allocated to a second cohort on the basis of receiving curative intent neoadjuvant FLOT chemotherapy (Figure 1). Patients with a known inherited oncogenic germline mutation or hereditary syndrome (*i.e.*, Familial Adenomatous Polyposis) were excluded.

Specimens were retrieved *via* endoscopic biopsy at the time of diagnosis, screening laparoscopy or at the time of surgical resection at the Walter C Mackenzie Health Sciences Centre or Royal Alexandra Hospital. Normal biopsies were obtained from gastric mucosa greater than 5 cm away from the cancerous lesion or associated gastritis. The initial study protocol retrieved two tissue biopsies for permanent pathology, however, following interim review four biopsies were retrieved thereafter. The presence of cancer in specimens was confirmed by a gastrointestinal pathologist. In the absence of cancer, clinical formalin-fixed paraffin-embedded pathology blocks were retrieved when available. In clinical samples with treatment effect, residual cancer cells were detected using anti-pan cytokeratin (Abcam, clone C-11, ab7753) IHC staining followed by the manual assembly of tissue microarray (TMA) blocks with 4mm cores of regions containing residual tumour.

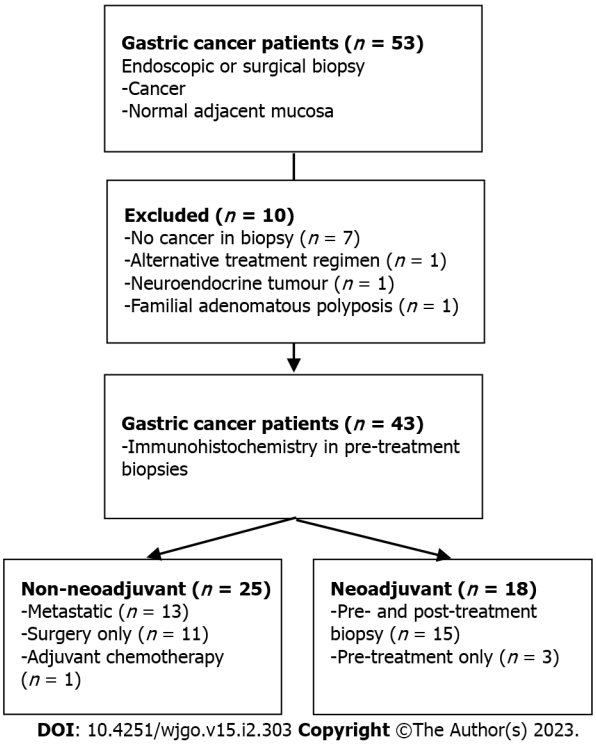

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Figure 1 Study overview. Flow chart outlining study design and patient allocation.

Our primary outcome for all patients was the difference in expression of selected biomarkers between normal and cancer tissue. In the subgroup of patients receiving neoadjuvant chemotherapy, our primary outcome was the difference in expression between tumour treatment response and incomplete treatment response. We also evaluated the difference in expression of biomarkers in paired samples before and after chemotherapy treatment.

Treatment response was retrieved from clinical pathology reports. The Tumour Regression Score was graded according to the College of American Pathologists and National Comprehensive Cancer Network protocol on a 4-point scale (0 = Complete response, 1 = near complete response, 2 = partial response, 3 = poor or no response)[40]. In accordance with prior studies, treatment response was expressed as a binary variable consisting of response and incomplete response categories[12]. Responsive tumours included complete and near-complete responses, whereas incomplete responses included partial, and poor no response. Patients who progressed to metastasis while receiving neoadjuvant treatment were classified as an incomplete response.

IHC

Tissue specimens of normal and cancer tissue were fixed in zinc-formalin (Z-Fixx, Sigma-Aldrich) for 24 h, washed three times and stored in 70% ethanol prior to preservation in paraffin. Briefly, 4 μm tissue sections were deparaffinized in Histoclear (National Diagnostics) and rehydrated. Endogenous peroxidases were quenched using 3% hydrogen peroxide in methanol for 5 min. Microwave heat induced epitope retrieval was performed using Sodium Citrate (pH 6, heated to 94 degrees Celsius in 1-min intervals followed by 9 min continuous heat) for E-cadherin and Tris-ethylenediaminetetraacetic acid (pH 9, heated to 94 degrees Celsius in 1-min intervals followed by 8 min 30 s continuous heat) for CD4 and CD8. Non-specific staining was blocked using 20% normal goat serum (Jackson Laboratories) for E-cadherin, CD4 and CD8 or 2% Fetal Bovine Serum (Gibco) in 1X phosphate buffered saline for Galectin-3 for 20 min followed by avidin and biotin blocking (Vector Laboratory, SP-2001) *per* manufacturer's protocol. Tissue sections were stained with primary antibodies anti-E-cadherin (1:25, 1.5 h room temperature, ThermoFisher Scientific, clone 4A2C7, 33-4000), anti-Galectin-3 (1:200, 30 min room temperature, Cedarlane, clone M3/38, CL8942AP), anti-CD4 (1:200, overnight at 4 degrees, Abcam, clone EPR6855, ab133616) or anti-CD8 alpha (1:200, overnight at 4 degrees Celsius, Abcam, ab4055). All biotinylated immunoglobulin G secondary antibodies were incubated at 1:200 for 30 min at room temperature, including rabbit anti-rat for Galectin-3 (Vector Laboratories, BA-4001), goat-anti-rabbit for CD4 (Vector Laboratories, BA-1000) and goat-anti-mouse for E-cadherin and CD8 (Jackson ImmunoResearch, 115-065-003). Antibody detection was performed using avidin-biotin complex/horseradish peroxidase (Vector Laboratories) and 3,3-diaminobenzidine tetrahydrochloride (DAB, Abcam, ab64238) *per* manufacturer's protocol. Stained tissue sections for E-Cadherin, CD4 and CD8 were counterstained with Harris' hematoxylin (Fisher Scientific) and Harris' hematoxylin and eosin (Fisher Scientific) for

Galectin-3.

Histology imaging and quantification

Histology images were captured at 20 times magnification using a Leica Aperio CS2 digital slide scanner. Digital pathology quantification of antibody expression was performed using QuPath version 0.3.1 (Figure 2A)[41]. Briefly, digital images were uploaded and the tumour and immediate tumour-host interface were annotated as a single region of interest. Stain vectors were estimated using default settings for each sample. For CD4, CD8 and Galectin-3, positive cells were detected using default nucleus DAB optical density settings. The CD4/CD8 ratio was calculated as the proportion of positively stained CD4 cells divided by the proportion of positively stained CD8 cells. For E-cadherin, both the proportion of positive cells and H-score was calculated. Annotated cell regions were assessed for accuracy and in the event of background or non-specific staining positive cell threshold values were adjusted to reflect true positive staining. The H-score provides a consensus scoring method for evaluating immunostaining across a gradient of intensity (Equation 1). As defined in McClelland *et al* [42], H, M and L denotes high, medium and low intensity staining. Cells without staining are denoted N for negative staining.

Statistical analysis

Statistical analyses were completed using R version 4.1.2[43]. The statistical methods of this study were reviewed by Dr. Ghosh and Dr. Skubleny from the University of Alberta. Differences between groups were assessed with a Wilcoxon two-sample test for independent samples and two-tailed paired Wilcoxon test for paired samples. Statistical significance was defined at alpha = 0.05. Multiple comparisons corrections were not made for our main outcomes given our prespecified analyses, but the possibility of false positive results is noted. Summary of continuous variables is expressed as median with interquartile range. Categorical variables are expressed as absolute number of cases and percent proportions.

The ability of biomarkers to predict treatment response was assessed using the caret package in R [44]. Briefly, out-of-sample resampling accuracy was estimated for each biomarker as well as the combination of all biomarkers using 1000 bootstraps with replacement. Continuous variables were centered and scaled. Logistic regression models were used for single biomarker estimates and a regularized ElasticNet model implemented in glmnet was used for estimates containing all biomarkers [45]. Model significance was tested using a one-sided binomial test comparing the estimated model accuracy to the No Information Rate (NIR). The NIR is defined as the largest proportion of observed classes, or the maximum accuracy of a classifier if it predicted the majority class every time.

Sample size calculations were performed using the MKpower package in R. Two-sample Wilcoxon distributions were generated using the mean and standard deviation from our pilot study sample. The normality of the distribution for each biomarker's expression levels were confirmed with a Shapiro-Wilk test. Random sampling from a truncated normal distribution constrained between 0 and 100 was performed for a series of samples sizes ranging from 10 to 120, in intervals of 10. The empirical power (beta) for each sample size was calculated using Monte-Carlo simulations with 1000 iterations for a specified type-I error rate (alpha = 0.05).

RESULTS

Patient demographics

Fifty-three patients were consented for this pilot study. Ten patients were excluded: One patient was diagnosed with Familial Adenomatous Polyposis, one was found to have neuroendocrine tumour pathology, one gastroesophageal junction tumour received alternative neoadjuvant therapy and seven patients were excluded due to inadequate tissue biopsies. Of note, an interim analysis of our protocol after enrolling the first 20 patients determined a biopsy accuracy rate of 60% for treatment naïve specimens and 25% for biopsy following neoadjuvant treatment. This prompted a change in study protocol to retrieve 4-8 tissue biopsies *per* sample.

A total of 43 patients were available for analysis, of which 18 (42%) underwent neoadjuvant chemotherapy during our study period. Baseline demographics are included in Table 1. Median age was 65 (60, 75) and the majority of patients were male (70%). Tumour pathology was represented by all TNM stages but a preponderance of high grade (72%), proximal stomach (60%) and diffuse type (63%) tumours were present. *H. pylori* status was available for 32 patients, of which the majority were negative (69%) and one was previously treated. Total gastrectomy was performed in nearly half of all patients and comprised 59% of all surgical resections.

Expression of biomarkers in normal and cancer tissues

Representative images of each IHC stain within the 75th and 25th percentile of expression is presented in Figure 2B. Staining for E-cadherin was only identified on cell membranes of gastric epithelium.

Table 1 Baseline demographics

Characteristic	*N* = 43[1]	*n*/*N* (missing %)
Age (yr)	65 (60, 75)	43 / 43 (0%)
Sex		43 / 43 (0%)
Female	13 (30%)	
Male	30 (70%)	
Stage		43 / 43 (0%)
I	11 (26%)	
II	10 (23%)	
III	8 (19%)	
IV	14 (33%)	
Grade		43 / 43 (0%)
G1	1 (2.3%)	
G2	10 (23%)	
G3	31 (72%)	
Gx	1 (2.3%)	
Tumour location		43 / 43 (0%)
Distal	14 (33%)	
Proximal	26 (60%)	
Whole stomach	3 (7.0%)	
Lauren classification		41 / 43 (4.7%)
Diffuse	26 (63%)	
Intestinal	13 (32%)	
Mixed	2 (4.9%)	
Signet ring cell (present)	26 (63%)	41 / 43 (4.7%)
H. pylori history		32 / 43 (26%)
Negative	22 (69%)	
Positive	9 (28%)	
Treated	1 (3.1%)	
Smoker		40 / 43 (7.0%)
Yes	9 (22%)	
No	14 (35%)	
Ex	17 (42%)	
Smoker (pack years)	7 (0, 32)	39 / 43 (9.3%)
Surgery		43 / 43 (0%)
Total gastrectomy	20 (47%)	
Distal gastrectomy	14 (33%)	
No resection	9 (21%)	
Neoadjuvant chemotherapy	18 (42%)	43 / 43 (0%)
CD4/CD8 ratio (% positive)	1.7 (1.2, 2.8)	42 / 43 (2.3%)
CD4 (% positive)	14 (7, 24)	43 / 43 (0%)
CD8 (% positive)	8 (5, 11)	42 / 43 (2.3%)
Galectin-3 (% positive)	46 (30, 57)	43 / 43 (0%)

E-cadherin (% positive)	18 (6, 28)	43 / 43 (0%)
E-cadherin H-score	22 (7, 40)	43 / 43 (0%)

[1]Median (IQR); *n* (%).

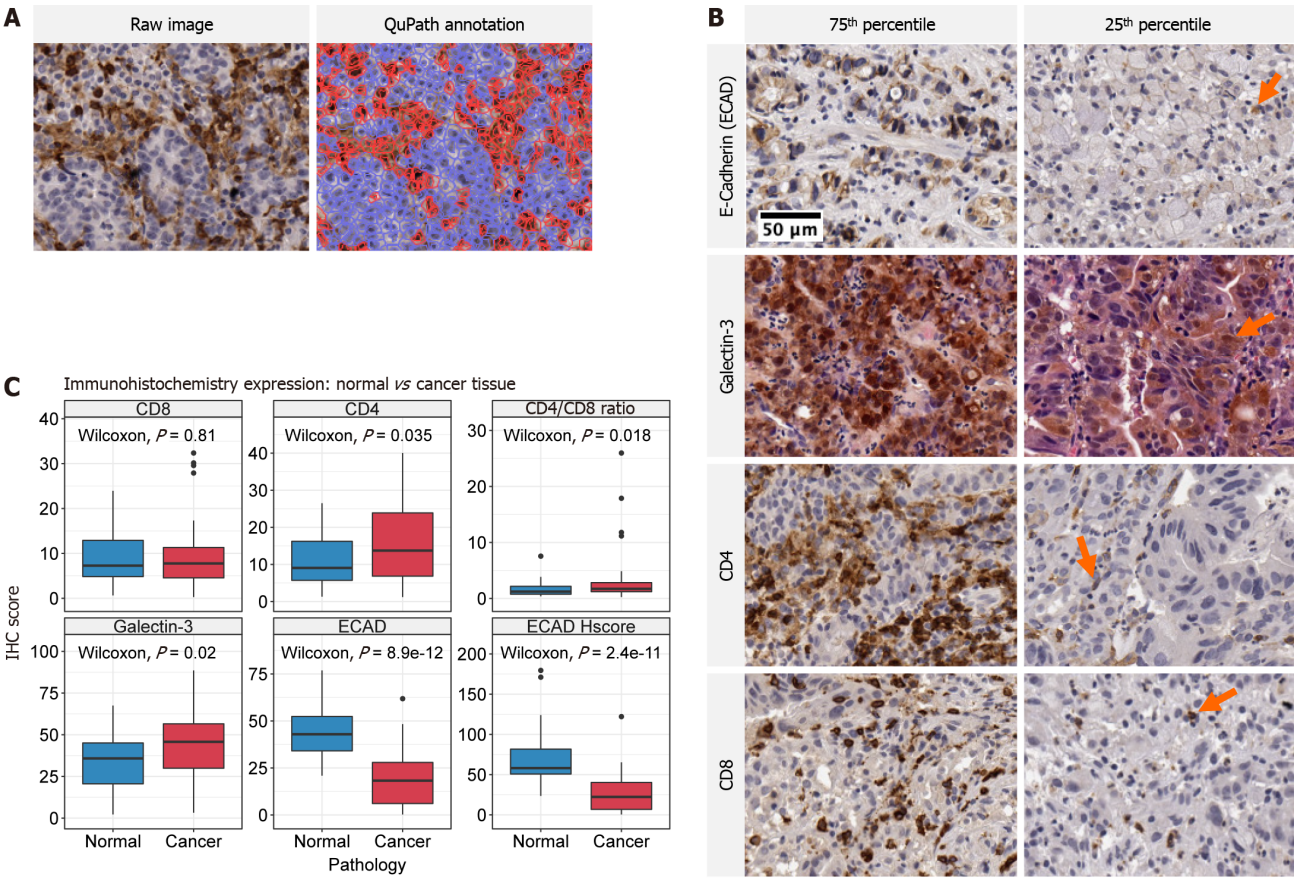

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Figure 2 Immunohistochemistry stains and expression of biomarkers in treatment naïve normal and cancer tissue. A: Representative images of QuPath digital pathology annotation using CD4 immunohistochemistry (IHC) at 10X magnification. Raw images (left) are processed and regions of interest are identified according to our methods. The annotated image (right) demonstrates the calculation of positive stained cells (red) and negative cells (blue); B: Representative IHC images taken at 10X magnification for each respective biomarker identified on the y-axis. Images of expression values within the 75th and 25th percentile are presented in the left and right columns, respectively. Arrows demonstrate positive staining in low expression specimens; C: Boxplot comparison of expression for each respective biomarker in treatment naive normal and cancer tissue. The IHC biomarker is labeled on the heading of each graph. The y-axis represents IHC score, which is the percent of positive stained cells for Galectin-3, CD4, CD8 and E-cadherin and the H-score for E-cadherin H-score plot. The x-axis labels the distribution corresponding to normal (blue) and cancer (red) tissue. The raw *P* value for Wilcoxon tests is annotated in each panel.

Galectin-3 exhibited heterogeneous staining and was identified in nuclei, cytoplasm, and surrounding tumour stroma. The presence of Galectin-3 was often sporadic with distinct regions representing intense positive stain followed by fairly abrupt transition to moderate positivity. CD4 and CD8 positive staining was identified on the cell membrane of lymphocytes.

Galectin-3 was the most abundant molecule with a median expression of 46% (30, 57), followed by E-cadherin, CD4 and CD8 (Table 1). The E-cadherin H-score (median 22 (7, 40)) closely approximated the proportion of E-cadherin positive cells (median 18 (6, 28)). Greater H-score values in the upper quartile reflected the presence of high staining intensity in positive cells.

Significantly increased expression of CD4, Galectin-3 and CD4/CD8 Ratio was identified in cancer tissue relative to normal adjacent tissue controls (Wilcoxon, $P = 0.035$, $P = 0.020$ and $P = 0.018$ respectively) (Figure 2C). The distribution of IHC scores between normal and cancer tissue for CD4 and Galectin-3 was relatively uniform, whereas differences in CD4/CD8 Ratios were dominated by sample outliers with large cancer IHC scores. In agreement with historical study, E-cadherin positivity and H-score was significant decreased in cancer tissue relative to normal. (Wilcoxon, $P < 0.0001$ and $P < 0.001$, respectively).

There were no statistically significant associations between relevant clinicopathologic factors and the expression of any biomarker for stage, lymphovascular invasion, perineural invasion, carcinomatosis, tumour grade or location (Supplementary material). The proportion of E-cadherin positive cells was significantly different according to Lauren Class, with relatively fewer positive cells present in diffuse and mixed type cancers (Kruskal-Wallis, $P = 0.043$).

Association of biomarker expression with exposure to neoadjuvant chemotherapy

We compared the expression of biomarkers in 15 paired tumour samples from the same patient before and after neoadjuvant FLOT to evaluate the effect of treatment on biomarker expression. All pre-treatment specimens were obtained by endoscopic biopsy and thus were restricted mainly to the mucosa and lamina propria. The majority of post-treatment samples were analyzed as TMA cores from surgical resection specimens (TMA cores = 87% *vs* biopsy = 13%) in which residual tumour was present in mucosa, submucosa and muscularis.

We found significantly increased association of tumour cells with $CD4^+$ and $CD8^+$ TILs following neoadjuvant chemotherapy (Paired Wilcoxon, $P = 0.002$ and $P = 0.008$, respectively) (Figure 3A). In contrast, E-cadherin positivity and H-score significantly decreased in post-treatment samples (Paired Wilcoxon, $P = 0.035$ and $P = 0.04$, respectively). This was likely in part due to differences in tumour cell depth of invasion between pre-treatment biopsy and post-treatment TMA cores. CD4/CD8 Ratio expression remained relatively stable within samples except for one patient (Figure 3A).

High CD4/CD8 ratio is associated with treatment response

Figure 3B outlines the relationship of biomarker expression to treatment response between pre- and post-treatment cancer specimens. For all analyses, we observed incomplete response in 14 patients (Partial = 9, Poor or No = 4, Progression to metastasis = 1) and response in 4 patients (Complete = 1, Near Complete = 3). Statistically greater CD4/CD8 Ratios were observed in pre-treatment cancer biopsies compared to incomplete responders (Wilcoxon, $P = 0.025$). Clinicopathologic characteristics were similar between treatment response groups (Table 2).

Next, we explored the utility of individual biomarkers (Models 1-6) and the combination of all biomarkers (Model 7) in predicting treatment response scores (Figure 3C). Given the small sample size and events *per* variable, we used out-of-sample estimates from 1000 bootstraps to limit bias by favouring pessimistic estimates of model accuracy. In this dataset, all biomarkers were effective at predicting incomplete tumour response (Sensitivity range 88-98%) but suffered from poor specificity (range 0-44%). CD4/CD8 Ratio was the only variable that provided significant model performance (Accuracy > NIR, one-sided binomial, $P < 0.001$). The ElasticNet model using CD4/CD8 Ratio, CD4, CD8, Galectin-3 and ECAD H-score as independent variables provided a mean accuracy greater than the NIR but failed to achieve statistical significance ($P = 0.26$).

The optimal glmnet model provided coefficients for all variables despite tuning parameters allowing for L2 regularization (alpha = 0). To guide future studies, we evaluated the contribution of all biomarker variables to the predictive model using the final regularized ElasticNet coefficients (Figure 3D). The absolute value of coefficients found CD4 /CD8 Ratio and Galectin-3 to provide the greatest influence in predicting favourable tumour response. Specifically, tumour response was associated with increasing CD4/CD8 Ratio and decreasing Galectin-3, respectively.

Sample size calculations

To inform future studies we performed sample size calculations using our pilot study sample distributions. In particular, we were interested in identifying the sample sizes required to evaluate the utility of biomarkers in explaining tumour response using a two-sample Wilcoxon test. In Figure 3E, we observe that CD8 and Galectin-3 require similar sample sizes of 30 and ~35 in each treatment response group to achieve adequate power. The relationship between sample size and empirical power was nearly identical for CD4 and E-cadherin, which were calculated to require ~70 and 80 samples in each group, respectively.

DISCUSSION

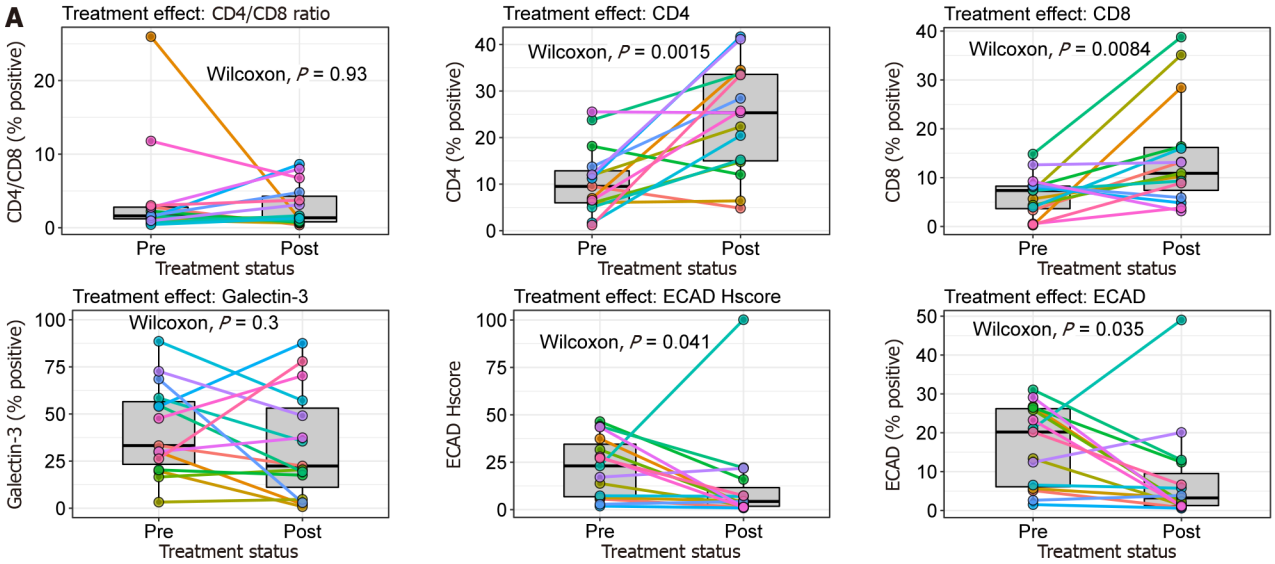
In this pilot study, we present the utility of IHC-based expression of Galectin-3, E-cadherin, CD4 and CD8 in predicting treatment response to the neoadjuvant chemotherapy regimen FLOT4. First, we establish that Galectin-3, CD4, E-cadherin and the CD4/CD8 Ratio expression are significantly different between cancer and normal adjacent tissue. These findings suggest that these markers are intrinsic to the tumour or tumour microenvironment and thus may provide prognostic or predictive yield. Next, we establish that the CD4/CD8 Ratio is significantly greater in tumours with complete or partial response to neoadjuvant chemotherapy. In preliminary univariate and multivariate machine learning models, the CD4/CD8 Ratio was the only significant predictive marker of treatment response with an accuracy of 86%. Finally, we demonstrate that the tumour-specific expression of CD4, CD8 and E-cadherin is significantly modified in paired tumour samples before and after chemotherapy.

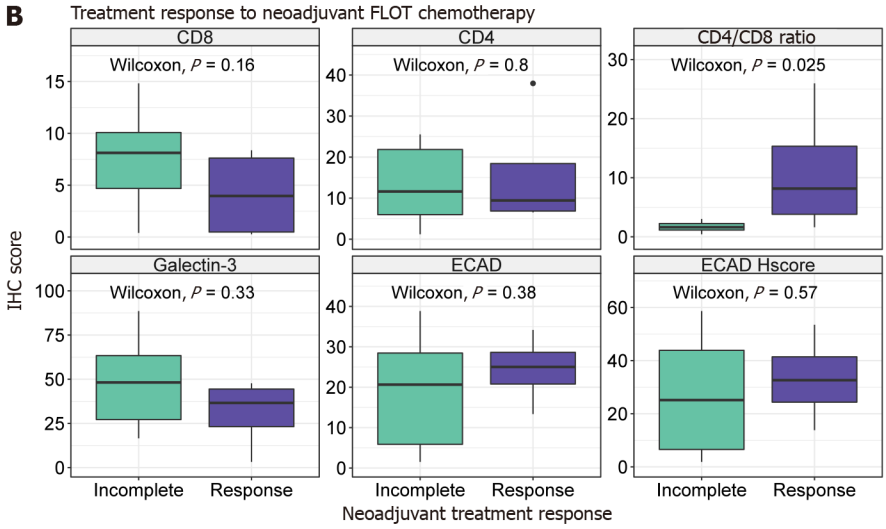
Table 2 Clinicopathologic factors according to treatment response

Characteristic	Incomplete response, *N* = 14[1]	Response, *N* = 4[1]	*P* value[2]
Age (yr)	60 (57, 63)	60 (52, 67)	> 0.9
Sex			0.3
F	5 (36%)	0 (0%)	
M	9 (64%)	4 (100%)	
Stage			0.6
I	2 (14%)	2 (50%)	
II	5 (36%)	1 (25%)	
III	6 (43%)	1 (25%)	
IV	1 (7.1%)	0 (0%)	
Grade			0.6
G1	1 (7.1%)	0 (0%)	
G2	2 (14%)	2 (50%)	
G3	10 (71%)	2 (50%)	
Gx	1 (7.1%)	0 (0%)	
Tumour location			> 0.9
Distal	3 (21%)	1 (25%)	
Proximal	10 (71%)	3 (75%)	
Whole stomach	1 (7.1%)	0 (0%)	
Lauren classification			0.5
Diffuse	10 (71%)	1 (33%)	
Intestinal	4 (29%)	2 (67%)	
Signet ring cell (present)	9 (64%)	1 (33%)	0.5
H. pylori history			> 0.9
Negative	8 (57%)	2 (50%)	
Positive	3 (21%)	1 (25%)	
Unknown	3 (21%)	1 (25%)	
Smoker			> 0.9
Yes	4 (33%)	2 (50%)	
No	4 (33%)	1 (25%)	
Ex	4 (33%)	1 (25%)	
Smoker (pack years)	13 (0, 40)	36 (25, 42)	0.5
Surgery			> 0.9
Total gastrectomy	10 (71%)	3 (75%)	
Distal gastrectomy	3 (21%)	1 (25%)	
No resection	1 (7.1%)	0 (0%)	

[1]Median (IQR); *n* (%).
[2]Wilcoxon rank sum test; Fisher's exact test.

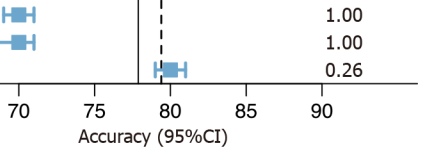
Several potentially useful approaches for determining treatment response have previously been recognized. Clinical or pathologic factors including age, tumour grade, signet cell pathology, serum carcinoembryonic antigen, various circulating lymphocyte populations and tumour size are significant predictors of tumour response[46-48]. The majority of predictive tumour biomarker research in gastric cancer has focused on identifying molecules associated with adjuvant chemotherapy response. For

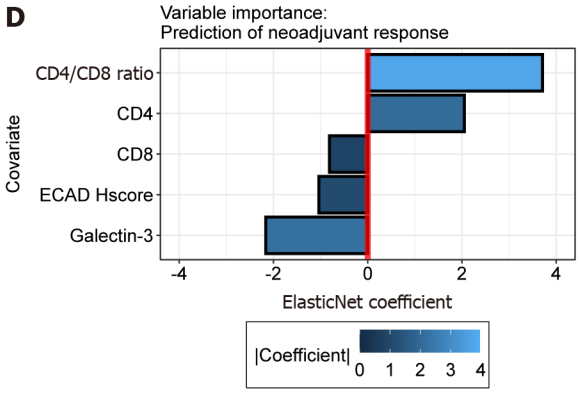




C

Model	Variables	Sensitivity (%)	Specificity (%)	Accuracy (95%CI)	*P* value
1	~ CD4/CD8 ratio	98	44	86 (85,87)	< 0.001***
2	~ CD4	94	0	73 (72,74)	1.00
3	~ CD8	90	16	74 (73,75)	1.00
4	~ Galectin-3	91	6	72 (71,73)	1.00
5	~ ECAD Hscore	89	1	70 (69,71)	1.00
6	~ ECAD	88	3	70 (68,71)	1.00
7	~ All IHC markers	92	31	80 (79,81)	0.26





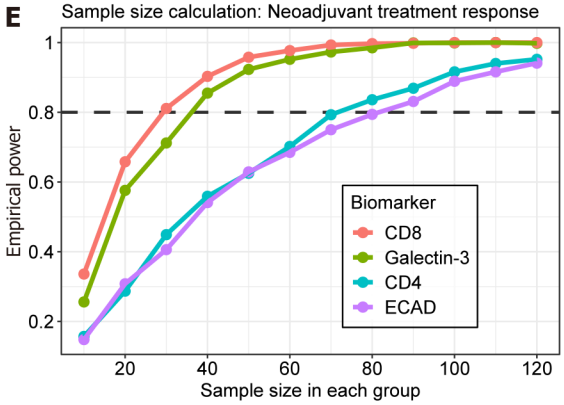


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Figure 3 Association between biomarker expression and neoadjuvant 5-fluorouracil, leucovorin, oxaliplatin and docetaxel chemotherapy.

A: Paired boxplots for biomarker expression pre- and post-neoadjuvant chemotherapy. Each coloured point and line correspond to a single patient. Boxplots in grey

represent the distribution of expression for all patients before and after chemotherapy. Paired Wilcoxon *P* value is present in each plot; B: Boxplot comparison of expression for each respective biomarker between treatment response (purple) and incomplete response (green). The immunohistochemistry (IHC) biomarker is labeled on the heading of each graph. The y-axis represents IHC score, which is the percent of positive stained cells for Galectin-3, CD4, CD8 and E-cadherin and the H-score for ECAD H-score plot; C: Forest plot for metrics of ElasticNet models. Models were constructed using the treatment response as the dependent variable and the corresponding independent variable(s) identified in the variable column. The plot represents the out-of-sample accuracy (blue square) and 95% confidence intervals (whiskers) for models estimated from 1000 bootstraps with replacement. The no information rate, defined as the maximum accuracy of a classifier if it predicted the majority class every time, is shown by the solid and dotted vertical lines for univariable and multivariable models, respectively; D: Barplot of model coefficients from multivariable glmnet model. The y-axis represents model covariates and the x-axis the coefficient value. Treatment response is related to increasing covariate values or decreasing covariate values for positive and negative coefficients, respectively. The absolute importance of the coefficient is shown in blue according to the scale legend; E: Lineplot illustrating monte-carlo simulations for two-sample Wilcoxon sample size calculations. The y-axis is the empirical power and the x-axis is the sample size in each group. Each coloured line corresponds to a biomarker labelled according to the legend.

example, a multivariable model utilizing the measurement of several TIL populations in 879 patients provided 3-year survival prediction accuracies of 79 and 84% for surgery alone and adjuvant chemotherapy populations, respectively[34]. In the neoadjuvant setting, a post-hoc analysis of 83 patients in the COMPASS trial identified several candidate gene expression based-biomarkers such as *TIMP1* and *DSG2* using quantitative real-time polymerase chain reaction[49]. Other studies to identify treatment response have used microRNAs, exosomes, inflammatory markers or medical imaging data [50]. Although predictive and prognostic factors identified in these studies show promise, there is limited external validity of these studies and clinical implementation is yet to be achieved.

This is the first study to evaluate the role of tumour-associated CD4/CD8 Ratio in gastric cancer neoadjuvant chemotherapy response. Increasing evidence has demonstrated the coordinated role of $CD4^+$ and $CD8^+$ T-cells in mediating tumour immune surveillance, immunotherapy response and cancer prognosis[51]. Sustained and effective tumour immune response requires $CD4^+$ T-cells, which potentiate effector $CD8^+$ response *via* secretion of cytokines such as interleukin-2, participate in direct anti-tumour effects *via* interferon-gamma and tumour necrosis factor, or facilitate antibody mediated humoral response from B-cells *via* CD40 Ligand binding[52]. Indeed, research evaluating chimeric antigen receptor (CAR) T-cell immune populations demonstrate increased anti-tumour activity with increasing CD4/CD8 ratio[53]. Yang *et al*[54] also demonstrated that $CD4^+$ CAR T cells are more effective at maintaining anti-tumour activity *in vivo* compared to CD8+ CAR T cells that are prone to exhaustion and apoptosis. Furthermore, in native tumour microenvironments increasing CD4/CD8 Ratio of the tumour-host interface in triple negative breast cancer is associated with improved overall and recurrence-free survival[51].

The dynamic increase in TIL expression following neoadjuvant chemotherapy in our pilot study also replicates previous findings. Significant work in breast cancer has implicated the pattern of TIL changes following chemotherapy to treatment response. In particular, greater $CD4^+$ T-cell expression is associated with pathologic complete response[55]. Also, decreased immune infiltration is a notable characteristic of residual tumours following neoadjuvant chemotherapy relative to pre-treatment biopsy [55]. Continued evaluation of the relationship of dynamic changes in CD4 and CD8 populations in gastric cancer are required to fully leverage these biomarkers.

Our study design is intended to provide a reproducible and externally valid method of biomarker analysis. Using IHC allows for easier clinical implementation given that common pathology workflows already include IHC analysis. Our use of open-source digital pathology software such as QuPath also provides a standardized basis to internally and externally validate our method in future studies. Digital pathology allows annotation and measurement of regions of interest within the software and thus eliminates the need for complex physical microdissection utilized in other biomarker studies.

The main limitation of this study is the low enrollment of curative intent patients. This is likely due to low disease incidence in our population but also may be related to the severe acute respiratory syndrome coronavirus 2 pandemic. Given our rate of patient enrollment, future study should prioritize increasing sample size by using a retrospective design in order to provide more accurate estimates for future multi-centre prospective study. Our sample size calculation suggests that a limited retrospective study with approximately eighty-five patients in each group will provide adequate power to assess these relationships.

CONCLUSION

The CD4/CD8 Ratio is a promising IHC-based biomarker with therapeutic implications for response to neoadjuvant chemotherapy in locally advanced gastric cancer. Future inquiry should focus on evaluating the prognostic value of these markers and the generation of a sufficient sample size to establish a predictive model for potential future clinical use.

ARTICLE HIGHLIGHTS

Research background

Neoadjuvant chemotherapy for gastric cancer is standard of care in western nations. Despite optimal therapy, only 40% of patients achieve complete or near complete treatment response. Treatment response following neoadjuvant chemotherapy is associated with overall survival. Thus, it is of critical importance to identify biomarkers capable of predicting which patients will achieve a favourable response to neoadjuvant chemotherapy in order to optimize survival outcomes.

Research motivation

Personalized medicine is predicated on providing the right treatment for the right patient at the right time. To achieve optimal outcomes treatment regimens now include complex decision-making processes surrounding the timing of chemotherapy and surgery. Recent research has demonstrated that some gastric cancer patients, such as those with tumours harbouring microsatellite instability, may be harmed by neoadjuvant chemotherapy. However, patients that achieve a good treatment response achieve superior clinical outcomes compared to adjuvant chemotherapy. Identifying specific subpopulations using tumour-based biomarkers is of critical importance to maximize outcomes.

Research objectives

We sought to characterize the expression of tumour immunohistochemistry (IHC)-based biomarkers CD4, CD8, Galectin-3 and E-cadherin in our Canadian population. Specifically, we evaluated these markers in comparison to their expression in normal gastric mucosa, as well as their relationship to neoadjuvant chemotherapy tumour response scores and expression in tumour biopsies before and after treatment. We successfully identified a biomarker, namely the CD4/CD8 T-cell ratio, with the potential to predict favourable treatment response. This pilot study serves as a foundation for future study to validate our preliminary findings.

Research methods

In this study, we evaluated IHC -based biomarkers in human gastric cancer specimens. Informed consent according to an approved ethics protocol was obtained for all patients. Samples were retrieved from endoscopic biopsy prior to treatment with neoadjuvant, adjuvant or palliative chemotherapy, as well as from pathology specimens following surgical resection. Using IHC, we quantified the expression of CD4, CD8, Galectin-3 and E-cadherin in gastric cancer tumours and adjacent normal mucosa. Quantification was performed on digitally scanned images using QuPath, which is an open-source and artificial intelligence-based digital pathology program. Statistical analysis was completed using R. Sample size calculations were performed using the MKpower package in R.

Research results

We demonstrate that an elevated CD4/CD8 ratio in gastric cancer tumours is significantly associated with complete or near complete response following FLOT chemotherapy. We identify that neoadjuvant chemotherapy is associated with increased infiltration of CD4 and CD8 T-cells in 15 paired samples assessed before and after exposure to chemotherapy. However, the dynamic increase in these lymphocyte populations does not associate with an increased CD4/CD8 ratio. To expand on the findings of this study, we performed a sample size calculation and identified that CD4, CD8, Galectin-3 and E-cadherin expression may be adequately evaluated with a future study population of 85 patients.

Research conclusions

For the first time, we identify that a high CD4/CD8 ratio within gastric cancer tumours is a promising biomarker that predicts favourable tumour response scores following neoadjuvant FLOT chemotherapy. To achieve this result, we use digital pathology technology and artificial intelligence-based quantification of biomarker staining.

Research perspectives

This study serves as a foundation for future research in validating the CD4/CD8 ratio as a reliable biomarker that is capable of predicting neoadjuvant treatment response. Our sample size calculations provide a framework for future study design.

FOOTNOTES

Author contributions: Skubleny D, McCall M, Ghosh S, Spratlin JL, Schiller D and Rayat G designed and coordinated the study; Skubleny D, Lin A, Garg S, McLean R performed experiments, generated and analyzed data; McLean R evaluated biopsy pathology and presence of cancer; Skubleny D, Ghosh S, Schiller D and Rayat G interpreted the data; Skubleny D wrote the manuscript with editorial assistance from Ghosh S, Spratlin JL, Schiller D and Rayat G.

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Data sharing statement: Raw data and code are available from the corresponding author at skubleny@ualberta.ca.

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ORIGINAL ARTICLE

Basic Study

microRNA-627-5p inhibits colorectal cancer cell proliferation, migration and invasion by targeting Wnt2

Dong-Yan Zhao, Teng-Fei Yin, Xi-Zhen Sun, Yuan-Chen Zhou, Qian-Qian Wang, Ge-Yujia Zhou, Shu-Kun Yao

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Abstract

BACKGROUND

microRNA-627-5p (miR-627-5p) dysregulation has been observed in several cancer types, such as hepatocellular carcinoma, oral squamous cell carcinoma, glioblastoma multiforme, and gastric cancer. The biological function of miR-627-5p in colorectal cancer (CRC) growth and metastasis is yet unclear.

AIM

To investigate the effects of miR-627-5p on the malignant biological properties of colorectal malignant tumour cells by targeting Wnt2.

METHODS

The levels of miR-627-5p in colorectal tumour tissues were assessed in Gene Expression Omnibus datasets. In order to identify Wnt2 transcript expression in CRC tissues, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used. Luciferase reporter tests were used to explore whether miR-627-5p might potentially target Wnt2. Wnt2 transcript and protein levels were detected in

CRC cells with high miR-627-5p expression. To learn more about how miR-627-5p affects CRC development, migration, apoptosis, and invasion, functional experiments were conducted. Cotransfection with the overexpression vector of Wnt2 and miR-627-5p mimics was utilized to verify whether overexpression of Wnt2 could cancel the impact of miR-627-5p in CRC. Western blot and qRT-PCR were conducted to investigate the effects of miR-627-5p on the Wnt/β-catenin signalling pathway.

RESULTS

miR-627-5p was notably decreased in colorectal tumour tissues, while the gene level of Wnt2 was notably upregulated. A dual luciferase reporter assay revealed that miR-627-5p specifically targets the 3′-untranslated regions of Wnt2 and miR-627-5p upregulation markedly reduced the protein and gene expression of Wnt2 in CRC cells. *In vitro* gain-of-function assays displayed that miR-627-5p overexpression decreased CRC cells' capabilities to invade, move, and remain viable while increasing apoptosis. Wnt2 overexpression could reverse the suppressive functions of miR-627-5p. Moreover, upregulation of miR-627-5p suppressed the transcript and protein levels of the downstream target factors in the canonical Wnt/β-catenin signalling, such as c-myc, CD44, β-catenin, and cyclinD1.

CONCLUSION

miR-627-5p acts as a critical inhibitory factor in CRC, possibly by directly targeting Wnt2 and negatively modulating the Wnt/β-catenin signalling, revealing that miR-627-5p could be a possible treatment target for CRC.

Key Words: miR-627-5p; Wnt2; Colorectal cancer; β-catenin; Progression

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Core Tip: It has been well established that miRNAs play vital roles in modulating cancer-related pathways, thereby regulating colorectal cancer (CRC) growth and metastasis. The study comprehensively explored the function of microRNA-627-5p (miR-627-5p), a rarely reported miRNA in CRC. miR-627-5p mimics restrained CRC cells invasion, migration, proliferation, and promoted cell apoptosis, indicated its suppressive effects on CRC development. A dual luciferase reporter test showed miR-627-5p directly binds with the 3'-untranslated region of Wnt2. Furthermore, miR-627-5p prevented the aggressive behaviours of cancer cells *via* inhibiting the activation of the canonical Wnt signalling. Strategies targeting the miR-627-5p/Wnt2/β-catenin signalling might be a new treatment option for CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent malignant tumours and has high incidence and mortality rates globally, posing a great threat to the health of human beings[1]. Despite great advances in the diagnosis and treatment of CRC over the span of a few decades, CRC is still incurable and often has a poor prognosis, especially in patients with advanced tumours, which necessities clarification of the underlying mechanisms of CRC[2,3]. Generally, a vast number of genetic and molecular changes, such as epigenetic aberrations, genetic alterations, microsatellite instability, and chromosomal instability, have been shown to be correlated with colorectal carcinogenesis and tumour metastasis[4,5]. All of these genomic events can contribute to the activation of important signalling pathways (Wnt/β-catenin, MAPK/PI3K, and SMAD/TGF-β) and the initiation of colorectal tumorigenesis[6]. Since the human genome has been extensively characterized, miRNAs have become a hot spot and have offered fresh understanding of the molecular mechanisms underpinning CRC progression[7].

microRNA (miRNA) is an endogenously derived non-coding RNA sequence composed of 19-25 nucleotides that can modulate the levels of downstream genes through direct binding to their 3′-untranslated regions (3′-UTRs) *via* cleavage or translational arrest[8]. Many published papers have implicated the role of microRNA-627-5p (miR-627-5p) in controlling the emergence and development of

a number of tumours[9-12]. For instance, Wang *et al*[9] reported that miR-627-5p acted as a inhibitory factor by reducing the expression of Bcl3 in hepatocellular carcinoma. miR-627-5p was also identified to be involved in suppressing the malignant behaviors of glioma cells by binding to the 3'-UTR of NR2C2 and downregulating its expression in glioblastoma multiforme[10]. These studies indicated the tumour inhibitory function of miR-627-5p. Furthermore, Shin *et al*[11] found miR-627-5p was observably upregulated in gastric cancer tissues than in normal controls, suggesting that miR-627-5p might stimulate gastric cancer progression. In other words, miR-627-5p may play different roles in different cancer types. A growing body of evidence has emphasized that abnormally expressed miRNAs play vital roles in promoting or suppressing the progression from normal to hyperproliferative epithelium, then to precancerous advanced adenoma (AA), and later invasive adenocarcinoma[13-15]. Nevertheless, the functions of miR-627-5p are largely unknown in colorectal carcinogen.

In the present study, TargetScan website speculated that miR-627-5p could be complementary with the 3'-UTR of Wnt2. Wnt2, an evolutionarily conserved secreted-type glycoprotein secreted by the Wnt signalling, performs a crucial role in promoting the malignant progression of gastrointestinal cancers through activation of the Wnt/β-catenin signalling[16-18]. Consequently, the current study's goal was to investigate how miR-627-5p and Wnt2 contribute to the emergence of CRC, and to assess the relationship between them.

MATERIALS AND METHODS

Bioinformation analysis

From the Gene Expression Omnibus (GEO) database, the original series matrix files of the miRNAs of patients with colorectal tumours and healthy controls (HCs) were gathered. GSE41655 and GSE18392 were employed to analyze the differential expression of miR-627 between CRC tissues and control tissues. GSE41655 contained 33 tumour tissues and 15 normal control tissues while GSE18392 included 116 tumour tissues and 29 control tissues.

Human tissue collection

A total of 30 patients with colorectal tumours, 33 AA and 20 HCs aged between 18 and 80 years were employed to explore the tissue levels of Wnt2 in the study. All patients diagnosed by histology as AA and colorectal adenocarcinoma were enrolled as case group. All healthy participants were recruited through advertisements and screened by careful history taking, physical examinations, essential laboratory examinations and colonoscopy. Individuals with negative colonoscopy results were selected as HC group. During the endoscopies, biopsy samples were taken from the rectosigmoid colon in the HCs. The excluding protocol for all subjects were indicated below: (1) Subjects with a history of other major organic diseases, malignant tumours in other organs, and psychiatric disorders; (2) pregnant or lactating female subjects; (3) patients with hereditary CRC or hereditary intestinal polyposis syndromes; (4) participants with a history of radiotherapy, chemotherapy, and major abdominal surgery; and (5) patients who presented with inflammatory or infective diseases. The clinicopathological characteristics of HCs and colorectal neoplasm patients are described in Supplementary Table 1. China-Japan Friendship Hospital's ethics committee gave the study's protocol approval under the number 2018-116-K85-1, and all participants gave their written informed permission.

Cell culture

The American Type Culture Collection was used to obtain a colonic epithelial cell line (FHC), human colonic malignant tumor cell lines (HCT116, RKO, and SW480), and a human embryonic kidney cell line (HEK-293T). For the purpose of investigating Wnt2 mRNA expression, FHC and three CRC cell lines were used. Gain of function tests were carried out using HCT116 and SW480 cells. Dual luciferase reporter tests were performed using HEK-293T cells because of their high transfection efficiency[19]. Incubation conditions included 5% CO_2 in the air and a minimum relative humidity of 95%, for the RPMI 1640 medium or Dulbecco's Modified Eagle's Medium in which cells were placed.

Cell transfection

miR-627-5p mimics, mimics negative control oligonucleotides (NC mimics), Wnt2 overexpressing plasmids (pcDNA-Wnt2) and matched negative controls (pcDNA-NC) were designed by GenePharma (Shanghai Province, China). In 6-well culture plates, SW480 and HCT116 cells were plated for 24 h. Following the manufacturer's instructions, LipofectamineTM 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, United States) was used to transfect a mixture of miR-627-5p mimics or NC mimics (150pmol) with pcDNA-Wnt2 or pcDNA-NC (3 g). In Supplementary Table 2, the oligonucleotide sequences are displayed.

Quantitative real-time polymerase chain reaction

Total RNA that included miRNA from tissues or cells was isolated by the RNAprep Pure Cell Kit

(Solarbio, Beijing, China). cDNA was obtained using the Hifair®II 1st Strand cDNA Synthesis Kit (YESEN, Shanghai, China). PCR amplification was conducted in a LineGene 9600 Plus Real-Time PCR system (Bioer Technology, Hangzhou, China) by using Hieff® qPCR SYBR® Green Master Mix (No Rox) (YESEN, Shanghai, China). The comparative threshold method was used to quantify the relative expression of miRNAs and mRNAs. In Supplementary Table 3, the primer sequences used in the study are displayed.

Western blotting

RIPA lysis buffer (Beyotime, Shanghai, China) was used for protein extraction, and the BCA Protein Assay Kit was used to measure the amount of protein (Solarbio, Beijing, China). Total proteins were then subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis and electrotransferred to PVDF membranes (Bio-Rad, Hercules, CA, United States). Nonspecific binding sites were blocked using 5% nonfat milk for 2 h and the membranes were incubated at 4 °C overnight with rabbit anti-Wnt2 antibody (1:1000; Affinity, Melbourne, United States), rabbit anti-c-myc antibody (1:1000; Bioss, Beijing, China), rabbit anti-CD44 antibody (1:1000; Affinity, Melbourne, United States), rabbit anti-cyclin D1 antibody (1:1000; Abcam, Cambridge, United Kingdom), and rabbit anti-β-tubulin antibody (1:4000; Proteintech, Chicago, United States). The PVDF membranes were then treated for 1 hour with a goat anti-rabbit secondary antibody that was HRP conjugated (1:5000; Bioss, Beijing, China). Enhanced chemiluminescence reagents (Beyotime, Shanghai, China) were used to visualize the bands. Image J was utilized to quantify the chemiluminescent signals of protein bands using β-tubulin as an internal control.

Cholecystokinin octapeptide assay

Cell viability was monitored using cholecystokinin octapeptide (CCK-8) reagents (Solarbio, Beijing, China). After transfection, CRC cells plated in 96-well plates were added to CCK-8 solution and incubated for 1 h. The number of viable cells was determined at a 24 h interval for four consecutive days following the manufacturer's instructions.

Matrigel invasion assay

Corning Transwell insert chambers (Corning Incorporated, New York, NY, United States) with a 6.5-μm pore size were used to assess invasive capability. Cancer cells were planted in the upper chamber, cultured with foetal bovine serum (FBS) free medium, and allowed to invade for 72 h. The lower chamber was added to culture medium comprising 10% FBS to attract the invaded cells. The invading cells that broke through the Matrigel were then fixed in paraformaldehyde, stained in crystal violet, and counted in five randomly selected high-power fields.

Scratch assay

Homogeneous single cell suspensions were plated in 6-well plates until a single layer formed before being wounded by scraping a straight line with a yellow micropipette tip. The plates were incubated with complete medium after 3 PBS solution washes. All lengthy wounds were captured on camera at 0 and 24 h after the wound.

Flow cytometry analysis

An Annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis kit (7seabiotech, Shanghai, China) was utilized to detect the proportion of apoptotic cells after transfection. All processes were performed following the manufacturer's protocols. Flow cytometry (BeckMan, United States) and FlowJo™ (Becton, New York, NY, United States) software were used to determine the cell apoptosis rates. Detailed experimental procedures are described in our previous study[20].

Dual luciferase reporter assay

The human Wnt2 3′-UTR comprising the expected complementary site of miR-627-5p (wild type), and its identical sequence with the mutant sequences of specific complementary sites of miR-627-5p (mutant) were inserted into the pmirGLO luciferase vector. HEK-293T cells were cotransfected with pmirGlo-Wnt2 3′-UTR wild type or pmirGLO-Wnt2 3′-UTR mutant and miR-627-5p mimics. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States).

Statistical analysis

All data are shown as mean ± SD or median (interquartile range). The Shapiro-wilk test was used to verify the normal distribution. Student's *t* test or Wilcoxon rank-sum test were employed to decide significant differences between two groups where appropriate. Spearman correlation analysis was used to calculate the relationship between Wnt2 gene expression and miR-627-5p expression in colorectal neoplasm tissues. All calculations were conducted with IBM SPSS (Chicago, IL, United States) and diagrams were described using GraphPad Prism (La Jolla, CA, United States). A *P* value of 0.05 or lower was considered significant.

RESULTS

The expression levels of miR-627 in CRC tissues

The miR-627 levels were contrasted between the CRC subgroup and the normal subgroup in two GEO datasets, GSE41655 (33 CRCs *vs* 15 HCs) and GSE18392 (116 CRCs *vs* 29 HCs), and the findings showed that CRC tissues had noticeably lower levels of miR-627 (Figure 1A and B). The AUCs of miR-627 in the GSE41655 and GSE18392 datasets were 0.90 ($P < 0.001$) and 0.67 ($P = 0.006$), respectively, according to receiver operating characteristic analysis (Figure 1C and D).

Relationship between miR-627-5p and Wnt2 expression in colorectal neoplasm tissues

The mRNA levels of Wnt2 in clinical tissues or in the colonic epithelial cells and cancer cells were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The findings indicated Wnt2 expression were sequentially upregulated from HC tissues and AA tissues to CRC tissues (Figure 2A). This observation was supported by the fact that Wnt2 expression in cancer cells was observably higher than it was in epithelial cell line (FHC) cells (Figure 2B). Our previous study examined miR-627-5p expression in the same clinical tissues[20] and we next analyzed the relationship among the expressions of miR-627-5p and Wnt2 in colorectal neoplasm tissues and found an inverse relationship between miR-627-5p and Wnt2 gene levels ($r = -0.61$, $P < 0.001$, Figure 2C).

Direct binding relationship between miR-627-5p and Wnt2

First, the possible miR-627-5p downstream genes were speculatively identified using the TargetScan website. As a result, a sequence located at bases 1287-1293 of the Wnt2 3′-UTR was binding to the seed sequence of miR-627-5p (Figure 3A). To verify that miR-627-5p and Wnt2 have a direct complimentary interaction, we conducted dual luciferase reporter assays. Then, the Wnt2 pmirGLO vector comprising wild type or mutant miR-627-5p target sites was constructed (Figure 3A). Then, the constructed vectors were transfected into 293T cells with the cotransfection of miR-627-5p mimics or scrambled controls. miR-627-5p overexpression reduced the luciferase levels of the reporter vector carrying the wild type sequence of Wnt2 3′-UTR (0.58 ± 0.04 *vs* 1.00 ± 0.05, $P < 0.001$), but not those of the reporter vector comprising the mutant target sequence in 293T cells (1.12 ± 0.09 *vs* 1.03 ± 0.11, $P = 0.35$, Figure 3B). Moreover, gain-of-function experiments were performed by transfection of miR-627-5p mimics in SW480 and HCT116 cells to verify whether miR-627-5p could influence the expression levels of Wnt2 in CRC cells. RT-PCR analysis demonstrated that miR-627-5p mimics markedly increased miR-627-5p level in SW480 (35.90 ± 3.09 *vs* 1.02 ± 0.20, $P < 0.001$) and HCT116 cells (31.30 ± 5.14 *vs* 1.02 ± 0.23, $P < 0.001$, Figure 3C). As depicted in Figure 3D-E, upregulation of miR-627-5p directly reduced the transcript expression (SW480, 0.09 ± 0.03 *vs* 1.01 ± 0.16, $P < 0.001$; HCT116, 0.02 (0.01-0.31) *vs* 0.93 (0.91-1.16), $P < 0.001$) and protein expression (SW480, 0.81 ± 0.01 *vs* 1.17 ± 0.10, $P = 0.004$; HCT116, 1.03 ± 0.01 *vs* 1.26 ± 0.03, $P < 0.001$) of Wnt2 in SW480 and HCT116 cells. In summary, miR-627-5p functions as a specific complement to Wnt2.

Role of miR-627-5p in CRC cells

Then, we investigated the biological function of miR-627-5p through gain-of-function tests in SW480 and HCT116 cells. As depicted in Figure 4A and D, wound healing assays showed that miR-627-5p overexpression contributed to a weakened ability of migrating cells (SW480, 23.63% ± 9.62% *vs* 139.11% ± 29.36%, $P < 0.001$; HCT116, 36.03% ± 15.15% *vs* 168.69% ± 31.75%, $P < 0.001$). Matrigel invasion assays demonstrated that exogenetic upregulation of miR-627-5p markedly blocked cancer cells' ability to invade (SW480, 112.00 ± 39.77 *vs* 236.20 ± 33.10, $P = 0.001$; HCT116, 144.60 ± 35.78 *vs* 335.20 ± 14.02, $P < 0.001$; Figure 4B and E). Next, we used flow cytometry analysis to verify whether miR-627-5p overexpression could influence cell apoptosis and the findings showed that miR-627-5p upregulation accelerated cell apoptosis (SW480, 33.91% ± 5.61% *vs* 17.08% ± 1.40%, $P = 0.007$; HCT116, 42.15% ± 1.00% *vs* 21.35% ± 0.61%, $P < 0.001$, Figure 4C and F). Furthermore, according to CCK-8 experiments, miR-627-5p overexpression attenuated cell growth (Figure 4G). Collectively, miR-627-5p inhibits CRC cells migration, invasion, and proliferation but promotes cell apoptosis.

Impact of the miR-627-5p/Wnt2 axis on the malignant behaviours of CRC cells

To explore the functional effects of the miR-627-5p/Wnt2 axis in CRC cells, we generated a Wnt2 overexpression vector (pcDNA-Wnt2) and designed rescue assays. As presented in Figure 5, pcDNA-Wnt2 effectively increased the gene [SW480, 44.84 ± 5.98 *vs* 1.00 ± 0.08, $P < 0.001$; HCT116, 51.39 (45.06-56.67) *vs* 1.15 (0.63-1.39), $P < 0.001$] and protein (SW480, 3.73 ± 0.16 *vs* 2.70 ± 0.10, $P = 0.001$; HCT116, 1.32 ± 0.01 *vs* 1.04 ± 0.05, $P = 0.001$) expression of Wnt2 in SW480 and HCT116 cells, proving that the construction of this vector was successful. Then, we used it to transfect CRC cells overexpression miR-627-5p. The results of wound healing and Matrigel invasion assays revealed that upregulation of Wnt partially canceled the suppressive functions of miR-627-5p on cell migration [SW480, 102.95% (96.05%-132.64%) *vs* 22.22% (15.48%-32.49%), $P = 0.008$; HCT116, 116.23% ± 20.46% *vs* 36.03% ± 15.15%, $P < 0.001$] and invasion (SW480, 265.80 ± 36.89 *vs* 112.00 ± 39.77, $P < 0.001$; HCT116, 322.00 ± 28.61 *vs* 144.60 ± 35.78, $P < 0.001$, Figure 4A, B, D, and E). Besides, flow cytometry analysis demonstrated that cell apoptosis

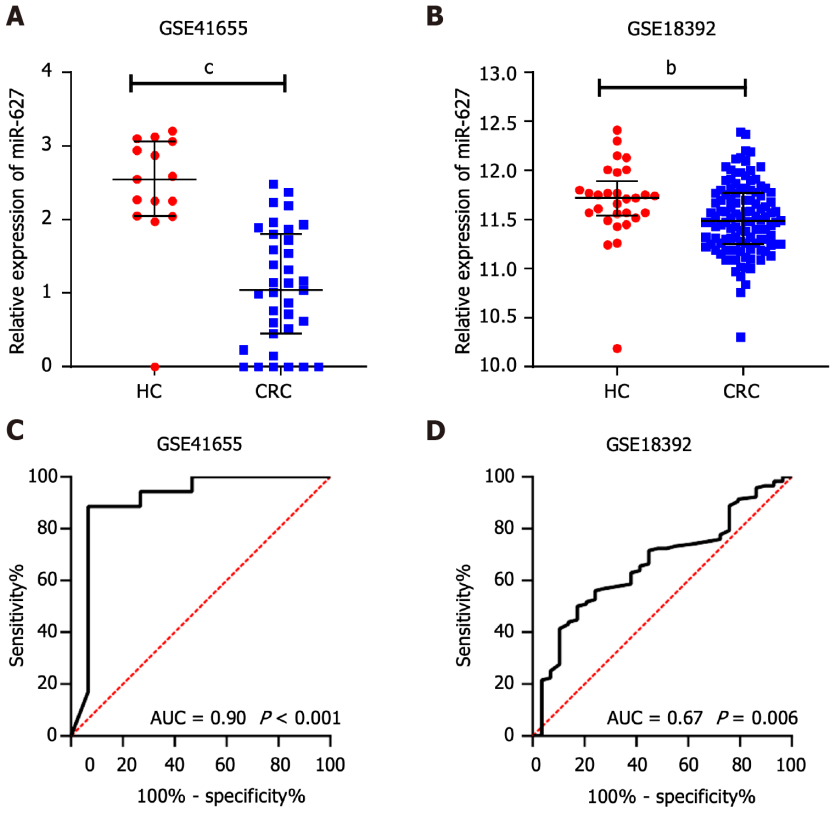

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Figure 1 Evaluation of the tissue expression and diagnostic utility of microRNA-627 in the GSE41655 and GSE18392 datasets. A: Tissue expression of microRNA-627 (miR-627) in the healthy controls (HCs) and colorectal cancer (CRC) patients in the GSE41655 dataset; B: Tissue expression of miR-627 in the HCs and CRC patients in the GSE18392 dataset; C: Receiver operating characteristic analysis of miR-627 to distinguish CRC patients from HCs in the GSE41655 dataset; D: Receiver operating characteristic analysis of miR-627 to distinguish CRC patients from HCs in the GSE18392 dataset. HCs: Healthy controls; CRC: Colorectal cancer. $^{b}P < 0.01$; $^{c}P < 0.001$.

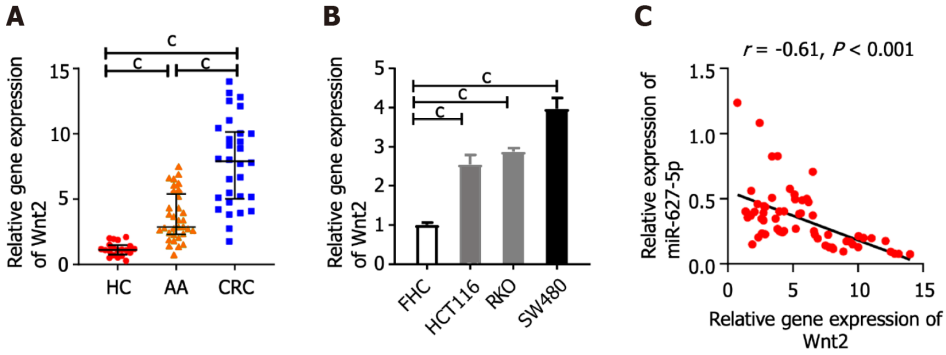

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Figure 2 Inverse correlation between Wnt2 and microRNA-627-5p expression in colorectal neoplasm tissues. A: The mRNA expression levels of Wnt2 in healthy control tissues, advanced adenoma tissues and colorectal cancer (CRC) tissues; B: The mRNA expression levels of Wnt2 in CRC cell lines (SW480, HCT116, and RKO cells) and epithelial cell line cell line; C: The relationship between miR-627-5p and Wnt2 mRNA expression in colorectal neoplasm tissues. HCs: Healthy controls; CRC: Colorectal cancer; AA: Advanced adenoma; FHC: Colonic epithelial cell line. $^{c}P < 0.001$.

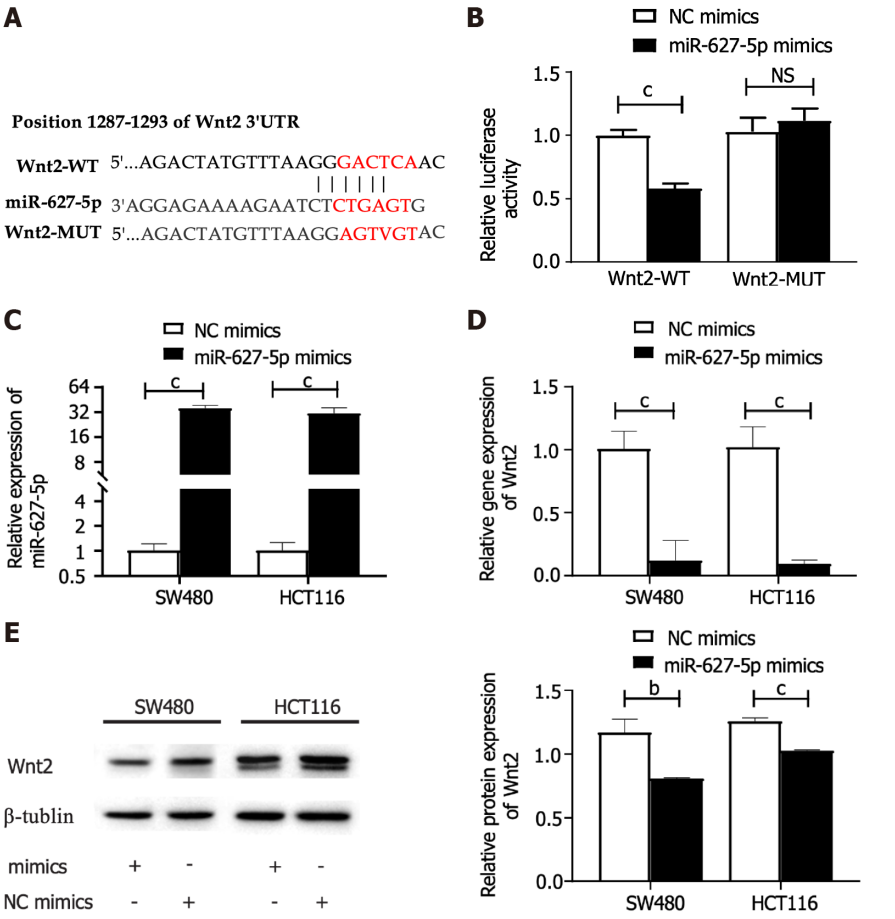

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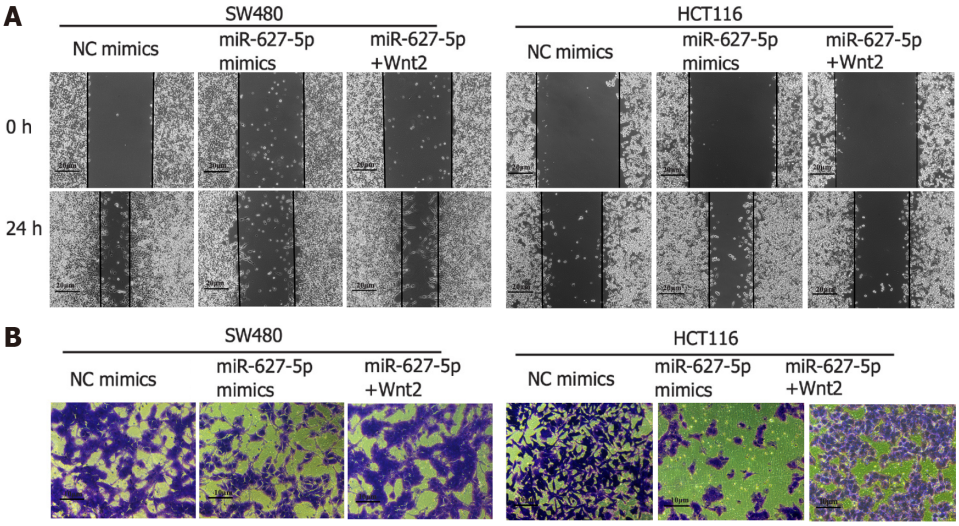
Figure 3 The direct binding relationship between microRNA-627-5p and Wnt2. A: Schematic illustration of the predicted binding sites between microRNA-627-5p (miR-627-5p) and Wnt2 mRNA; B: Dual luciferase reporter assays in HEK-293T cells. Experimental group: NC mimics + pcDNA-Wnt2-WT, miR-627-5p mimics + pcDNA-Wnt2-WT, NC mimics + pcDNA-Wnt2-MUT, miR-627-5p mimics + pcDNA-Wnt2-MUT; C: The transfection efficiency of miR-627-5p mimics in SW480 and HCT116 cells; D: The effects of miR-627-5p overexpression on the transcript expression levels of Wnt2 in SW480 and HCT116 cells; E: The effects of miR-627-5p overexpression on the protein expression levels of Wnt2 in SW480 and HCT116 cells. 3' UTR: 3'-untranslated region; NS: Not significant; $^{b}P < 0.01$; $^{c}P < 0.001$.



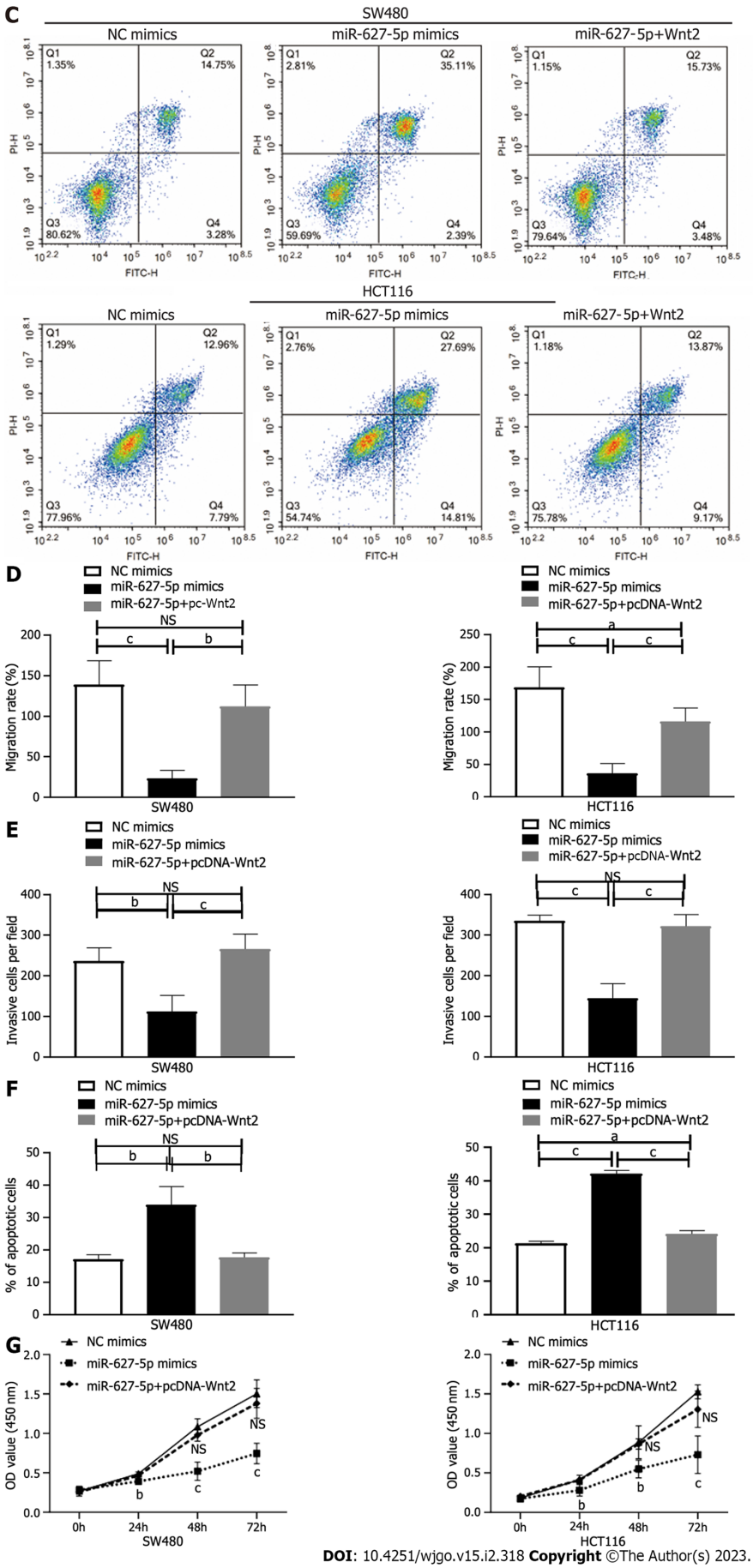


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Figure 4 Cellular behaviours induced by microRNA-627-5p mimics and Wnt2 overexpression plasmids in SW480 and HCT116 cells.

Experimental group: NC mimics, microRNA-627-5p (miR-627-5p) mimics, miR-627-5p mimics + pcDNA-Wnt2. A and D: Scratch assay was used to detect the migration of colorectal cancer (CRC) cells in each group; B and E: Matrigel invasion assay was used to detect the invasive capability of CRC cells in each group; C and F: Flow cytometry analysis was used to evaluate the apoptosis of CRC cells in each group; G: Cholecystokinin octapeptide (CCK-8) assay was used to detect the viability of CRC cells in each group. NS: Not significant; CRC: Colorectal cancer. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$.

induced by miR-627-5p could be attenuated by Wnt2 overexpression (SW480, 17.69% ± 1.35% *vs* 33.91% ± 5.61%, $P = 0.008$; HCT116, 24.17% ± 1.00% *vs* 42.15% ± 1.00%, $P < 0.001$, Figure 4C and F). CCK-8 assays demonstrated that the decreased in cell viability caused by miR-627-5p could be attenuated by Wnt2 overexpression (Figure 4G).

Function of miR-627-5p in the Wnt/β-catenin signalling pathway

To clarify the signalling pathways influenced by the miR627-5p/Wnt2 axis in CRC cells, we conducted qRT-PCR and western blot analysis to evaluate dysregulated genes in the classical Wnt signalling. In Figures 6 and 7, miR-627-5p overexpression in SW480 and HCT116 cells led to a sharp decline in the transcript and protein levels of β-catenin, c-myc, CD44, and cyclin D1. Next, we monitored the expression of β-catenin, c-myc, CD44, and cyclin D1 by treating miR-627-5p overexpressing CRC cells with pcDNA-Wnt2 or scramble vector. The findings illustrated that the transcript and protein levels of β -catenin, c-myc, CD44, and cyclin D1 were rescued at least partly by the overexpression of Wnt2. Consequently, it was suggested miR-627-5p reduces the gene and protein levels of downstream Wnt/β-catenin signalling components *via* Wnt2.

DISCUSSION

In our study, we concentrated on whether miR-627-5p, a rarely reported miRNA in CRC, could exert a suppressive effect on CRC development. First, we selected two GEO datasets to compare the expression of miR-627 in colorectal tumour patients and HCs, and the findings showed the decreased levels of miR-627 in cancer tissues in both GEO datasets. Consistent with our results, a study published in 2013 found the expression of miR-627 were observably downregulated in CRC tissues when compared to those in control tissues[21]. Unfortunately, this study did not distinguish the 5p and 3p forms of miR-627. In our previous study, we collected CRC and AA tissues to assess the expression of miR-627-5p and showed significantly decreased expression in CRC and AA tissues compared to HC tissues. Besides, miR-627-5p was found to be deceased in CRC cell lines in comparison with those in FHC cells[20]. According to these results, miR-627-5p expression was reduced in CRC.

Next, we conducted functional experiments using SW480 and HCT116 cells by transfecting miR-627-5p mimics or NC mimics to clarify the biological function of miR-627-5p in colorectal tumour. According to the findings, miR-627-5p greatly reduced cancer cells' ability to migrate, invade, and proliferate while also accelerating apoptosis, which was in accordance with past researches in other cancer types. For instance, miR-627-5p is markedly reduced in hepatocellular carcinoma and negatively correlated with the prognosis of cancer patients. miR-627-5p silencing promotes cell multiplication and cell cycle progression of hepatocellular carcinoma cells[9]. In oral squamous cell carcinoma, LINC00958 promotes tumour cell growth, delays apoptosis, and accelerates cell migration and epithelial-mesenchymal transition by suppressing the expression of miR-627-5p[12]. Thus, miR-627-5p is regarded as a tumour suppressor and could serve as a target for the treatment of cancer in the future.

A variety of literature have elucidated that the loss or enhancement of miRNA function is mainly involved in cancer carcinogenesis and progression by targeting the expression of cancer-causing or cancer-suppressing genes[22]. To clarify the key mechanism of miR-627-5p in suppressing CRC growth, we used an online tool to excavate the possible downstream genes of miR-627-5p. We subsequently discovered that miR-627-5p might have a complementary site within the Wnt2 3′-UTR. A series of studies have claimed that Wnt2 contributes to the development of numerous malignant malignancies [17,23-26]. For example, the Wnt2 gene is almost undetectable in the normal gastrointestinal tract but is highly upregulated in precancerous adenomas, primary colorectal tumours and liver metastases[27]. High expression of Wnt2 is implicated as a critical factor in promoting the invasive and metastatic potential of CRC cells[26]. In concordance with past researches, the current study confirmed that Wnt2 mRNA levels were considerably elevated in colorectal neoplasm tissues and inversely related to miR-627-5p levels, suggesting that miR-627-5p might participate in intestinal carcinogenesis by regulating the expression of Wnt2.

To explore whether miR-627-5p is directly complementary to Wnt2, we conducted a series of experiments *in vitro*. According to the results of luciferase reporter tests, miR-627-5p upregulation inhibited the luciferase level of the reporter vector comprising the wild type sequence of Wnt2 3′-UTR, but no obvious change on the reporter vector comprising the mutation sequence. In addition, ectopic expression of miR-627-5p significantly reduced the gene and protein expression of Wnt2 in CRC cell lines. The above findings suggested that Wnt2 is a specific target of miR-627-5p. However, whether

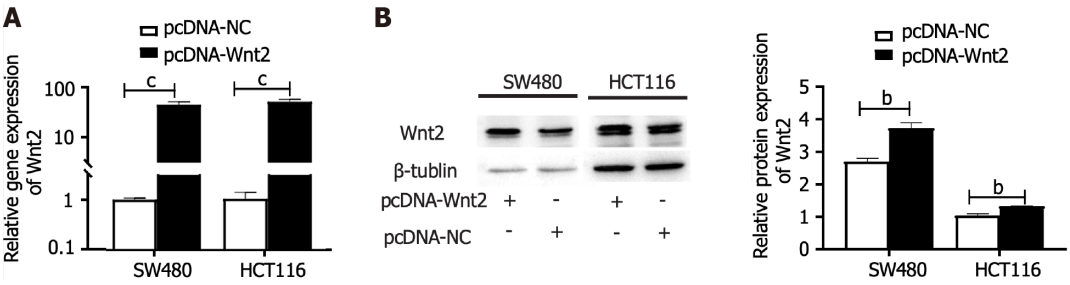

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Figure 5 Identification of the transfected efficiency of Wnt2 overexpression plasmids. A: The mRNA expressions of Wnt2 in SW480 and HCT116 cells after the transfection of Wnt2 overexpression plasmids; B: The protein expressions of Wnt2 in SW480 and HCT116 cells after the transfection of Wnt2 overexpression plasmids. $^{b}P < 0.01$; $^{c}P < 0.001$.

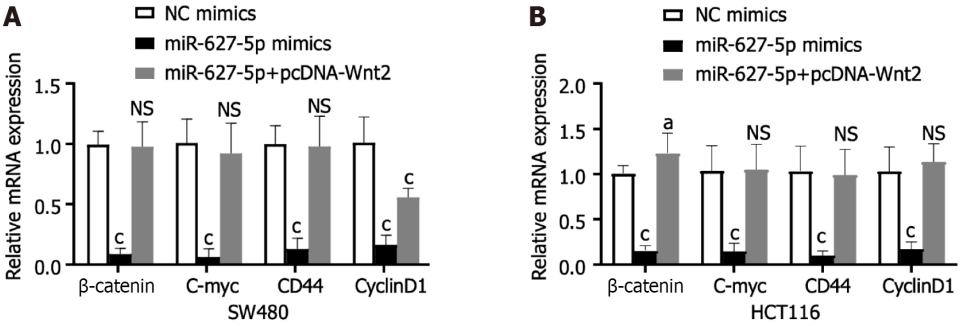

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Figure 6 The mRNA expression alterations of downstream target genes in the Wnt/β-catenin signalling induced by miR-627-5p mimics and Wnt2 overexpression plasmids in SW480 and HCT116 cells. A: SW480 cells; B: HCT116 cells. Experimental group: NC mimics, miR-627-5p mimics, miR-627-5p mimics + pcDNA-Wnt2. NS: Not significant; $^{b}P < 0.01$; $^{c}P < 0.001$.

miR-627-5p exerts its tumour inhibitory effect through directly regulating the expression of Wnt2 remains unknown. To answer this question, we conducted rescue experiments and cotransfected miR-672-5p mimics and Wnt2 overexpression plasmids into CRC cells. Rescue experiments showed that the cells' survival, motility, and invasion were enhanced and the proportion of apoptotic cells were decreased when compared to transfection of miR-627-5p mimics alone. Thus, miR-627-5p decreased CRC cell proliferation, motility, and invasion while promoting death *via* Wnt2.

As is well-known, Wnt2 is one of the critical ligands that regulates the activity of the Wnt/β-catenin signalling, while aberrant activation of the classical Wnt signalling is a major driver of colorectal carcinogenesis[28,29]. Herein, we hypothesized that miR-627-5p might regulate Wnt2 expression, thereby modulating the Wnt/β-catenin signalling, and exerting its tumour suppressive function on CRC *in vitro*. Normally, β-catenin, the essential element of the canonical Wnt pathway, is continually eliminated by the destruction complex (AXIN, GSK3β, CK1, and APC) without canonical Wnt ligands. The constant degradation of β-catenin leads to low level of free β-catenin in the cytoplasm and the repression of Wnt target genes. Conversely, the Wnt pathway is activated when canonical Wnt ligands bind to their receptors on the cell surface and subsequently cause the aggregation of the degradation complex, resulting in the accumulation of β-catenin in the cytoplasm. Then, β-catenin gradually migrates to the nucleus, where it serves as a co-activator for T-cell specific factor/lymphoid enhancer-binding factor to activate Wnt target genes such as cyclinD1[30], CD44[31], and c-myc[32], which are identified to be involved in the malignant tendency of cancer cells, including stemness, tumorigenicity, metastasis, and chemoresistance[33-35]. To verify this hypothesis, we investigated how miR-627-5p affected the expression of the Wnt/β-catenin signalling like β-catenin, cyclinD1, c-myc, and CD44. Our current study revealed that upregulation of miR-627-5p could effectively decrease the mRNA and protein expression of β-catenin, cyclinD1, c-myc and CD44, whereas the suppressive effects of miR-627-5p could be partially canceled by Wnt2 overexpression. These results suggested that miR-627-5p/Wnt2 regulates the canonical Wnt pathway in CRC cells.

There are some limitations in the study. First, we performed transient transfection to increase the levels of miRNAs in CRC cell lines. Since miRNAs do not integrate into the cellular genome, the typical effects can only last for several days, and we could not assess the long-term effects of miRNAs. Stable transfection of miRNAs is required to achieve the long-term effects of miR-627-5p on tumour progression. Second, the translocation of cytoplasmic β-catenin to the nucleus is a crucial step in the activation of Wnt signaling. However, the current study only detected alterations in β-catenin in whole

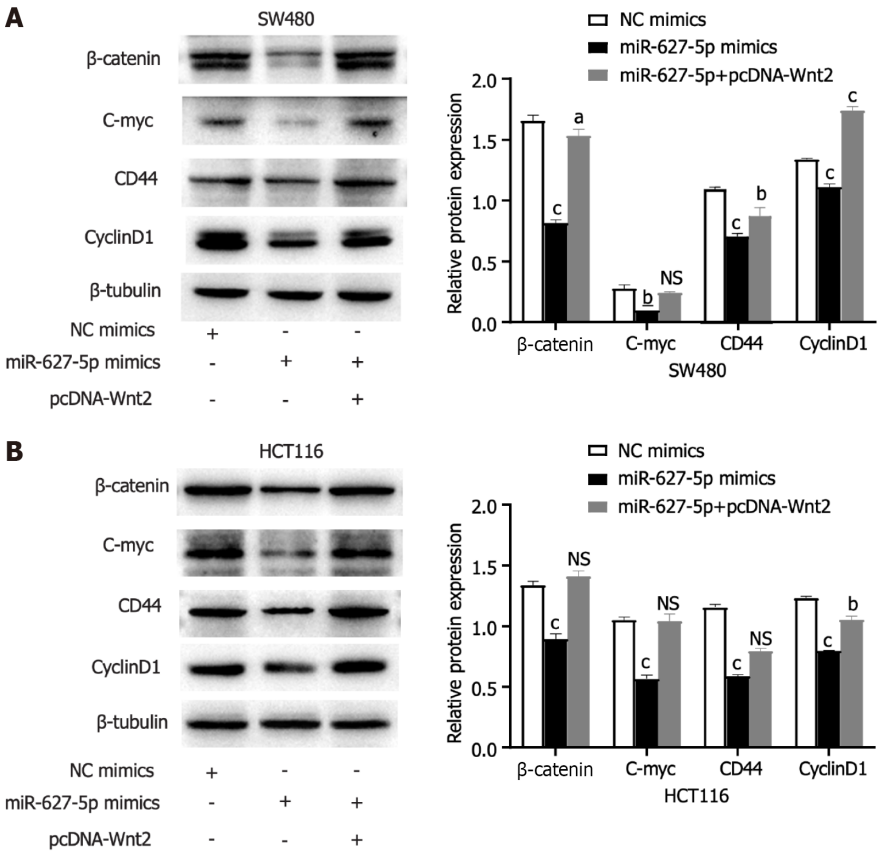

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Figure 7 The protein expression alterations of downstream target genes in the Wnt/β-catenin signalling induced by microRNA-627-5p mimics and Wnt2 overexpression plasmids in SW480 and HCT116 cells. A: SW480 cells; B: HCT116 cells. Experimental group: NC mimics, miR-627-5p mimics, miR-627-5p mimics + pcDNA-Wnt2. NS: Not significant. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001.

cells, not nuclear alterations. Finally, to learn more about the impact of miR-627-5p on the development of tumors in vivo, nude mouse carcinogenesis tests are necessary.

CONCLUSION

In summary, miR-627-5p could inhibit the malignant tendencies of CRC cells by directly inhibiting Wnt2 expression. The tumour suppressive effects were mainly achieved by inhibiting the activation of the classical Wnt/β-catenin signalling and the levels of its downstream target factors. These findings not only advance our understanding of the pathogenesis of CRC, but also provide evidence for an exploitable therapeutic target for CRC patients.

ARTICLE HIGHLIGHTS

Research background

Population aging has given rise to the incidence rate of colorectal cancer (CRC) worldwide. Better elucidation of the mechanisms underlying the formation and growth of CRC is very helpful for the development of new therapy.

Research motivation

Latest studies have shown that miRNAs generally regulate the expression of oncogenes or tumour suppressor genes and exert integral roles in modulating cancer-related pathways and mediating the formation and progression of CRC. However, whether miR-627-5p is involved in the tumorigenesis of colorectal tumours or not is largely unexplored.

Research objectives
This current study is designed to verify the function of miR-627-5p in colorectal tumorigenesis by targeting Wnt2/β-catenin signalling pathway.

Research methods
The levels of miR-627-5p and Wnt2 were detected in CRC tissues. Functional experiments, including CCK-8, flow cytometry, Matrigel invasion, and scratch assays, were conducted to elucidate the function of miR-627-5p and wnt2 in colorectal tumour cells. Dual luciferase reporter tests were carried out to investigate how miR-627-5p and Wnt2 interact. The critical signalling pathway modulated by miR-627-5p was further identified.

Research results
Wnt2 transcript expression was markedly increased in colorectal tumour tissues and negatively correlated with miR-627-5p level. Upregualtion of miR-627-5p inhibited cancer cells' abilities to invade growth and migrate by directly restraining Wnt2 expressions. Furthermore, miR-627-5p exerted the suppressive role in CRC *via* inactivating the Wnt2/β-catenin signalling.

Research conclusions
miR-627-5p restrained the malignant biological properties of CRC cells *via* directly inhibiting Wnt2 expression to modulate the classical Wnt/β-catenin signalling.

Research perspectives
miRNA-627-5p/Wnt2/β-catenin may have potential therapeutic application for CRC.

FOOTNOTES

Author contributions: Zhao DY designed and performed the study, analyzed the data, and drafted the manuscript; Yin TF and Sun XZ collected colorectal samples from subjects, and provided guidance on experimental procedures; Zhou YC, Wang QQ, and Zhou GYJ collected the clinical data and colorectal samples from the subjects; Yao SK designed the study, supervised the study performance, revised the manuscript, and obtained the funding; all authors read and approved the final manuscript.

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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

Data sharing statement: No additional data are available.

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ORIGINAL ARTICLE

Basic Study

Potent bromodomain and extraterminal domain inhibitor JAB-8263 suppresses MYC expression and exerts anti-tumor activity in colorectal cancer models

Xin-Mo Liu, Shao-You Xia, Wei Long, Hai-Jun Li, Gui-Qun Yang, Wen Sun, Song-Yan Li, Xiao-Hui Du

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Abstract

BACKGROUND

The overexpression of the *MYC* gene plays an important role in the occurrence, development and evolution of colorectal cancer (CRC). Bromodomain and extraterminal domain (BET) inhibitors can decrease the function BET by recognizing acetylated lysine residues, thereby downregulating the expression of MYC.

AIM

To investigate the inhibitory effect and mechanism of a BET inhibitor on CRC cells.

METHODS

The effect of the BET inhibitor JAB-8263 on the proliferation of various CRC cell lines was studied by CellTiter-Glo method and colony formation assay. The effect of JAB-8263 on the cell cycle and apoptosis of CRC cells was studied by propidium iodide staining and Annexin V/propidium iodide flow assay, respectively. The effect of JAB-8263 on the expression of c-MYC, p21 and p16 in

CRC cells was detected by western blotting assay. The anti-tumor effect of JAB-8263 on CRC cells *in vivo* and evaluation of the safety of the compound was predicted by constructing a CRC cell animal tumor model.

RESULTS

JAB-8263 dose-dependently suppressed CRC cell proliferation and colony formation *in vitro*. The MYC signaling pathway was dose-dependently inhibited by JAB-8263 in human CRC cell lines. JAB-8263 dose-dependently induced cell cycle arrest and apoptosis in the MC38 cell line. SW837 xenograft model was treated with JAB-8263 (0.3 mg/kg for 29 d), and the average tumor volume was significantly decreased compared to the vehicle control group ($P < 0.001$). The MC38 syngeneic murine model was treated with JAB-8263 (0.2 mg/kg for 29 d), and the average tumor volume was significantly decreased compared to the vehicle control group ($P = 0.003$).

CONCLUSION

BET could be a potential effective drug target for suppressing CRC growth, and the BET inhibitor JAB-8263 can effectively suppress c-MYC expression and exert anti-tumor activity in CRC models.

Key Words: Bromodomain; Bromodomain and extraterminal domain inhibitor; Colorectal cancer; JAB-8263; MYC; p21

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Core Tip: After treating colorectal cancer (CRC) cells with the bromodomain and extraterminal domain (BET) inhibitor JAB-8263, we found that MC38 cells undergo cell cycle arrest and apoptosis. In multiple human CRC cell lines, we found that JAB-8263 downregulated c-MYC expression and upregulated p21 and p16 expression, which is associated with the highly potent antiproliferative effects of JAB-8263. JAB-8263 effectively inhibited CRC growth with acceptable tolerance in tumor mouse models. Our studies suggested that BET can be a potential effective drug target for suppressing CRC growth, and JAB-8263 can effectively suppress c-MYC expression and exert anti-tumor activity in CRC models.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors, and its morbidity and mortality ranks third among all tumor patients[1], which seriously threatens human health. Traditional treatment methods include surgery, chemotherapy and radiotherapy. However, these treatments are invasive and are often accompanied by side effects[2]. In recent years, targeted therapy and immunotherapy have also developed rapidly as new treatment methods. With the deepening of tumor research, it has been found that the occurrence and development of colorectal tumors are related to the dysregulation of the epigenome[3], and one of the major areas of interest in epigenetic targets is the bromodomain and extraterminal domain (BET).

BET proteins belong to the acetyl-lysine-binding bromodomain (BRD) protein family and have four members, BRD2, BRD3, BRD4 and BRDT[4,5]. BET proteins have two N-terminal bromodomains (BD1 and BD2) that interact with acetylated lysine residues in histones. Then, it binds to transcription factor P-TEFb and RNA polymerase II and induces transcription[6]. BET protein acts as an epigenetic regulator and transcriptional cofactor, and it is closely associated with gene transcription, cell cycle and apoptosis, invasion and metastasis. BET proteins promote aberrant expression of many oncogenes such as *MYC*, *CCND1* and *BCL2L1*[7,8].

MYC is a proto-oncogene that is activated by amplification and chromosomal translocation rearrangement. The overexpression of MYC plays an important role in the occurrence, development and evolution of CRC[9,10]. Overexpression of MYC and dysregulation of MYC target genes can be found in most CRC cells[11]. BET inhibitors bind to the BET protein, occupying the space where it binds to acetylated lysines, thus inhibiting the transcription of its downstream *MYC* oncogenes and MYC-dependent genes[12,13]. A study showed the small molecule BET inhibitor JQ1 occupies the bromodomain pocket of BRD4, resulting in downregulation of *MYC* mRNA and MYC protein[14]. This

provides a rationale for the idea that BET inhibitors may exert anti-tumor activity in CRC cells.

The BET inhibitor JAB-8263 used in this study is a new type of BET inhibitor, which has a strong affinity with BET proteins and can significantly inhibit BET downstream signals c-MYC and N-MYC at a concentration of less than 1 nmol/L. It can significantly inhibit the proliferation of various tumor cells and induce the expression of cleaved PARP and the activation of caspase3/7, thereby inhibiting the proliferation of tumor cells and inducing apoptosis. Previous *in vivo* studies have shown that JAB-8263 has strong anti-tumor effects in various tumor models such as hematological tumors and small cell lung cancer through the MYC pathway. The pharmacology tests on safety show that JAB-8263 has no adverse effects on the cardiovascular system, respiratory system and central nervous system.

We predicted that JAB-8263 can suppress CRC cells *in vitro* and *in vivo*, and the purpose of this study was to explore the mechanism of its inhibitory effect on CRC cells.

MATERIALS AND METHODS

Cell proliferation

All CRC cell lines (HT29, DLD1, Colo205, H716, SW837, H508 and MC38) used in this study were purchased from ATCC and kept in our laboratory. The CellTiter-Glo method was used in this experiment. CRC cells were plated in cell culture plates and cultured in a cell culture incubator at 37 °C, 5% CO_2 or 100% air and 95% humidity. Compounds were added the next day and incubated for 5 d, and cell viability was detected with the CellTiter-Glo kit. The data were analyzed using GraphPad Prism software, and a four-parameter equation was used to fit a concentration-response curve, from which the IC_{50} of the compound concentration corresponding to 50% cell viability on the curve was calculated. Cell viability (%) = ($Lumi_{test\ compound}$-$Lumi_{blank\ control}$)/($Lumi_{solvent\ control}$-$Lumi_{blank\ control}$) × 100%. Compound information: BET inhibitor JAB-8263 (Jacobio Pharmaceuticals, Beijing, China), purity: 99.10%, storage condition: 4 °C.

Colony formation assay

The cell suspension was serially diluted, and 1000 cells were inoculated in each group of cells per dish, cultured in a cell incubator at 37 °C, 5% CO_2 or 100% air and 95% humidity and stained with crystal violet solution after 5 d. Cells exposed to the drug were compared to controls (treated with DMSO) assayed in triplicate.

Cell cycle analysis

Six-well plates were seeded with MC38 cells in logarithmic growth phase, 5 × 10^5 cells per well. Diluted JAB-8263 compound was added to each well, and 0.1% DMSO was added to the control group; the incubation time was 3 d and 5 d, respectively. Cells were then trypsinized, washed with PBS and stained with propidium iodide (PI) solution for 30 min in a dark room. Cell DNA content was analyzed by flow cytometry in triplicate.

Apoptosis assay

Six-well plates were seeded with MC38 cells in logarithmic growth phase, 5 × 10^5 cells per well. Diluted JAB-8263 compound was added to each well, and 0.1% DMSO was added to the control group; the incubation time was 3 d and 5 d, respectively. Cells were then trypsinized and washed with PBS. Cells were stained (Thermo Annexin V Apoptosis Detection Kit, APC) and incubated for 30 min at room temperature in a dark room. Analysis was performed in triplicate using a drain cytometer in triplicate.

Western blotting

Cells were harvested, and cellular protein collection was performed after addition of lysate. The protein concentration was detected according to the BCA instructions. The samples added to loading buffer were electrophoresed by discontinuous SDS-PAGE denaturing gel. The protein was transferred to PVDF membrane and detected by ECL exposure. Antibodies information: Anti-c-MYC antibody (ab32072, Abcam, United Kingdom); p21 Waf1/Cip1 (12D1) Rabbit mAb (#2947, GST, United States); p16 INK4A (E6N8P) Rabbit mAb (#18769, GST, United States); GAPDH (D16H11) XP® Rabbit mAb (#18769, GST, United States).

In vivo studies

All animal care and use-related experimental protocols and changes to the experimental protocols of animals in this experiment were reviewed, approved and guided by the Jacobio Animal Care and Use Management Committee.

SW837 xenograft mouse model: 12 female NOD-SCID mice were subcutaneously inoculated with 1 × 10^7 SW837 cells on the right back. When the tumor grew to an average of 121 mm^3, the mice were randomly divided into two groups according to tumor size and body weight. The experiment was

divided into a vehicle control group and a JAB-8263 0.3 mg/kg treatment group. The JAB-8263 0.3 mg/kg treatment group and vehicle control group were administered by gavage once every 2 d. The anti-tumor activity was evaluated according to the relative tumor growth inhibition (TGI) rate. TGI (%) = $(1-T_{RTV})/C_{RTV} \times 100\%$ (T_{RTV}: mean RTV of the treatment group; C_{RTV}: mean RTV of the vehicle control group; RTV = V_t-V_0, V_0 is the volume of the subcutaneous transplanted tumor of the mouse at the time of grouping, and V_t is the volume of the subcutaneous tumor of the mouse after treatment). The safety was evaluated according to the changes in animal body weight, drug withdrawal and death.

MC38 syngeneic murine model: 16 female C57BL/6 mice were subcutaneously inoculated with 1×10^6 MC38 cells on the right back. When the tumors grew to an average of 103 mm^3, they were randomly divided into two groups according to the tumor size and the weight of the mice. The experiment was divided into a vehicle control group and a JAB-8263 0.2 mg /kg treatment group. JAB-8263 0.2 mg/kg treatment group and vehicle control group were administered by gavage once every 2 d. The anti-tumor activity was evaluated according to the relative TGI rate, and the safety was evaluated according to the changes in animal body weight, drug withdrawal and death.

A single-dose MC38 model: In addition, we used the above method to establish a single-dose MC38 model. Nine female C57BL/6 mice were randomly divided into two groups according to the tumor size and the weight of the mice. The experiment was divided into a vehicle control group, a JAB-8263 0.1 mg/kg treatment group and a JAB-8263 0.2 mg/kg treatment group. One hour after the treatments were administered, the experiment was terminated, all mice were euthanized, and tumor tissues were collected.

Statistical analysis

All experimental results were expressed as mean ± SD. The *t*-test method was used to compare the data of the treatment group and the control group for statistical differences. All data were analyzed with SPSS 22.0, and $P < 0.05$ was considered statistically significant.

RESULTS

JAB-8263 dose-dependently suppressed CRC cell proliferation and colony formation in vitro

We found seven CRC cell lines that were sensitive to JAB-8263 in cell proliferation assays, including human CRC cell lines (HT29, DLD1, Colo205, H716, SW837 and H508, Figure 1A) and murine CRC cell line (MC38, Figure 1B). The IC_{50} values of six human CRC cell lines including HT-29, DLD-1, Colo205, H716, SW837 and H508 were 0.09-1.24 nmol/L, and the IC_{50} of the murine CRC cell line MC38 was 1.25 nmol/L.

In the colony formation assay, five groups of CRC cell lines were sensitive to the JAB-8263 compound. Compared with the control group (DMSO), the colony formation of the cell lines in each group was significantly reduced with increasing drug concentration (Figure 1C). Taken together, these data suggest that JAB-8263 dose-dependently suppressed CRC cell proliferation and colony formation *in vitro*.

JAB-8263 suppressed CRC cell MYC expression and promoted p21 and p16 expression

Western blot assays on MYC, p21 and p16 levels were performed in human CRC cell lines with JAB-8263 treatment. Compared with the control group (DMSO), the expression of MYC was downregulated in all cell lines with the treatment of different concentrations of JAB-8263 (1 nmol/L, 10 nmol/L and 100 nmol/L). The p21 expression of MC38, DLD-1, H508, HT29, SW837 and Colo205 was upregulated, and the expression of p16 in H716, HT29 and colo205 was upregulated (Figure 2A-C). This data suggest that JAB-8263 dose-dependently downregulated the expression of c-MYC in CRC cells and upregulated the expression of p21 and p16 in some of the CRC cell lines.

JAB-8263 dose-dependently induced cell cycle arrest and apoptosis in the MC38 cell line

We conducted further cell cycle and apoptosis assays on the murine CRC cell line MC38 to explore the mechanism of JAB-8263 suppressed CRC cell proliferation. In the cell cycle assay, the MC38 cell cycle was arrested in the subG0 phase compared with the control group after 3 d and 5 d of treatment with JAB-8263 in different concentrations. JAB-8263 dose-dependently decreased the G2/M phase ratio and increased the subG0 prophase ratio in MC38 cells, indicating that JAB-8263 induced cell cycle arrest in the G0 phase. (Figure 3A and B). In the apoptosis assay, the apoptotic ratio of MC38 was increased compared with the control group after 3 d and 5 d of treatment with JAB-8263. Furthermore, the apoptotic ratio increased with the compound concentration (Figure 3C and D). This data indicates that JAB-2485 suppressed tumor cell activity in two ways by inducing MC38 cell cycle arrest and apoptosis.

JAB-8263 suppressed in vivo CRC growth

After 29 d of treatment in the SW837 xenograft model, the average tumor volume in the vehicle control

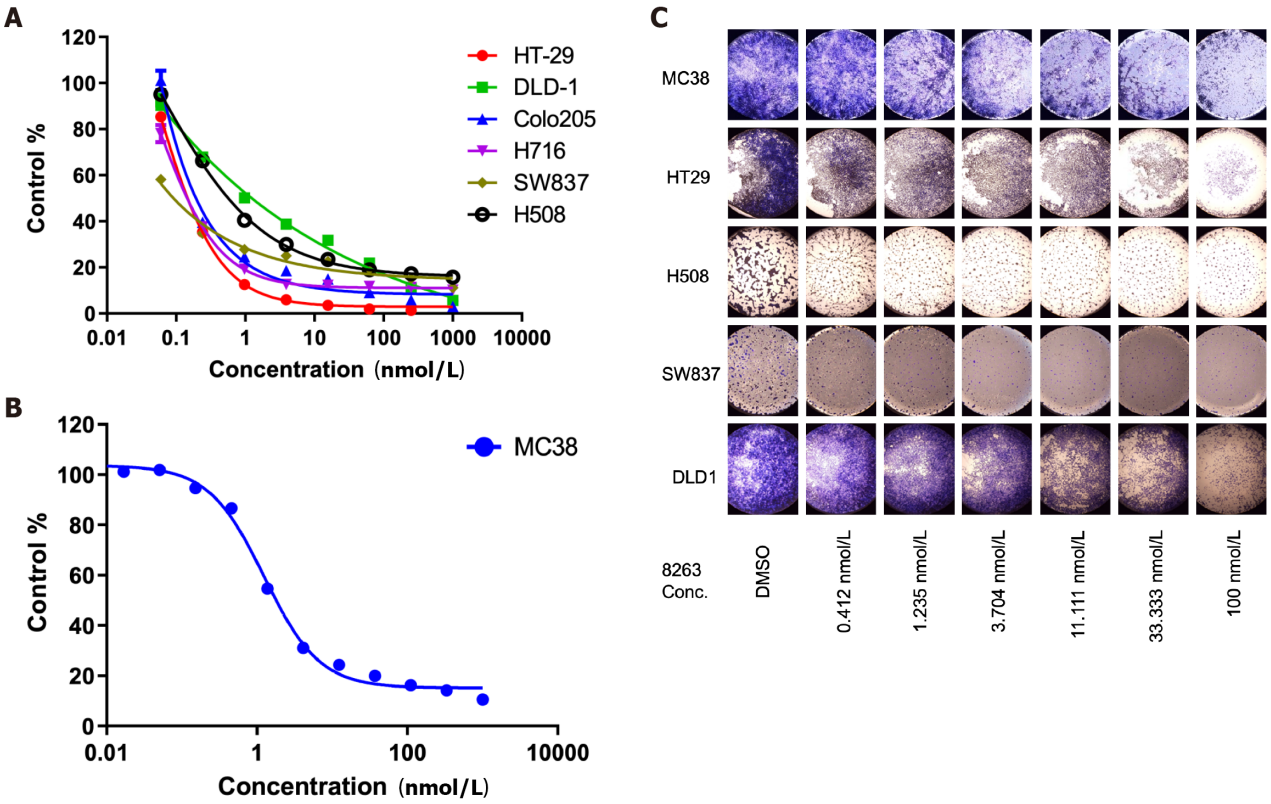

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Figure 1 JAB-8263 dose-dependently suppressed colorectal cancer cell proliferation and colony formation *in vitro*. A: Human colorectal cancer cell lines including HT29, DLD1, Colo205, H716, SW837 and H508 were treated with JAB-8263 for 6 d, and the proliferation was dose-dependently suppressed; B: The MC38 mouse cell line was treated with JAB-8263 for 6 d, and the proliferation was dose-dependently suppressed; C: The IC_{50} values of HT-29, DLD-1, Colo205, H716, SW837, H508 and MC38 were 0.15, 1.24, 0.19, 0.09, 0.57, 0.14 and 1.25 μmol/L. Colony formation assays for six colorectal cancer cell lines including MC38, HT29, H508, SW837 and DLD1 were treated with various concentrations of JAB-8263 for 5 d. Cell proliferation in all cell lines was dose-dependently suppressed. All experiments were performed in triplicate. Conc.: Concentration.

group was 895 mm^3, and the average tumor volume in the JAB-8263 0.3 mg/kg treatment group was 283 mm^3, which was statistically significant compared to the vehicle control group. The relative tumor inhibition rate TGI (%) was 79.0% (Figure 4A). Only one animal in the JAB-8263 treatment group lost 16.6% of body weight at the end of the trial, and animals in the other groups tolerated it well without discontinuation or death (Figure 4B). After 18 d of treatment in the MC38 syngeneic model, the average tumor volume in the vehicle control group was 2580 mm^3, and the average tumor volume in the JAB-8263 0.2 mg/kg treatment group was 686 mm^3. Compared with the vehicle control group, there was a significant statistical difference (P = 0.003) (Figure 4C), and the relative tumor inhibition rate TGI (%) was 76.5%. The body weight change of each treatment group was controlled within 15%, no drug discontinuation or death occurred, and the animals tolerated the treatment well (Figure 4D).

The tumor tissue of the single-dose MC38 model was further subjected to the western blot assay to evaluate the underlying mechanism, and it was found that the expression of c-MYC was significantly decreased by a single dose of JAB-8263 administration (P = 0.013 and P = 0.011) (Figure 4G and H). All data showed that JAB-8263 downregulated the expression of c-MYC in tumor tissue from the single-dose MC38 model.

DISCUSSION

In recent years, BET protein inhibitors have received extensive attention in the application of tumors, and many BET inhibitors have been used in clinical trials, but most are focused on hematological tumors and some solid tumors such as lung cancer and prostate cancer[15-19]. Some previous studies have used JQ1 and other compounds in the study of CRC cells[20,21], but due to the short half-life of most compounds, they are quite challenging for further clinical application. JAB-8263 used in this study has stronger protein affinity, high affinity for BET protein *in vitro*, and the IC_{50} is less than 1 nmol/L.

In the *in vitro* cell proliferation and colony formation experiments, we found that JAB-8263 had an inhibitory effect on CRC cells. To further study its mechanism of action, we performed cell cycle and apoptosis experiments. However, only the mouse CRC cell line MC38 obtained ideal positive results,

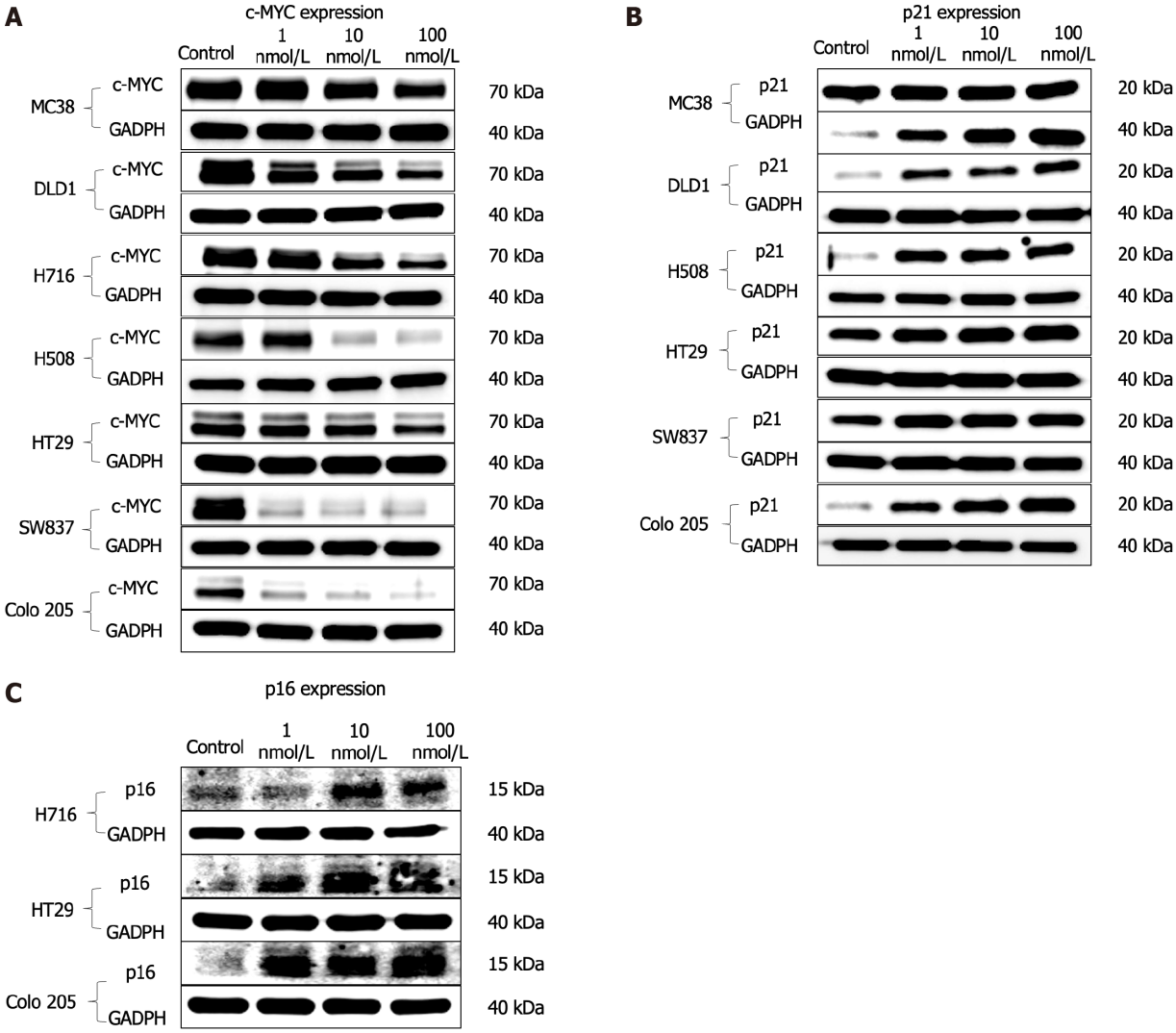

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Figure 2 MYC signaling pathway was dose-dependently inhibited by JAB-8263 in human colorectal cancer cell lines. A-C: Multiple colorectal cancer cell lines were treated with different concentrations of JAB-8263 (1 nmol/L, 10 nmol/L and 100 nmol/L) for 16 h. Western blotting assay was performed to detect levels of MYC, p21 and p16. The expression of MYC were downregulated (A), and the expression of p21 (B) and p16 (C) were upregulated in multiple colorectal cancer cell lines.

and the human CRC cell lines did not have a significant difference from the control group. We conducted the western blot experiments to further explore this result.

MYC plays an important role in the cell cycle, cell death, cellular senescence, and tumorigenesis of CRC cells[9]. Myc-related lnc-RNAs such as MYCLo-2 are overexpressed in CRC cells and have oncogenic functions[14]. Through the *in vitro* and *in vivo* studies of this study, we found that JAB-8263 can effectively suppress the expression of c-MYC and finally suppress CRC cells.

The tumor suppressor genes *p21* and *p16* are regulated by the *MYC* gene[14]. Therefore, we further investigated whether the expression of these two genes was affected by BET inhibitors. p21 (CDKN1A) is involved in the regulation of cell cycle and cellular senescence[22]. In 1993, it was reported that p21 can suppress multiple tumors such as CRC by activating wild-type p53[23]. Moreover, studies have shown that p21 can also suppress tumor growth by inhibiting cyclin kinase complexes and proliferating cell nuclear antigen[24]. JAB-8263 achieves an anti-tumor effect by inducing CRC cell cycle arrest by upregulating p21. However, at the same time, some studies have suggested that p21 has an anti-apoptotic effect, and the apoptosis of hCT116 colon cancer cells can be inhibited by inhibiting p21[25, 26]. This might be one reason why JAB-8263 did not have ideal results in the apoptosis experiments, which also requires further study.

p16 (CDKN2A) can inhibit the function of CDK4, and the combination of CDk4 and cyclin D1 plays a key regulatory role in the G1→S phase of the cell cycle, thereby suppressing the malignant proliferation of cells[27]. The inactivation or decreased expression of the *p16* gene can lead to the malignant proliferation of cells and lead to tumorigenesis[28,29]. JAB-8263 inhibits CDK4 function by upregulation of p16, thereby suppressing CRC cells.

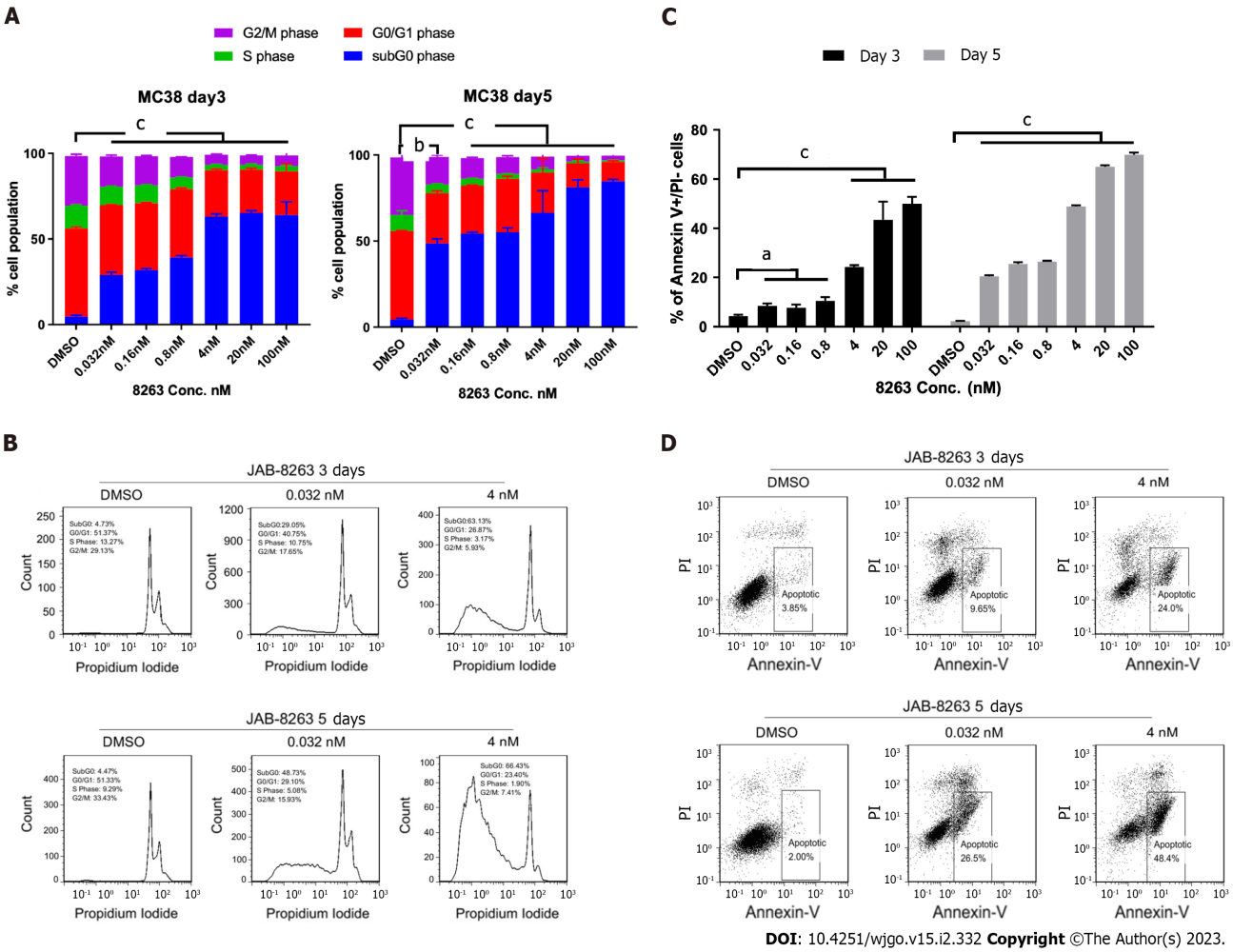

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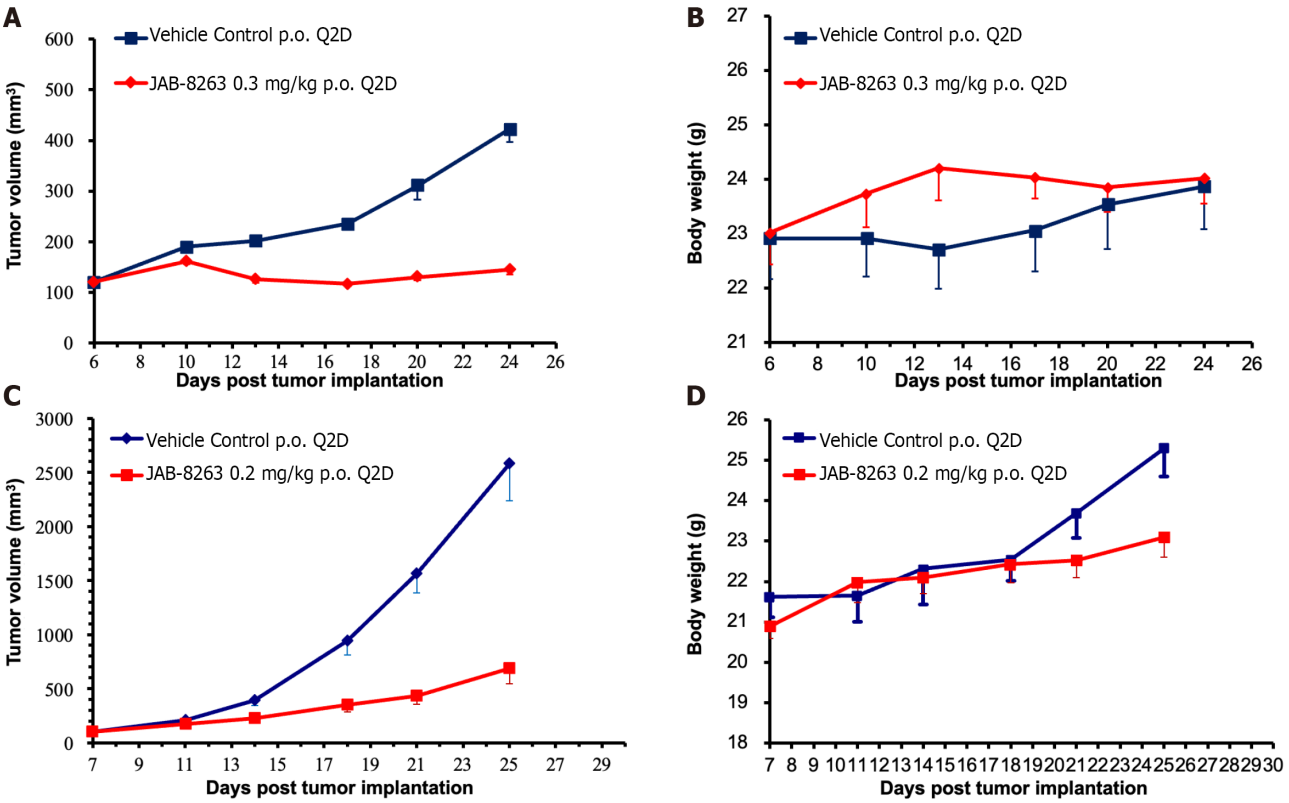
Figure 3 JAB-8263 dose-dependently induced cell cycle arrest and apoptosis in the MC38 cell line. After incubation with different concentrations of JAB-8263 (0, 0.032, 0.16, 0.8, 4, 20 and 100 nmol/L) for 3 d and 5 d, MC38 cells were collected and analyzed for cell cycle and apoptosis assays by flow cytometry. A and B: JAB-8263 dose-dependently decreased the G2/M phase ratio and increased the G0 prophase ratio in MC38 cells, indicating that JAB-8263 induced cells to arrest in the G0 phase; C and D: JAB-8263 dose-dependently induced apoptosis in MC38 after treatment for 3 d and 5 d. All experiments were performed in triplicate. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* DMSO control. PI: Propidium iodide.

Finally, we verified that JAB-8263 has a significant tumor inhibitory effect compared with the control group in the SW837 and MC38 animal models. The animals in the treatment group tolerated the drug well. Since c-MYC expression is disturbed in long-term dosing models, we established a single-dose model. The detection of tumor tissue in single-dose MC38 model also showed that c-MYC was downregulated. This is consistent with the conclusions we obtained in the *in vitro* studies.

According to the conclusion of this study, the BET inhibitor JAB-8263 can inhibit CRC cells mainly by inhibiting the expression of c-MYC. But at the same time, we found that the inhibition of BET inhibitors on CRC has many mechanisms other than the *MYC* gene. Further directions include whether the BET inhibitors still have an anti-tumor effect in cells that do not overexpress MYC, which will provide a theoretical basis for the indications of CRC treatment in future clinical applications.

CONCLUSION

The JAB-8263 compound inhibited the BET target. The expression of BET downstream signaling protein MYC was repressed by JAB-8263, resulting in downregulation of c-MYC and upregulation of p21 and p16. It induced cell cycle arrest, promoted apoptosis of CRC cells and displayed anti-tumor activity. *In vivo*, JAB-8263 was effective in CRC models.


A
Vehicle Control p.o. Q2D
JAB-8263 0.3 mg/kg p.o. Q2D
Tumor volume (mm³)
Days post tumor implantation
B
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JAB-8263 0.3 mg/kg p.o. Q2D
Body weight (g)
Days post tumor implantation
C
Vehicle Control p.o. Q2D
JAB-8263 0.2 mg/kg p.o. Q2D
Tumor volume (mm³)
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D
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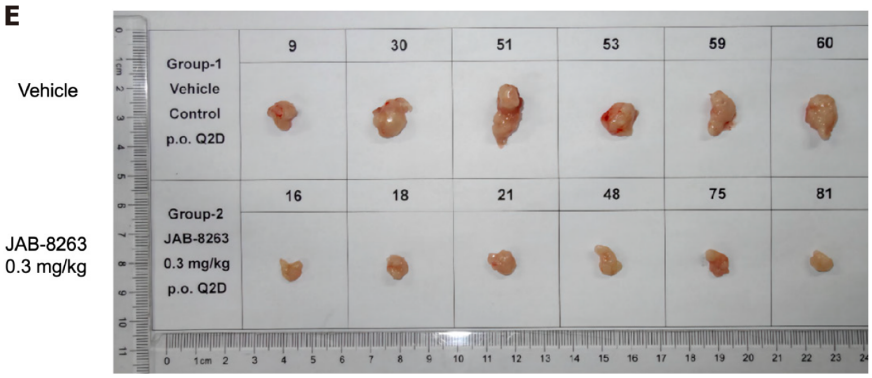
JAB-8263 0.2 mg/kg p.o. Q2D
Body weight (g)
Days post tumor implantation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Control
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9
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60
Group-2
JAB-8263
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p.o. Q2D
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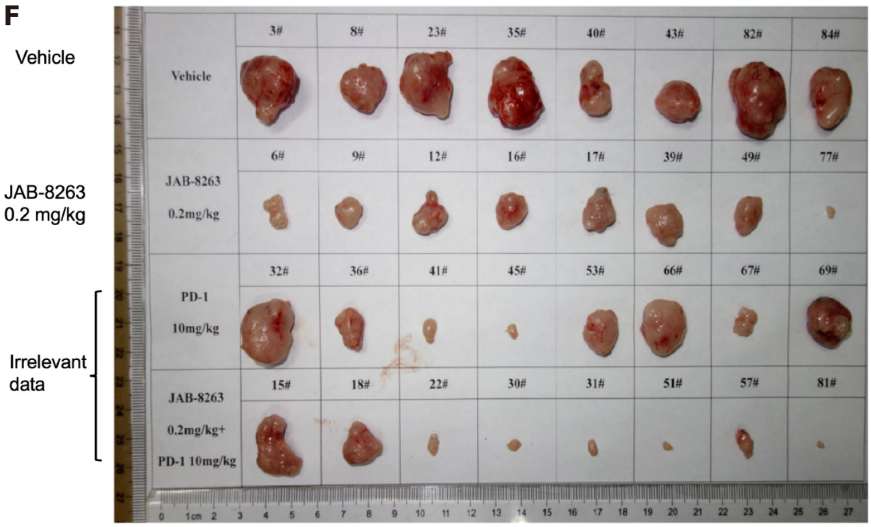
81


F
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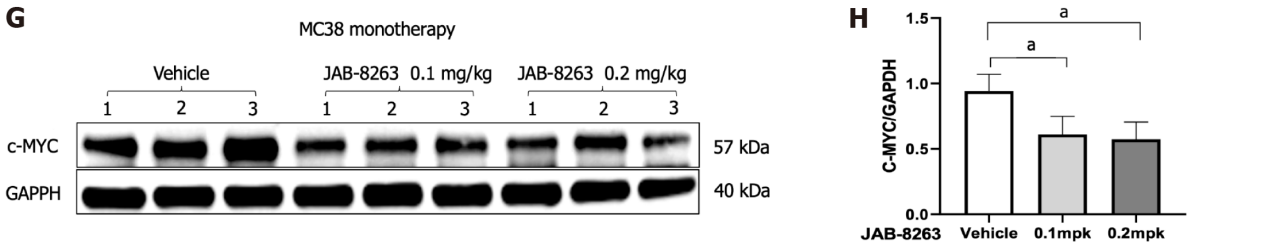

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Figure 4 JAB-8263 suppressed tumor growth in colorectal cancer murine xenograft models. A and E: The SW837 xenograft model (6 mice for each group) was treated with JAB-8263 0.3 mg/kg for 29 d. The average tumor volume was significantly decreased compared to the vehicle control group, $P < 0.001$; B: There was no significant difference in body weight change between groups; C and F: The MC38 syngeneic murine model (8 mice for each group) was treated with JAB-8263 0.2 mg/kg for 29 d. The average tumor volume was significantly decreased compared to the vehicle control group, $P = 0.003$; D: There was no significant difference in body weight change between groups; G and H: The tumor tissue of the MC38 syngeneic murine model (3 mice for each group) after a single dose of JAB-8263 treatment was collected for the Western blotting assay. Compared with the control group, the expression of c-MYC in the treatment group was downregulated, $^{a}P < 0.05$.

ARTICLE HIGHLIGHTS

Research background

The overexpression of the *MYC* gene plays an important role in the occurrence, development and evolution of colorectal cancer (CRC). Bromodomain and extraterminal domain (BET) inhibitors decrease the function of BET, which is the recognition of acetylated lysine residues, thereby downregulating the expression of MYC.

Research motivation

BET proteins are an important target in solid tumors, hematologic tumors and myelofibrosis. The development of BET small-molecule inhibitors has promising therapeutic value.

Research objectives

The study aimed to investigate the inhibitory effect and mechanism of a BET inhibitor on CRC cells.

Research methods

The effect of the BET inhibitor JAB-8263 on the proliferation of various CRC cell lines was studied by the CellTiter-Glo method and colony formation assay. The effect of JAB-8263 on the cell cycle and apoptosis of CRC cells was studied by propidium iodide staining and Annexin V/propidium iodide flow assay, respectively. The effect of JAB-8263 on the expression of c-MYC, p21 and p16 in CRC cells was detected by western blot. To predict the anti-tumor effect of JAB-8263 on CRC cells *in vivo* and to evaluate the safety of the compound, a CRC cell animal tumor model was developed.

Research results

JAB-8263 dose-dependently suppressed CRC cell proliferation and colony formation *in vitro*. The MYC signaling pathway was dose-dependently inhibited by JAB-8263 in human CRC cell lines. JAB-8263 dose-dependently induced cell cycle arrest and apoptosis in the MC38 cell line. The SW837 xenograft model was treated with JAB-8263 0.3 mg/kg for 29 d. The average tumor volume was significantly decreased compared to the vehicle control group, $P < 0.001$. The MC38 syngeneic murine model was treated with JAB-8263 0.2 mg/kg for 29 d. The average tumor volume was significantly decreased compared to the vehicle control group, $P = 0.003$.

Research conclusions

BET can be a potential effective drug target for suppressing CRC growth, and the BET inhibitor JAB-8263 can effectively suppress c-MYC expression and exert anti-tumor activity in CRC models.

Research perspectives

BET proteins are an important target in solid tumors, hematologic tumors and myelofibrosis. The development of BET small-molecule inhibitors has promising therapeutic value. Our study results are encouraging and will motivate further clinical evolution.

FOOTNOTES

Author contributions: Liu XM performed experiments and data analysis and wrote the paper; Xia SY performed experiments; Long W and Li H collected data; Yang GQ and Sun W performed the data analysis; Du XH and Li SY designed and revised the manuscript; All authors contributed to the article and approved the submitted version.

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

Data sharing statement: The data used to support the findings of this study are available from the corresponding author upon reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic value of claudin 18.2 expression in gastric adenocarcinoma

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Abstract

BACKGROUND

Claudin 18.2 (CLDN18.2) is a cell surface protein expressed by gastric cancer cells. The monoclonal antibody zolbetuximab binds CLDN18.2-positive cancer cells and causes cancer cell death. A few studies researched the prognostic effect of CLDN18.2 expression in metastatic gastric adenocarcinoma.

AIM

To identify the prognostic value of CLDN18.2 expression in patients with metastatic gastric adenocarcinoma.

METHODS

This study was conducted with 65 patients over the age of 18 who were diagnosed with metastatic gastric adenocarcinoma. We investigated the effect of CLDN18.2 expression on clinicopathological characteristics (age, sex, histological grade, Lauren classification, family history, metastatic site, HER2 expression) and prognosis for patients with metastatic gastric adenocarcinoma.

RESULTS

CLDN18.2 expression was positive in 73.8% (48) of the patients. During the median 17.7-mo follow-up period, 89.2% (58) of the patients died. Median progression-free survival and overall survival (OS) were 6 mo (95% confidence interval: 1.6-10.4) and 12 mo (95% confidence interval: 7.5-16.5). There was no

statistically significant correlation between CLDN18.2 expression and clinicopathological characteristics of the patients. In univariate and multivariate Cox regression analysis, there was no correlation between clinicopathological characteristics of patients and progression-free survival or OS.

CONCLUSION

CLDN18.2 expression was quite high in patients with gastric adenocarcinoma, identifying the proportion of the patients in whom zolbetuximab would be efficacious. There is no statistically significant correlation with clinicopathological characteristics and OS. CLDN18.2 is not a prognostic marker in patients with gastric adenocarcinoma, although it is predictive.

Key Words: Gastric adenocarcinoma; Claudin 18.2; Overall survival; Clinicopathological characteristics

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Core Tip: Zolbetuximab is a new antibody drug targeting the cell surface protein claudin 18.2 (CLDN18.2) expressed by gastric cancer cells. CLDN18.2 expression, identifying the patient population who are susceptible to zolbetuximab, is discordant in different studies. The present study aimed to research the expression ratio of CLDN18.2 and its prognostic value for overall survival in patients with gastric adenocarcinoma in a single center located in Turkey.

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INTRODUCTION

Stomach cancer represents the third most common cause of cancer-related mortality globally and caused 768793 deaths in 2020 (7.7% of all cancer deaths)[1]. Most people with stomach cancer in its early stages show no symptoms. The majority of patients (60%) receive diagnosis at the advanced stage following the emergence of symptoms[2]. In light of phase 2 and 3 studies from Europe, perioperative chemotherapy (ChT) has become standard for patients with stage 2 and 3 gastric cancer, but the 5-year overall survival (OS) is still approximately 36%[3,4]. The prognosis for locally advanced, unresectable, or metastatic gastric cancer is poor; in clinical trials evaluating the effectiveness of ChT, the median survival time was typically less than 1 year[5].

Claudin (CLDN) 18, a member of the cell surface protein claudin family, has two isoforms: CLDN18.1 expressed in lung tissue and CLDN18.2 expressed specifically in gastric tissue. CLDN18.2 is also expressed by gastric cancer cells, showing that it is not lost during malignant transformation[6]. The monoclonal antibody zolbetuximab binds CLDN18.2-positive cancer cells and causes cancer cell death by antibody-dependent cellular toxicity and complement-dependent cytotoxicity. In MONO phase 2a study of zolbetuximab as a single agent, CLDN18.2-positive patients with metastatic gastric and gastroesophageal junction (G/GEJ) adenocarcinoma received a minimum of one line of ChT and showed a 23% response rate[7]. The phase 2 FAST study of zolbetuximab plus ChT (epirubicin, oxaliplatin, capecitabine) *vs* ChT (epirubicin, oxaliplatin, capecitabine) showed superior OS and progression-free survival (PFS), defining CLDN18.2 as a new target for cancer therapy[8].

We investigated the effect of CLDN18.2 expression on clinicopathological characteristics and prognosis of patients with metastatic gastric adenocarcinoma undergoing ChT.

MATERIALS AND METHODS

Patients admitted to the medical oncology clinic of Suleyman Demirel University hospital between January 2013 and December 2021 with metastatic gastric adenocarcinoma were enrolled in this study. All cases were histopathologically confirmed according to the 5th edition of the World Health Organization classification of digestive system tumors[9]. The Protocol for the Examination of Specimens from Patients with Cancers of the Stomach 2022 of the College of American Pathologists was used to identify histopathologic subtype, tumor location, tumor grade, and HER2 for gastric adenocarcinoma[10]. From the hospital database, the following clinical data were obtained: age, sex, histological

type and grade, family history of gastric cancer, metastatic site, HER2 expression, PFS, and OS. The ethics committee of Suleyman Demirel University approved the study with date and number 01/04/2022-102. Patients who accepted participation in the study, who were older than 18-years-old, followed up in the medical oncology clinic of Suleyman Demirel University hospital, and whose paraffin blocks for diagnosis of gastric adenocarcinoma could be reached were enrolled in the study.

Immunohistochemistry

Hematoxylin and eosin sections representing the tumor of patients diagnosed with gastric adenocarcinoma were re-examined. The best paraffin block was selected for immunohistochemistry staining. Sections with 4-micron thickness were taken from paraffin blocks and transferred onto an adhesive coated slide system. The following method was used for immunohistochemical staining with streptavidin-biotin. Sections were incubated at 56 °C for 12 h for deparaffinization. Three percent hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was performed in a microwave oven for 20 min using 0.01 mol/L Tris/EDTA buffer pH 9.0. Sections were coated with primary antibodies including CLDN18.2 (rabbit monoclonal antibody, Clone EPR19202, at 1:500 dilution, Abcam, United Kingdom) and incubated at room temperature for 2 h. Sections were incubated for another 20 min at room temperature after the addition of binding (secondary) antibody (Goat Anti-Rabbit IgG H&L (HRP) kit, Abcam, United Kingdom). The streptavidin-biotin complex was added. 3,3′-Diaminobenzidine was used as chromogen for visualization. CLDN18.2 non-tumor gastric tissues were used as positive controls for each staining session.

Evaluation of immunohistochemical staining

Pathology slides were reviewed by two expert pathologists (ROY and NK) who did not know patient treatments and outcomes. Tumor cells were scored positive for CLDN18.2 if they showed definite membranous staining and negative if tumor nuclei and cell membrane did not have immunoreactivity. Staining intensity was scored between 0 and 3 (absent: 0, weak: 1, moderate: 2, strong: 3).

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences 26.0 (SPSS Inc., Chicago, IL, United States). Age and clinical characteristics were compared between patients with expression of CLDN18.2 using the Mann-Whitney *U*-test for individual samples. In patient tumor samples with expression of CLDN18.2, sex, localization, family history, Lauren classification, grade, sites of metastasis, liver and lung metastases, and history of adjuvant and neoadjuvant ChT were compared using Pearson's χ^2 test. The correlation between CLDN18.2 and HER2 was determined with the Spearman correlation test. OS and PFS were estimated using the Kaplan-Meier method, and a log-rank test was used to compare study groups in terms of survival. Multivariate analyses were performed using Cox regression analysis. A P value of < 0.05 was considered statistically significant.

RESULTS

Sixty-nine patients were screened, and sixty-five were included in the study. The mean age was 64.6 years ± 12.9 years. Among the patients, 49 (75.4%) were male, and 16 (24.6%) were female. Table 1 shows the demographic and clinicopathologic characteristics of the patients according to CLDN18.2 expression. Immunohistochemical staining was used to screen 65 metastatic gastric adenocarcinoma cases for the pathological significance of CLDN18.2 expression (Figure 1). CLDN18.2 expression was positive in 73.8% (48) of the patients.

During the median 17.7-mo follow-up period, 89.2% (58) of the patients died. Median PFS and OS were 6 mo (95% confidence interval: 1.6-10.4) and 12 mo (95% confidence interval: 7.5-16.5). There was no statistically significant correlation between CLDN18.2 expression and clinicopathological characteristics of the patients (Figure 2). In univariate and multivariate Cox regression analysis for PFS, there was no correlation between clinicopathological characteristics of patients and PFS (Table 2). In univariate and multivariate Cox regression analysis for OS, older age was an independent risk factor for poor OS (Table 3).

DISCUSSION

Gastric cancer is common and fatal. With targeted agents and immunotherapy, the median OS of patients with metastatic gastric cancer has reached 13.8-14.4 mo[11,12]. Novel therapies are critical for extending the survival of gastric adenocarcinoma patients. CLDN18.2 is a tight junction molecule found on the surface of gastric mucosa epithelium and gastric cancer cells[6]. In metastatic gastric cancer patients, the monoclonal antibody zolbetuximab targeting CLDN18.2 contributes to OS alone and when combined with ChT. It had tolerable side effects such as nausea and vomiting[7,8]. Worldwide clinical

Table 1 Clinicopathological characteristics of patients with gastric adenocarcinoma based on claudin 18.2 expression

Parameter	Number of cases		CLDN18.2 score								*P* value
			0		1		2		3		
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	
Age in yr											
< 65	30	46.2	4	13.3	10	33.3	9	30.0	7	23.3	0.091
≥ 65	35	53.8	13	37.1	10	28.6	5	14.3	7	20.0	
Sex											
Male	49	75.4	14	28.6	15	30.6	11	22.4	9	18.4	0.314
Female	16	24.6	3	18.8	5	31.3	3	18.8	5	31.3	
Lauren classification											
Intestinal	41	63.1	9	22.0	12	29.3	10	24.4	10	24.4	0.221
Diffuse	24	39.9	8	33.3	8	33.3	4	16.7	4	16.7	
Tumor grade											
G1	30	46.2	9	30.0	8	26.7	7	23.3	6	20.0	0.889
G2	8	12.3	1	12.5	3	37.5	2	25.0	2	25.0	
G3	27	41.5	7	25.9	9	33.3	5	18.5	6	22.2	
Localization											
Cardia	18	27.7	3	16.7	9	50.0	3	16.0	3	16.7	0.307
Corpus	10	18.5	4	33.3	5	41.7	1	8.3	2	16.7	
Antrum	12	15.4	3	30.0	3	30.0	2	20.0	2	20.0	
Pylorus	2	2.1	0	0.0	0	0.0	2	100.0	0	0.0	
Antropyloric	23	35.4	7	30.4	3	13.0	6	26.1	7	30.4	
Her2Neu											
Negative	54	83.1	13	24.1	15	27.8	13	24.1	13	24.1	0.116
Positive	11	16.9	4	36.4	5	45.5	1	9.1	1	9.1	
Family history											
No	40	61.5	13	32.5	9	22.5	10	25.0	8	20.0	0.751
Yes	14	21.5	2	14.3	6	42.9	2	14.3	4	28.6	
Unknown	11	16.9	2	18.2	5	45.5	2	18.2	2	18.2	
Liver metastasis											
No	33	50.8	8	25.0	10	31.3	6	18.8	8	25.00	0.703
Yes	32	49.2	9	27.3	10	30.3	8	24.2	6	18.2	
Lung metastasis											
No	48	73.2	9	52.9	3	17.6	2	11.8	3	17.6	0.053
Yes	17	26.8	8	16.7	17	35.4	12	25.0	11	22.9	
Metastasis sites											
Liver	16	24.6	2	12.5	7	43.8	2	12.5	5	31.3	0.050
Lung	4	6.2	2	50.0	0	0.0	0	0.0	2	50.0	
Peritoneum	11	16.9	0	0.0	5	45.5	3	27.3	3	27.3	
LAP	14	21.5	4	28.6	2	14.3	6	42.90	2	14.30	
Brain	2	3.1	2	100.0	0	0.0	0	0.0	0	0.0	
Liver + lung	17	26.2	7	41.2	6	35.0	2	11.8	2	11.8	

Ovary	1	1.5	0	0.0	0	0.0	1	100.0	0	0.0	
Adjuvant chemotherapy											
No	29	44.6	8	27.6	9	31.0	6	20.7	6	20.7	0.793
Yes	36	55.4	9	25.0	11	30.6	8	22.2	8	22.2	
Neoadjuvant chemotherapy											
No	61	91.8	14	23.0	20	32.8	13	21.3	14	23.0	0.097
Yes	4	8.2	3	75.0	0	0.0	1	25.0	0	0.0	
Exitus											
No	7	10.8	0	0.0	3	42.9	3	42.9	1	14.3	0.401
Yes	58	89.2	17	29.3	17	29.3	11	19.0	13	22.4	

CLDN18.2: Claudin 18.2; LAP: Lymphadenopathy.

Table 2 Univariate and multivariate analysis of baseline characteristics for progression-free survival

Parameter	Progression-free survival univariate analysis				Progression-free survival multivariate analysis		
	HR	95%CI	*P*		HR	95%CI	*P*
Age in yr	1.36	0.80-2.30	0.26	Age in yr	1.29	0.71-2.33	0.41
Sex	1.40	0.76-2.56	0.28	Sex	1.49	0.73-3.05	0.28
Lauren classification	0.89	0.52-1.53	0.67	Lauren classification	0.93	0.39-2.22	0.87
Tumor grade	0.85	0.49-1.47	0.56	Tumor grade	0.91	0.58-1.43	0.69
Family history	0.87	0.45-1.77	0.75	Family history	0.51	0.20-1.28	0.15
Liver metastasis	1.09	0.64-1.85	0.75	Liver metastasis	1.07	0.60-1.91	0.82
Lung metastasis	0.93	0.52-1.66	0.79	Lung metastasis	0.98	0.49-1.94	0.95
Localization	0.94	0.42-2.11	0.88	Localization	1.01	0.81-1.24	0.98
Metastasis sites	0.83	0.27-2.60	0.75	Metastasis sites	0.99	0.84-1.18	0.94
Her2Neu	0.81	0.40-1.64	0.56	Her2Neu	0.85	0.37-1.93	0.69
CLDN18.2	1.22	0.54-2.32	0.77	CLDN18.2	1.30	0.54-3.19	0.56

CI: Confidence interval; CLDN18.2: Claudin 18.2; HR: Hazard ratio.

trials of zolbetuximab in the first-line setting, in combination with ChT and immunotherapy, are ongoing for G/GEJ adenocarcinoma (NCT03505320, NCT03504397, and NCT03653507).

Histopathological subtype was diffuse in 36.9% (24) of patients and intestinal in 63.1% (41), and there was no correlation with CLDN18.2 expression. In a study including 481 patients with gastric cancer, there was no correlation between histopathological subtype per Lauren classification and CLDN18.2 expression, as in our study[13]. However, in a study including 263 Japanese patients with gastric adenocarcinoma, diffuse histopathological subtype was associated with strong CLDN18.2 expression [14]. In another study of 85 patients with gastric adenocarcinoma, intestinal subtype was associated with strong CLDN18.2 expression[15]. There was no correlation between grades of tumors and CLDN18.2 expression in a study including 485 patients with esophageal adenocarcinoma[16]; however, grade 3 tumors were associated with strong CLDN18.2 expression in two studies[13,14].

HER2 expression was positive in 16.9% (11) of patients, and there was no correlation between HER2 and CLDN18.2 expression. In three different studies, there was no correlation between HER2 and CLDN18.2 expression[13,15,17], while there was an inverse correlation in a study including patients with esophageal adenocarcinoma[16].

In the present study, CLDN18.2 expression was detected in 73.8% (48) of patients, with moderate to strong expression (≥ 2+) in 43.1% (n = 28). CLDN18.2 expression was detected in 87%, with moderate and strong expression in 51.5%, of Japanese patients in a study conducted by Rohde *et al*[14], and moderate to strong expression was present in 49% of patients with G/GEJ adenocarcinoma in the FAST study conducted by Sahin *et al*[6]. There was no correlation between clinicopathological characteristics

Table 3 Univariate and multivariate analysis of baseline characteristics for overall survival

Paragraph	Overall survival univariate analysis			Overall survival multivariate analysis			
	HR	95%CI	*P*		HR	95%CI	*P*
Age in yr	2.46	1.39-4.33	0.01	Age in yr	3.17	1.45-6.92	0.01
Sex	1.10	0.61-1.99	0.75	Sex	0.65	0.26-1.59	0.34
Lauren classification	1.28	0.66-1.94	0.66	Lauren classification	1.23	0.24-6.16	0.81
Tumor grade	0.41	0.15-1.07	0.07	Tumor grade	0.29	0.08-1.06	0.06
Family history	0.83	0.36-1.97	0.67	Family history	2.14	0.68-6.71	0.19
Liver metastasis	0.94	0.56-1.59	0.82	Liver metastasis	0.74	0.33-1.65	0.46
Lung metastasis	0.71	0.39-1.27	0.25	Lung metastasis	0.58	0.22-1.52	0.27
Localization	0.91	0.46-1.79	0.78	Localization	2.14	0.68-6.71	0.19
Metastasis sites	1.36	0.30-6.09	0.69	Metastasis sites	1.88	0.52-6.82	0.34
Her2Neu	1.11	0.56-2.22	0.77	Her2Neu	0.90	0.35-2.34	0.83
CLDN18.2	1.68	0.81-3.50	0.12	CLDN18.2	2.78	0.85-9.07	0.09

CI: Confidence interval; CLDN18.2: Claudin 18.2; HR: Hazard ratio.

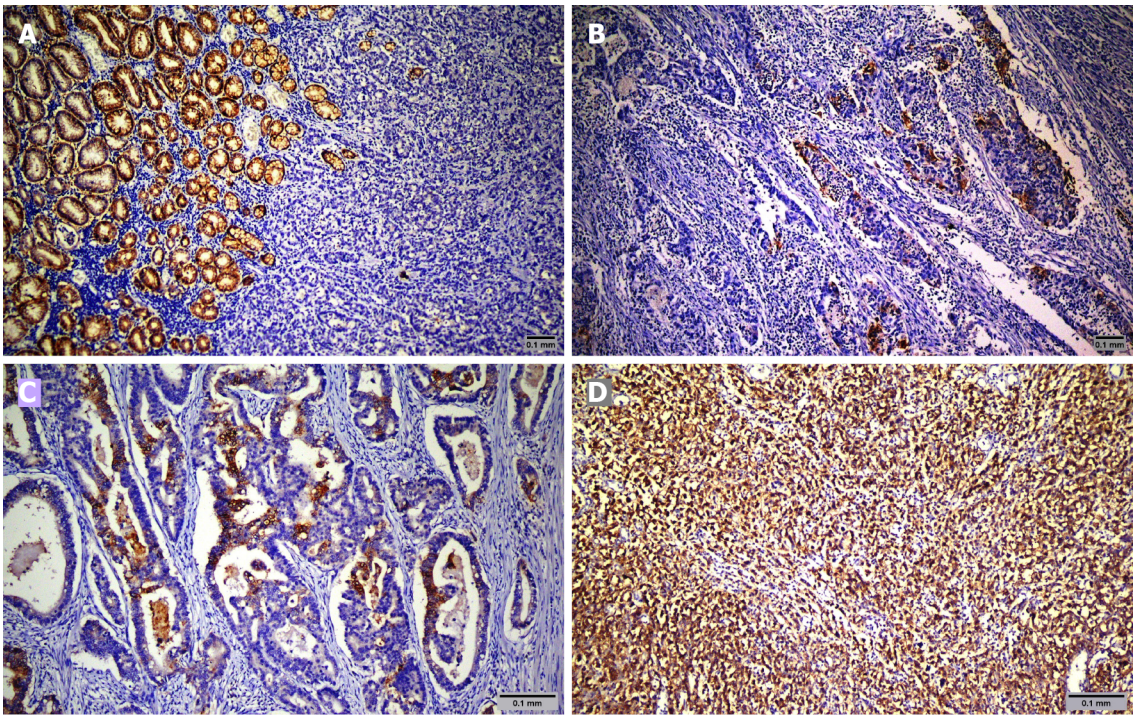

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Figure 1 Representative images of claudin 18.2 immunohistochemical staining in gastric adenocarcinoma. A: Score 0; B: Score 1+; C: Score 2+; D: Score 3+.

of the patients and OS in the present study, consistent with other studies[13,15,16].

Inconsistent with the present study, Türeci *et al*[7] and Sahin *et al*[8] detected CLDN18.2 expression in only 17.1% and 14.1% of patients, respectively. This could be due to the different patient cohorts in the studies as well as the different kits used to detect CLDN18.2 expression. Few studies have been published regarding the expression of CLDN18.2 in gastric adenocarcinoma. Conflicting results exist about the CLDN18.2 expression ratios and the relationship between these parameters and the clinicopathological characteristics of patients with gastric adenocarcinoma; however, the studies are consistent in showing there is no correlation between CLDN18.2 expression and OS, as in the present study. The proportion of patients with gastric adenocarcinoma in whom zolbetuximab was efficacious was determined by the MONO and FAST trials. Our findings are consistent with these studies.

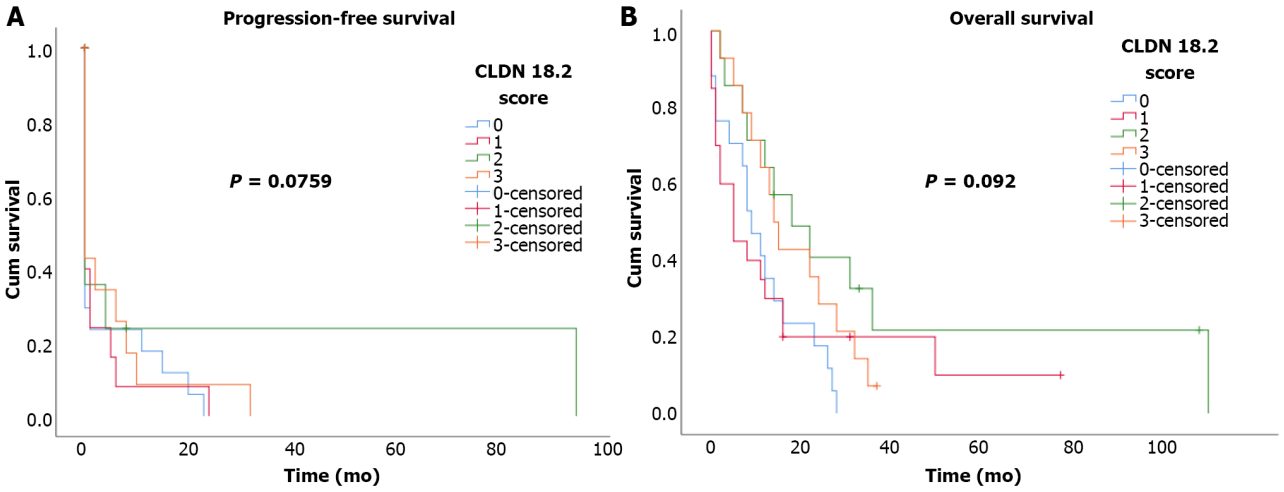

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Figure 2 Kaplan-Meier curves for according to claudin 18.2 expression scores. A: Progression-free survival; B: Overall survival according to claudin 18.2 expression (CLDN 18.2) scores.

The limitations of this study included the relatively small number of patients analyzed and the retrospective character. Additional studies with a larger number of patients are needed to define the effect of CLDN18.2 expression on OS.

CONCLUSION

CLDN18.2 expression is quite high in patients with gastric adenocarcinoma, identifying the proportion of the patients in whom zolbetuximab would be efficacious. There is no statistically significant correlation with clinicopathological characteristics and OS. CLDN18.2 is not a prognostic marker in patients with gastric adenocarcinoma.

ARTICLE HIGHLIGHTS

Research background

Claudin 18.2 (CLDN18.2) is a cell surface protein expressed by gastric cancer cells and a new target for the monoclonal antibody named zolbetuximab.

Research motivation

It is unknown whether CLDN18.2 expression on gastric cancer cells is prognostic.

Research objectives

To identify the prognostic value of CLDN18.2 expression in patients with metastatic gastric adenocarcinoma.

Research methods

We investigated the effect of CLDN18.2 expression on clinicopathological characteristics (age, sex, histological grade, Lauren classification, family history, metastatic site, and HER2 expression) and prognosis for patients with metastatic gastric adenocarcinoma.

Research results

There was no statistically significant correlation between CLDN18.2 expression and clinicopathological characteristics of the patients. In univariate and multivariate Cox regression analysis, there was no correlation between clinicopathological characteristics of patients and progression-free survival or overall survival. The expression of CLDN18.2 was predictive for zolbetuximab in metastatic gastric adenocarcinoma, but it is not prognostic.

Research conclusions

CLDN18.2 expression is high in metastatic gastric adenocarcinoma and predictive for zolbetuximab, but it is not prognostic.

Research perspectives

Detection of new prognostic and predictive markers will make gastric cancer more manageable.

FOOTNOTES

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META-ANALYSIS

Immune-related adverse events associated with immune checkpoint inhibitors for advanced gastric and gastroesophageal junction cancer: A meta-analysis

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Abstract

BACKGROUND

Immune checkpoint inhibitors (ICIs) have shown promising efficacy in treatment and clinical management of advanced gastric and gastroesophageal junction cancer. However, the inhibitors also cause immune-related adverse events (irAEs). The current systematic review and meta-analysis study aimed to investigate the incidence and nature of irAEs caused by ICIs.

AIM

To investigate the incidence and nature of irAEs in advanced gastric and gastroesophageal junction cancer.

METHODS

This systematic review was registered with PROSPERO (Reg. number: CRD42020152291). Data included in this study were collected from patients diagnosed with advanced gastric cancer or gastroesophageal junction cancer and treated with ICIs. A systematic literature search was conducted using the PubMed, EMBASE, and Cochrane Library databases. Meta-analysis was carried out using the single sample rate method. Synthesis and analysis of the data was conducted using Stata/SE and Review Manager Software.

RESULTS

The patients enrolled in the present study included 14 patients from 14 case reports, 326 patients from 6 case series, and 1249 patients from 8 clinical trials. It was found that the overall incidence of irAEs was 16% [95% confidence interval (CI): 11-20] for all grades and 3% (95%CI: 2-4) for the severe grade. It was evident that the incidence of irAEs varied with the type of inhibitor and organs. A comparative study of the anti-programmed cell death receptor-1 (PD-1) and anti-programmed death receptor-ligand 1 (PD-L1) treatments showed that the anti-

PD-1 group had a higher overall incidence of irAEs (20%) as compared with that of the anti-PD-L1 group (13%). Results of this study showed that the endocrine system experienced the highest incidence of organ-specific irAEs (7.4%), including hypothyroidism, hyperthyroidism, thyroiditis, diabetes, and adrenal insufficiency, followed by gastroenterology (2.2%), pulmonology (1.8%), neurology (1.4%), dermatology (1.4%), hematology (0.8%), and hepatology (0.7%). In clinical trials, it was found that the incidence of death related to irAEs was 1% (95%CI: 0-2.0), whereby colitis and interstitial lung diseases were the leading causes of death.

CONCLUSION

It was evident that the incidence and nature of irAEs are both organ- and inhibitor-specific. The anti-PD-1 group had the highest incidence of all irAEs grades including the severe grades of irAEs. Early identification and management of irAEs allows clinical oncologists to effectively consider the pros and cons and hence enables them to strike a balance.

Key Words: Immune checkpoint inhibitors; Advanced gastric and gastroesophageal junction cancer; Systematic review; Meta-analysis.

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Core Tip: This systematic review shows that there is an increasing number of immune-related adverse events (irAEs) associated with immune checkpoint inhibitors that are being reported in patients with gastric cancer or gastroesophageal junction cancer. This is particularly severe organ-specific irAEs and death because of irAEs, which poses significant challenges for clinical oncologists. Therefore, to help clinicians effectively identify and manage irAEs as well as strike a balance, a comprehensive understanding, systematic prediction, and appropriate management of the adverse events are critical.

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INTRODUCTION

Gastric cancer (GC) is one of the most prevalent malignancies of the digestive tract in the world whereby the global incidence and mortality of GC ranks fifth and fourth of the malignancies, respectively[1]. Furthermore, it has been found that the global incidence and mortality rates of GC are 15.59 *per* 100000 and 11.88 *per* 100000, respectively, as well as 30.64 *per* 100000 and 21.72 *per* 100000, respectively in China[2]. Although the most effective treatment for GC or gastroesophageal junction cancer (GEJC) is a surgical operation, the majority of patients cannot undergo radical surgery because of the advanced stage of the disease at the time of diagnosis. Instead, the patients receive chemotherapy, targeted therapy, and other medical treatment. Recently, immune checkpoint inhibitors (ICIs) have also made significant progress in the treatment and management of GC/GEJC.

The first ICI, ipilimumab [Yervoy, anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)], was approved by the Food and Drug Administration (FDA) in 2011 for the treatment of metastatic melanoma[3]. Following the approval of the first programmed cell death receptor-1 (PD-1)/programmed death receptor-ligand 1 (PD-L1) inhibitor (pembrolizumab) in 2014, several ICIs have later been utilized in clinical practice[4-5]. In 2021, the FDA approved two anti-PD-1 drugs (pembrolizumab and nivolumab) for treatment of different forms of GC. Generally, ICIs are divided into three categories: inhibitors of PD-1, PD-L1, and CTLA-4. Activation of PD-1 or PD-L1 signaling acts as the principal mechanism by which tumors evade antigen-specific T-cell immunologic responses. However, antibody blockage of PD-1 or PD-L1 reverses this process and enhances anti-tumor immune activity[6].

CTLA-4 possesses structural homology with CD28 and binds to the B7 molecules on APC with a higher affinity than the CD28. This results in a competitive inhibition of costimulatory CD28 signaling and damage to the T cell signaling[7-8]. ICIs exert anti-tumor effects by damaging co-inhibitory T cell signaling (Figure 1, source: Beida Pharmaceutical official website).

ICIs offer patients with GC or GEJC a glimmer of hope. A previous study suggested that pembrolizumab monotherapy may provide a potential treatment benefit for GC or GEJC[9]. However, ICIs result in severe or even fatal immune-related adverse events (irAEs) whereby they cause immune

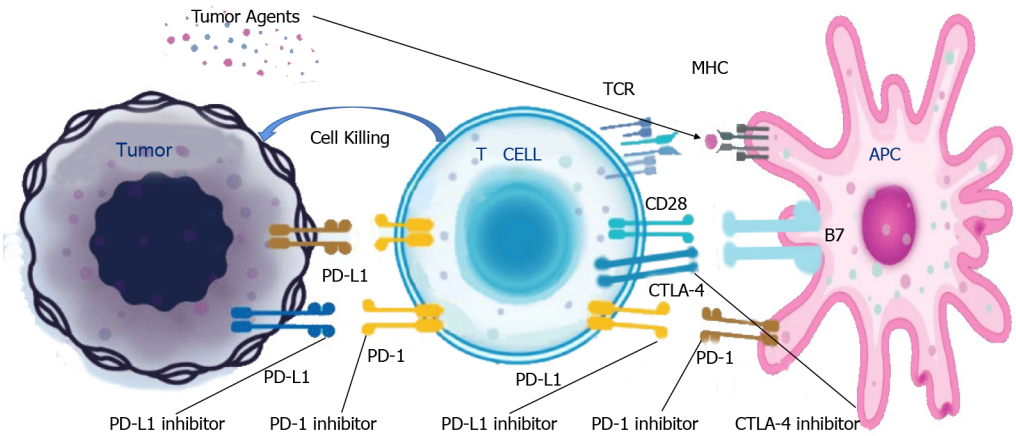

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Figure 1 The action mechanism of programmed cell death receptor-1, programmed death receptor-ligand 1, and cytotoxic T lymphocyte-associated antigen-4 inhibitors. PD-1: Programmed cell death receptor-1; PD-L1: Programmed death receptor-ligand 1; CTLA-4: Cytotoxic T lymphocyte-associated antigen-4; TCR: T-cell receptor; MHC: Major histocompatibility complex; APC: Antigen-presenting cell.

system hyperactivation in the normal tissues, which may be the underlying cause of irAEs[10]. Organ-specific irAEs associated with ICIs mainly occur in endocrinopathy, gastroenterology, hepatology, neurology, hematology, dermatology, pulmonology, nephrology, cardiology, and rheumatic immunology.

The irAEs can result in a reduction in dosage, drug withdrawal, a decrease in compliance, delayed treatment, organ function damage, and eventual death. These adverse events have been reported in other tumors, but there has been no systematic review of the events in GC or GEJC. Therefore, this meta-analysis was aimed to assess the incidence and nature of irAEs by conducting a systematic review of their adverse events in patients with GC or GEJC. The objective of the current systematic review and meta-analysis study was to assist clinicians in effective identification, and strike a balance by considering the pros and cons in management approaches of irAEs.

MATERIALS AND METHODS

Literature sources and searches

Three major databases (PubMed, EMBASE, and Cochrane Library) were used to perform a systematic literature search for the present study. The search was conducted for the studies published between January, 2000 and January, 2022. Population, Intervention, Comparison, Outcome, and Study design was utilized as a framework to conduct the literature search (Table 1). The relevant searching terms corresponded to terms of the Medical Subject Heading. In addition, the searches were immediately repeated before the final analyses to identify any additional studies for inclusion[11]. This study adhered to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses and was registered with PROSPERO (Registration number: CRD42020152291)[12]. Detailed search strategies in the three major databases (PubMed, EMBASE, and Cochrane Library) were as shown in Supplementary Tables 1-3. The retrieved documents were lastly managed using the EndNote 20.

Eligibility criteria and study selection

Inclusion criteria of the participants of this systematic review and meta-analysis study were: Adults diagnosed with advanced GC, GEJC, and treated with ICIs. On the other hand, the included studies were randomized controlled trials (RCTs), case series, and case reports published in peer-reviewed journals without language or time restrictions. In addition, there were no set restrictions on sex, race, ethnicity, education, and economic status in the study.

Exclusion criteria of this systematic review and meta-analysis study were: Patients receiving other therapies such as chemotherapy, radiotherapy, targeted therapy, or other immunotherapy. Further, the studies excluded were: Cohort studies, case-control studies, cross-sectional studies, and other nonrandomized studies.

First, duplications were filtered using the automatic screening function of EndNote 20. The unqualified documents were then filtered after reading the title and abstract. Finally, the studies were further filtered by reading their full text *via* the online databases and school libraries. Corresponding authors were also contacted for further clarification during the filtering process. The search was carried out by two independent reviewers. Differences were resolved through consensus after discussion and

Table 1 Population, Intervention, Comparison, Outcome, and Study design

Query	Search term
#1	P (Neoplasm, Stomach[Title/Abstract] OR Stomach oplasm[Title/Abstract] OR Neoplasms, Stomach[Title/Abstract] OR Gastric Neoplasms[Title/Abstract] OR Gastric Neoplasm[Title/Abstract] OR Neoplasm, Gastric[Title/Abstract] OR Cancer of Stomach[Title/Abstract] OR Stomach Cancers[Title/Abstract] OR Gastric Cancer[Title/Abstract] OR Cancer, Gastric[Title/Abstract] OR gastroesophageal junction cancer[Title/Abstract] OR gastroesophageal junction adenocarcinoma[Title/Abstract] OR adenocarcinoma gastroesophageal junction[Title/Abstract])
#2	I (Checkpoint Inhibitors, Immune[Title/Abstract] OR Immune Checkpoint Inhibitor[Title/Abstract] OR PD-L1 Inhibitors[Title/Abstract] OR PD L1 Inhibitors[Title/Abstract] OR PD-L1 Inhibitor[Title/Abstract] OR PD L1 Inhibitor[Title/Abstract] OR CTLA-4 Inhibitors[Title/Abstract] OR CTLA 4 Inhibitors[Title/Abstract] OR ipilimumab[Title/Abstract] OR ticilimumab[Title/Abstract] OR nivolumab[Title/Abstract] OR pembrolizumab[Title/Abstract] OR pidilizumab[Title/Abstract] OR atezolizumab[Title/Abstract] OR durvalumab[Title/Abstract] OR avelumab[Title/Abstract])
#3	O (immune-related adverse events[Title/Abstract] OR immune related adverse events checkpoint inhibitors[Title/Abstract] OR immune related adverse events checkpoint blockade[Title/Abstract] OR management of immune related adverse events[Title/Abstract] OR immune related adverse events in patients[Title/Abstract] OR immune related adverse events systemic immunosuppression[Title/Abstract])
#4	S ("randomized controlled trial"[pt] OR "controlled clinical trial"[pt] OR randomized[tiab] OR placebo[tiab] OR "drug therapy"[sh] OR randomly[tiab] OR trial[tiab] OR groups[tiab] OR "randomized controlled trial"[pt] OR "controlled clinical trial"[pt] OR "clinical trials as topic"[mesh] OR "random allocation"[mesh] OR "double-blind method"[mesh] OR "single-blind method"[mesh])
#5	#1 AND #2 AND #3 AND #4

PD-1: Programmed cell death receptor-1; PD-L1: Programmed death receptor-ligand 1; CTLA-4: Cytotoxic T lymphocyte-associated antigen-4.

consultation with a senior third party.

Outcomes

Incidences of irAEs and organ-specific adverse events associated with ICIs in the treatment of GC/GEJC were documented in the present meta-analysis. The irAEs were described using version 2, 3, or 4 of the Common Terminology Criteria for Adverse Events of the National Cancer Institute. Adverse events were graded on a scale of 1 to 5 and grades 3 to 5 were regarded as the severe grade.

Data extraction

The data for each study were independently extracted and recorded by the two reviewers. The data collected for clinical trials were: Author(s), year, clinical trial information, study design, enrollment size, types of tumors, type and dose of monoclonal antibodies, version of the Common Terminology Criteria for Adverse Events, frequency of irAEs and organ-specific irAEs, and the median time.

The data collected for case reports and case series were: Patient characteristics, previous oncologic treatment, cancer outcome (oncologic response or progressive disease), the nature of each irAE, as well as irAE onset, treatment, and outcome. The final results were cross-checked and any disagreements (Kappa score: 0.76) were resolved through consensus after discussion or consultation with a senior third party.

Quality assessment

The Cochrane Risk of Bias Tool was used to assess the risk of bias and quality of the RCTs[13]. The tool consists of seven aspects: Random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other biases. Each aspect of the Cochrane Risk of Bias Tool was assigned a high, low, or unclear risk of bias[13]. Quality assessment was conducted using Review Manager Software (version 5.4.1). This quality assessment was independently conducted by two reviewers who reached an agreement through consensus.

Data synthesis and analysis

All analyses in the present systemic review and meta-analysis were performed using Stata/SE (version 12.0) and Review Manager (version 5.4.1) software. The following was the procedure involved in conducting the statistical analysis in the study.

Effect values combination

First, the incidence of irAEs was determined in each study based on the sample size and total number of irAEs. The incidence of irAEs was then combined, and the effect value was determined based on a meta-analysis of sample rate and standard error. Stata/SE software (version 12.0) was used to draw the forest map and obtain the 95% confidence intervals (CIs) for the weighted average of all studies[14]. The combined effect value was conducted using Stata/SE (version 12.0) with the metan and metafunnel

commands of meta-analysis.

Heterogeneity test

Statistical heterogeneity between the selected studies was analyzed using the Q test and I^2 statistic. When the *P*-value of Q statistic was > 0.10 or $I^2 \leq 50\%$, there was no heterogeneity or acceptable heterogeneity between the studies. Further, when the *P* value was ≤ 0.10 or $I^2 > 50\%$, there was a greater degree of heterogeneity between the studies[15]. The random-effect model, which accounts for the heterogeneity between the studies was used to examine the effect size because the heterogeneity between the studies was greater[16]. A Galbraith plot for heterogeneity was drawn to evaluate heterogeneity in the present study. The heterogeneity test was conducted using Stata/SE with the galbr command of meta-analysis.

Publication bias test

Initially, the risk of publication bias was evaluated using a funnel plot with pseudo 95% confidence limits and the publication bias was then assessed in the present study by observing the symmetry of the funnel plot. Furthermore, the funnel plot was evaluated using both the Begg and Egger methods. Therefore, the funnel plot was quantified and publication bias was assessed by examining the P-value. The test of publication bias was conducted using Stata/SE with the metabias command of meta-analysis.

Subgroup analysis

Subgroup analysis is a common method for addressing heterogeneity. The studies in the present review were grouped according to the types of ICIs and organ-specific adverse events studied. The analysis was conducted using Stata/SE with the metan command of meta-analysis.

Sensitivity analysis

A new meta-analysis was conducted to determine whether the effect size had changed whenever research was deleted. However, the deleted study was considered when result of the new meta-analysis differed from that of the previous one to influence the total effect size. Influence analysis was conducted using Stata/SE with the metaninf command of meta-analysis.

RESULTS

Study selection

The literature search was conducted in the current systematic review based on the pre-established strategy. A total of 285 pieces of literature were searched including 38, 146, and 101 in the PubMed, EMBASE, and Cochrane databases, respectively. Initially, a total of 62 duplicated literature were excluded. A total of 155 articles that did not meet the criteria were then excluded after reading their titles and abstracts. A total of 28 articles (8 clinical trials, 14 case reports, and 6 case series) that met the inclusion criteria were finally selected after reading the full text (Figure 2). A reference list of all the excluded studies and reasons for their exclusion was as shown in the Supplementary Table 4.

Nature of irAEs: Data from clinical trials

General characteristics: A total of 8 clinical trials were included in this meta-analysis including 2 PD-1 (pembrolizumab)[17-18], 5 PD-L1 (avelumab)[19-23], and 1 CTLA-4 (ipilimumab)[24]. All reviewed trials showed total irAEs, with 6 of them describing organ-specific irAEs. The remaining 2 trials only reported total irAEs. The general characteristics of the included studies were as shown in Table 2, which included a total of 1249 participants from 8 clinical trials. All trials included in the meta-analysis were open-label, multicenter, and randomized trials. Further, it was found that there was only one phase II clinical trial, three phase I clinical trials, and four phase III clinical trials. The average immunotherapy duration for the all included trials was 2.9 mo [interquartile range (IQR): 2.4 to 3.1 mo], whereas the median follow-up time was 15.5 mo (IQR: 9.9 to 20.5 mo). The median overall survival (OS) of these trials ranged between 4.6 (95%CI: 3.6 to 5.7) to 12.7 mo (95%CI: 10.5 to 18.9) whereas the median PFS ranged between 1.4 (95%CI: 1.4 to 1.5) and 3.2 mo (95%CI: 2.8 to 4.1).

Global incidence of irAEs: The global incidence of irAEs for overall grades was 20% (95%CI: 16 to 23) in the anti-PD-1 group, 13% (95%CI: 8 to 19) in the anti-PD-L1 group, and 18% (95%CI: 8 to 27) in the anti-CTLA-4 group, whereas 4% (95%CI: 2 to 5) in the anti-PD-1 group and 3% (95%CI: 1 to 4) in the anti-PD-L1 group for severe grade (Supplementary Figures 1-3). It was found that the anti-PD-1 group had the highest incidence of irAEs at all grades and severe grades as compared with that of the other three inhibitors. In addition, the overall incidence of irAEs was 16% (95%CI: 11 to 21) for all grades and 3% (95%CI: 2 to 4) for severe grade in the anti-PD-1 combined with the anti-PD-L1 group (Supplementary Figure 4), which was comparable with that in the anti-PD-1 combined with anti-PD-L1,

Table 2 Study characteristics of included clinical trials

Trial	Design	Design details	Cancer types	Enrollment size, *n*	ICIs	Dose, mg/kg	IrAEs, all grades, *n*	IrAEs, severe grades, *n*	mOS	mPFS
Shitara *et al*[17], 2018	RCT	Open-label, multicenter, phase III	Advanced GC/GEJC	294	PD-1 (pembrolizumab)	200 mg, q3w	61	10	9.1 mo (95%CI: 6.2 to 10.7 mo)	1.5 mo (95%CI: 1.4 to 2.0 mo)
Fuchs *et al* [18], 2022	RCT	Open-label, multicenter, phase III	Advanced GC/GEJC	294	PD-1 (pembrolizumab)	200 mg, q3w	55	11	NA	NA
Moehler *et al*[19], 2021	RCT	Open-label, multicenter, phase III	Advanced GC/GEJC	249	PD-L1 (avelumab)	10, q2w	32	8	10.4 mo (95%CI: 9.1 to 12.0 mo)	3.2 mo (95%CI: 2.8 to 4.1 mo)
Doi *et al* [20], 2019	RCT	Open-label, multicenter, phase I	Advanced GC/GEJC	40	PD-L1 (avelumab)	10, q2w	9	0	9.1 mo (95%CI: 7.2 to 11.2 mo)	2.4 mo (95%CI: 1.4 to 2.8 mo)
Doi *et al* [21], 2018	RCT	Open-label, multicenter, phase I	Advanced GC/GEJC	40	PD-L1 (avelumab)	10, q2w	5	0	9.1 mo (95%CI: 7.2 to 11.2 mo)	2.5 mo (95%CI: 1.4 to 2.8 mo)
Chung *et al*[22], 2019	RCT	Open-label, multicenter, phase I	Advanced GC/GEJC	90	PD-L1 (avelumab)	10, q2w	17	2	NA	NA
Bang *et al* [23], 2018	RCT	Open-label, multicenter, phase III	Advanced GC/GEJC	185	PD-L1 (avelumab)	10, q2w	12	4	4.6 mo (95%CI: 3.6 to 5.7 mo)	1.4 mo (95%CI: 1.4 to 1.5 mo)
Bang *et al* [24], 2017	RCT	Open-label, multicenter, phase II	Advanced GC/GEJC	57	CTLA-4 (ipilimumab)	10, q3w	10	0	12.7 mo (95%CI: 10.5 to 18.9 mo)	2.7 mo

ICIs: Immune checkpoint inhibitors; irAEs: Immune-related adverse events; mOS: Median overall survival; mPFS: Median progression-free survival; RCT: Randomized controlled trial; GC: Gastric cancer; GEJC: Gastroesophageal junction cancer; NA: Not available; PD-1: Programmed cell death receptor-1; PD-L1: Programmed death receptor-ligand 1; CTLA-4: Cytotoxic T lymphocyte-associated antigen-4.

and anti-CTLA-4 groups (Figure 3A and B).

Incidence of organ-specific irAEs: Organ-specific irAEs and their incidence were described as shown in Figure 3C. It was found that only one article documented an irAEs associated with dermatology[21]. In addition, it was noted that there were only two articles that showed the incidence of total irAEs without describing organ-specific irAEs[22-23]. The most common organ-specific irAEs occurred in the endocrine system, accounting for 7.4% (95%CI: 5.9-8.8), and included hypothyroidism, hyperthyroidism, thyroiditis and diabetes, followed by gastroenterology, pulmonology, neurology, and dermatology, accounting for 2.2% (95%CI: 1.4-3.1), 1.8% (95%CI: 1.0-2.5), 1.4% (95%CI: 0.8-2.1) and 1.4% (95%CI: 0.8-2.1), respectively.

On the other hand, it was found that organ-specific irAEs with a lower incidence occurred in hematology and hepatology, accounting for 0.8% (95%CI: 0.3-1.3) and 0.7% (95%CI: 0.3-1.2), respectively. However, it was evident that the incidence of severe grade organ-specific irAEs was higher in hematology and hepatology than in other systems which was comparable with the results observed in the anti-PD-1 group (Figure 3C). In the group of anti-PD-1 combined with the anti-PD-L1, it was found that the most common organ-specific irAEs occurred in endocrinology accounting for 7.7% (95%CI: 6.2-9.2), whereas the rarest organ-specific irAEs occurred in hepatology and accounted for 0.7% (95%CI: 0.4-1.0). Incidence of organ-specific irAEs related to endocrinopathy, gastroenterology, hepatology, neurology, hematology, dermatology, pulmonology, nephrology, cardiology, and rheumatic immunology was displayed in forest plots for all grade and severe grade in anti-PD-1, anti-PD-L1, anti-CTLA-4, or anti-PD-1 combined with anti-PD-L1 groups (Supplementary Figures 5-23).

Incidence of death related to irAEs: Results of this study found that the incidence of death related to irAEs was 1% (95%CI: 0-2.0) in all the included trials and a total of 6 deaths were reported in the anti-PD-1 group (Supplementary Figure 24). Further, it was evident that the main causes of death were

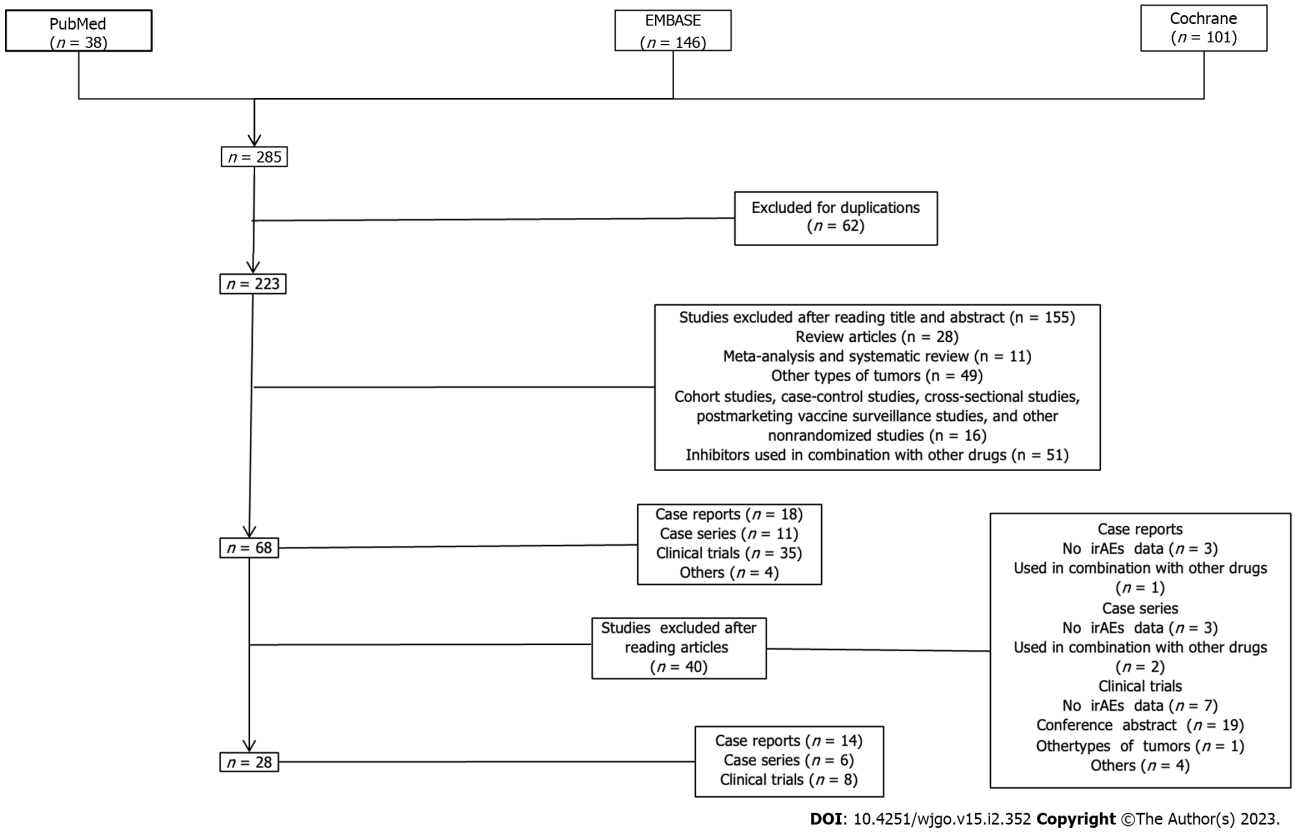

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Figure 2 Flowchart of study selection and design.

colitis and interstitial lung disease.

Nature of irAEs: Data from case reports and case series

General characteristics: A total of 14 case reports[25-38] and 6 case series[39-44] were included in this meta-analysis. In the case of reports, one patient received pembrolizumab treatment and thirteen received nivolumab treatment. The general characteristics of the patients were as shown in Attached file 4 (Supplementary Table 5). The average age of the enrolled patients was 70 years and 79% of them were male. Before receiving the anti-PD-1 therapy, it was found that 13 patients (93%) had failed at least one course of chemotherapy. Twelve patients (86%) reported occurrence of irAEs in a single system[25,27-33,35-38], whereas two patients (14%) reported occurrence of irAEs in more than one system.

Results of case reports indicated an average immunotherapy duration of 14.6 cycles (IQR: 5.5 to 17.5) and a mean onset time of 8.2 mo (IQR: 3.0 to 6.0). Four patients (29%) continued to receive anti-PD-1 treatment despite irAEs[25,27,29-30]. In the case series, all 326 patients received nivolumab treatment and their general characteristics were as shown in Table 3. Furthermore, the median OS in these case series ranged from 2.5 mo (95%CI: 0 to 5.0) to 7.9 mo (95%CI: 5.9 to 13.5), and the median PFS ranged from 1.0 mo (95%CI: 0.9 to 1.1) to 2.3 mo (95%CI: 0.5 to 24.8).

Incidence and nature of irAEs: Results of the present study showed that organ-specific irAEs in the case of reports were as described in the Supplementary Table 6. It was found that the endocrine system had the highest incidence of organ-specific irAEs, accounting for 36% (n = 5), including hyperthyroidism (n = 1)[37], thyroiditis (n = 2)[34,38], ACTH deficiency (n = 2)[30,38], and diabetes (n = 1)[31]. On the other hand, neurology and dermatology had the lowest incidence of organ-specific irAEs, accounting for 7% for each (n = 2)[26,29], including dizziness, nausea, truncal ataxia, rash, and sequential herpes zoster virus activation.

One patient experienced irAEs in multiple systems[26], including hematology, nephrology, dermatology, cardiology, and pulmonology. Although the patient was cured of irAEs, after receiving steroid treatment, he later suffered severe irAEs[38], developed grade 3 thyroiditis, and ACTH deficiency. It was found that treatment with anti-PD-1 induced an oncologic response in three patients (21%), and disease progression in five patients (36%). A total of 11 (79%) patients with irAEs were treated with steroids, 6 (43%) had cured irAEs, 3 (21%) had persistent irAEs, and 2 (14%) remained uncertain. Two (14%) of the 3 patients who were not treated with steroids developed persistent irAEs, and 1 (7%) died.

Table 3 Characteristics of the included case series studies

Case series	Enrollment size, *n*	Cancer types	ICIs	IrAEs, all grades, *n*	IrAEs, severe grades, *n*	mOS	mPFS
Suzuki *et al*[44], 2021 (Low ascites burden)	50	AGC	PD-1 (nivolumab)	9	1	5.3 mo (95%CI: 3.4 to 7.3 mo)	1.5 mo (95%CI: 1.0 to 2.0 mo)
Suzuki *et al*[44], 2021 (High ascites burden)	22	AGC	PD-1 (nivolumab)	5	0	2.5 mo (95%CI: 0 to 5.0 mo)	1.0 mo (95%CI: 0.9 to 1.1 mo)
Ohta *et al*[43], 2020	15	AGC	PD-1 (nivolumab)	5	0	6.3 mo	NA
Namikawa *et al*[42], 2020	29	AGC	PD-1 (nivolumab)	10	0	5.6 mo (95%CI: 0.6 to 26.8 mo)	2.3 mo (95%CI: 0.5 to 24.8 mo)
Kono *et al*[41], 2021	52	AGC	PD-1 (nivolumab)	13	1	7.9 mo (95%CI: 5.9 to 13.5 mo)	1.9 mo (95%CI: 1.4 to 3.0 mo)
Booka *et al*[40], 2021	50	GEA/ESCC	PD-1 (nivolumab)	13	5	NA	NA
Ando *et al*[39], 2021	108	AGC	PD-1 (nivolumab)	17	5	3.6 mo (95%CI: 3.0 to 5.3 mo)	1.4 mo (95%CI: 1.2 to 1.8 mo)

ICIs: Immune checkpoint inhibitors; irAEs: Immune-related adverse events; mOS: Median overall survival; mPFS: Median progression-free survival; NA: Not available; PD-1: Programmed cell death receptor-1; AGC: Advanced gastric cancer; GEA: Gastroesophageal adenocarcinoma; ESCC: Esophageal squamous cell carcinoma.

Results showed that the overall incidence of irAEs in the case series was 22% (95%CI: 17 to 27) for all grades and 3% (95%CI: 1 to 6) for severe grade (Figure 4). It was noted that[39] the overall incidence of organ-specific irAEs was reported in only one article but did not describe organ-specific irAEs. Further, 7.1% of all grade organ-specific irAEs occurred in the endocrine system, including hypothyroidism (n = 6), hyperthyroidism (n = 2), thyroiditis (n = 3), hypopituitarism (n = 2), hyperglycemia (n = 1), thyroid insufficiency (n = 3), type 1 diabetes mellitus (n = 1) and others (n = 5). This was then followed by pulmonology (4.3%, n = 14), gastroenterology (3.7%, n = 12), and dermatology (3.4%, n = 11), whereas organ-specific irAEs with a lower incidence included, myocarditis, infusion reaction, arthritis, liver insufficiency, loss of appetite, taste disorder, myopathy, adrenal insufficiency, and mucositis.

Interstitial pneumonia and myocarditis were the most common organ-specific irAEs for severe grade. It was found that two patients died due to severe myocarditis and interstitial pneumonia. Furthermore, one article[43] reported that the incidence of irAEs in patients with advanced GC and a high ascites burden was 23% (95%CI: 5 to 40), as compared with 18% (95%CI: 7 to 29) in patients with a low ascites burden. In addition, the median OS in the high and low ascites burden groups was 2.5 mo (95%CI: 0 to 5.0) and 5.3 mo (95%CI: 3.4 to 7.3), respectively. Comparatively, the median PFS in the high and low ascites burden groups were 1.0 mo (95%CI: 0.9 to 1.1) and 1.5 mo (95%CI: 1.0 to 2.0), respectively.

Quality of included studies and sensitivity analysis: Quality assessment: The risk of bias in each of the included RCTs was as shown in the attached file 5. The risk of selection bias was rated as high in 3 studies (37.5%) whereas the risk of reporting bias was rated as high in 5 studies (62.5%) (Supplementary Figures 25 and 26).

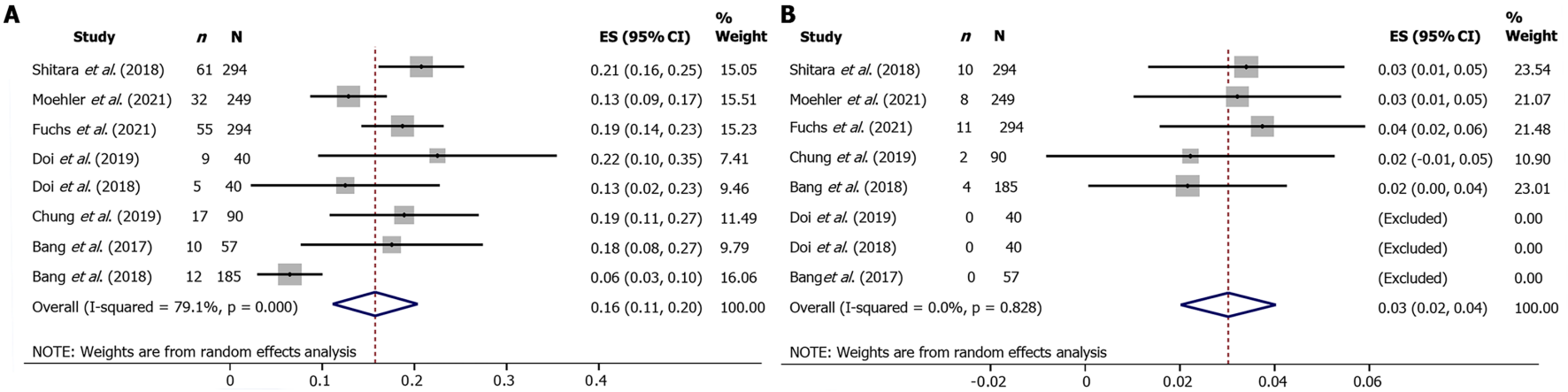
Sensitivity analysis: The sensitivity analysis of all clinical trials was as shown in Attached file 5. It was found that the influence of a single study on the total merger effect was not significant except for one study[22] (Supplementary Figure 27).

Heterogeneity test: Galbraith plot indicated that there existed heterogeneity between the included studies (Supplementary Figure 28). Therefore, sensitivity analysis was used to explain the source of heterogeneity and the random effect model was used to determine the effect quantity.

Publication bias test: The Begg's funnel plot and Egger's publication bias plot showed that there was existence of publication bias among the included studies (Supplementary Figures 29 and 30).

DISCUSSION

This meta-analysis analyzed the irAEs of ICIs for advanced GC/GEJC according to different targets, tumor types, drug types, doses, and organ specificity to improve the understanding of the safety and efficacy of the emerging cancer drugs. A total of 8 clinical trials, 14 case reports, and 6 case series were included in this study. It was evident that the overall incidence of irAEs was high in patients with advanced GC/GEJC, at a rate of 16% (95%CI: 11 to 21) in clinical trials and 22% (95%CI: 17 to 27) in case series. It noted that the most common organ-specific irAEs were endocrine system disorders, including



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		IRAES,(95%CI)													
	Intervention	**Endocrinopathy**		**Gastroenterology**		**Hepatology**		**Neurology**		**Hematology**		**Dermatology**		**Pulmonology**	
Drug		Any (%)	Severe (%)	Any (%)	Severe (%)	Any (%)	Severe (%)	Any (%)	Severe (%)	Any (%)	Severe (%)	Any (%)	Severe (%)	Any (%)	Severe (%)
Pembrolizumab (Anti-PD-1)	588	13.9 (11.1-16.7)	0.9 (0.1-1.6)	3.7 (2.2-5.3)	0.7 (0-1.3)	1.4 (0.4-2.3)	1.4 (0.4-2.3)	3.1 (1.7-4.5)	0.2 (-0.2-0.5)	1.7 (0.7-2.7)	1.2 (0.3-2.1)	0.3 (-0.1-0.8)	0.3 (-0.1-0.8)	2.7 (1.4-4.0)	0.7 (0-1.3)
Avelumab (Anti-PD-Ll)	604	1.7 (0.6-2.7)	0	NA	NA	NA	NA	NA	NA	NA	NA	2.6 (1.4-0.03.9)	0	1.0 (0.2-1.8)	0
Anti-PD-1 and anti-PD-Ll	1192	7.7 (6.2-9.2)	0.4 (0.1-0.8)	1.8 (1.1-2.6)	0.3 (0-0.7)	0.7(0.2-1.1)	0.7 (0.2-1.1)	1.5 (0.8-2.2)	0.1 (-0.1-0.2)	0.8 (0.3-1.4)	0.6 (0.2-1.0)	1.5 (0.8-2.2)	0.2 (-0.1-0.4)	1.8 (1.1-2.6)	0.3 (0-0.7)
Ipilimumab (Anti-CTLA-4)	57	NA	NA	10.5 (2.6-18.5)	0	1.8 (-1.7-5.2)	0	NA	NA	NA	NA	NA	NA	NA	NA
Total	2441	7.5 (6.5-8.6)	0.4 (0.2-0.7)	2.0 (1.5-2.6)	0.3 (0.1-0.6)	0.7 (0.4-1.0)	0.7 (0.3-1.0)	1.5 (1.0-2.0)	0.1 (0-0.2)	0.8 (0.5-1.2)	0.6 (0.3-0.9)	1.5 (1.0-2.0)	0.2 (0-0.3)	1.8 (1.3-2.3)	0.3 (0.1-0.6)
Author															
Shitara *et al.* (2018)	294	13.6 (9.7-17.5)	0.7 (-0.3-1.6)	6.5 (3.7-9.3)	0.7 (-0.3-1.6)	1.4 (0-2.7)	1.4 (0-2.7)	6.1 (3.4-8.9)	0.3 (-0.3-1.0)	3.4 (1.3-5.5)	2.4 (0.6-4.1)	0.3 (-0.3-1.0)	0.3 (-0.3-1.0)	2.7 (0.9-4.6)	0.7 (-0.3-1.6)
Fuchs *et al.* (2021)	294	14.3 (10.3-18.3)	1.0 (-0.1-2.2)	1.0 (-0.1-2.2)	0.7 (-0.3-1.6)	1.4 (0-2.7)	1.4 (0-2.7)	NA	NA	NA	NA	0.3 (-0.3-1.0)	0.3 (-0.3-1.0)	2.7 (0.9-4.6)	0.7 (-0.3-1.6)
Moehler *et al.* (2021)	249	2.8 (0.8-4.9)	0	NA	NA	NA	NA	NA	NA	NA	NA	2.0 (0.3-3.8)	0	2.4 (0.5-4.3)	0
Doi *et al.* (2019)	40	7.5 (-0.7-15.7)	0	NA	NA	NA	NA	NA	NA	NA	NA	15.0 (3.9-26.1)	0	NA	NA
Doi *et al.* (2018)	40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	12.5 (2.3-22.7)	0	NA	NA
Chung *et al.* (2019)	90	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Bang *et al.* (2017)	57	NA	NA	10.5 (2.6-18.5)	0	1.8 (-1.7-5.2)	0	NA	NA	NA	NA	NA	NA	NA	NA
Bang *et al.* (2018)	185	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total	1249	7.4 (5.9-8.8)	0.4 (0.1-0.8)	2.2 (1.4-3.1)	0.3 (0-0.6)	0.7 (0.3-1.2)	0.6 (0.2-1.1)	1.4 (0.8-2.1)	0.1 (-0.1-0.2)	0.8 (0.3-1.3)	0.6 (0.1-1.0)	1.4 (0.8-2.1)	0.2 (-0.1-0.4)	1.8 (1.0-2.5)	0.3 (0-0.6)

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Figure 3 Incidence of global immune-related adverse events associated with anti- programmed cell death receptor-1, anti-programmed death receptor-ligand 1, and anti-cytotoxic T lymphocyte-associated antigen-4. A: All grade; B: Severe grade; C: Incidence of organ specific immune-related adverse events, value are percentage (95% confidence intervals). Any: includes all Common Terms classified by Clinical Adverse Events grades; Severe: includes CTCAE grades 3,4, or 5. NA: Not available; PD-1: Programmed cell death receptor-1; PD-L1: Programmed death receptor-ligand 1; CTLA-4: Cytotoxic T lymphocyte-associated antigen-4.

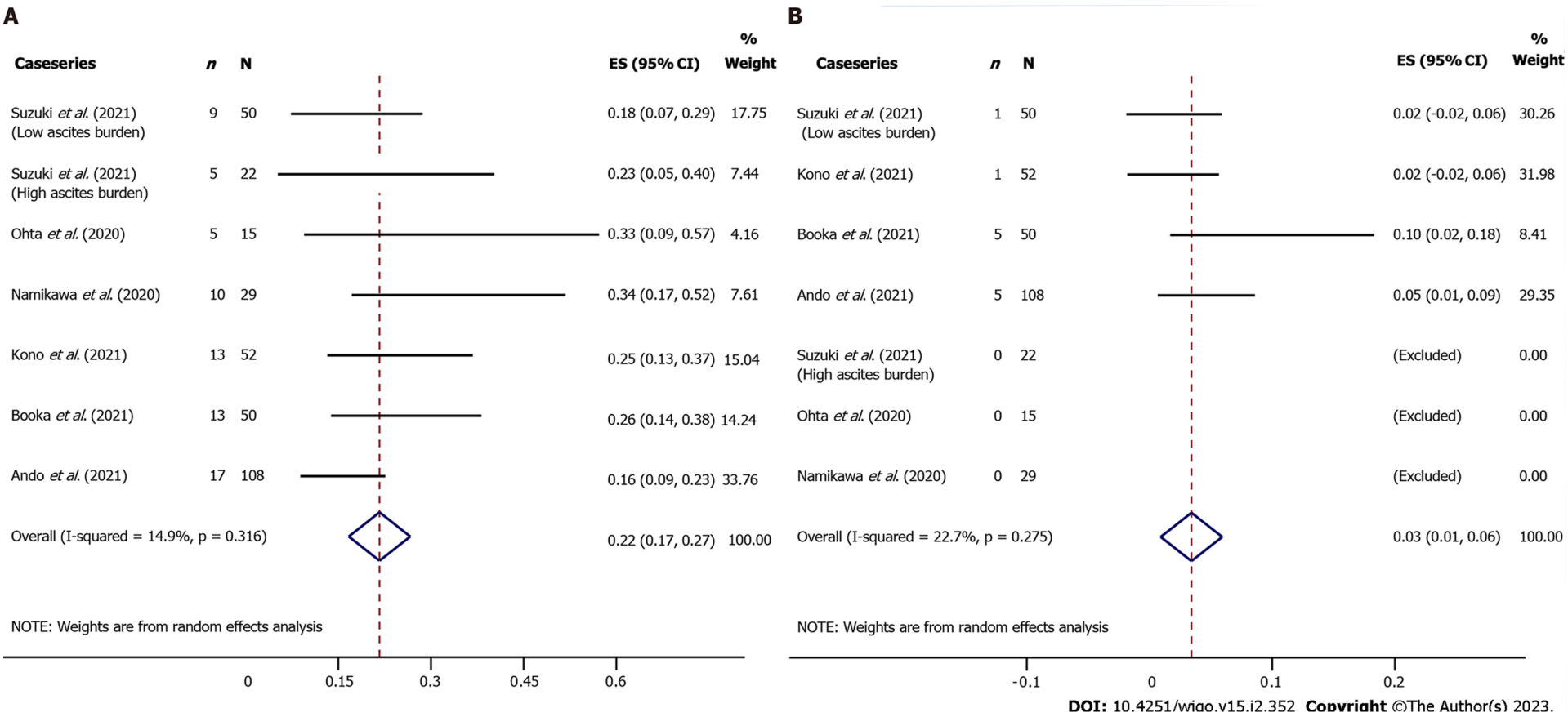

Figure 4 Incidence of immune-related adverse events associated with anti-anti- programmed cell death receptor-1 (nivolumab) in case series. A: All grade; B: Severe grade.

hypothyroidism, hyperthyroidism, thyroiditis, and diabetes. The incidence of irAEs for severe grade (3%), and especially the death rate (1%) were relatively low whereas the interstitial pneumonia was the leading cause of death.

Consistent with results of a previous study, it was found that treatment with anti-PD-1 was significantly associated with a higher prevalence of all irAEs grades and severe grade irAEs as compared with that of anti-PD-L1 treatment[45]. This could be because the variation in the irAEs associated with anti-PD-1 and anti-PD-L1. Anti-PD-1 drugs may increase the risk of immune-related pneumonia whereas anti-PD-L1 drugs may increase the risk of hypothyroidism[45]. However, results of the current research showed that both anti-PD-1 and anti-PD-L1 drugs were associated with an increase in risk of endocrinopathy, which could be caused by different types of cancer.

In addition, findings of a previous meta-analysis showed that the overall incidence of irAEs with anti-CTLA-4 treatment was 72% (95%CI: 65 to 79) for all grade and 24% (95%CI: 18 to 30) for severe grade [46], which was higher than 18% (95%CI: 8 to 27) and 0% in the present meta-analysis. This conclusion may have been caused by insufficient sample sizes of our study, or that the definition of irAEs required to be further clarified. Based on the findings of this study, there is need for additional research on irAEs with a particular focus on comparing anti-PD-1 and anti-PD-L1 medications to provide future guidance for clinical practices.

The relationship between irAEs and efficacy of ICI is the subject of current debate. IrAEs have been associated with improved outcomes and high heterogeneity[47]. A previous meta-analysis showed that anti-PD-1 or anti-PD-L1 treatment improved the clinical benefits of long-term OS and prolonged duration of response in the patients as compared with that of chemotherapy[48]. The median OS for these trials was 9.2 mo and the median PFS was 2.3 mo which was higher than the best supportive therapy or placebo. However, irAEs cannot be ignored when ICIs improve the clinical outcome of oncology. It was found that the overall incidence of irAEs was particularly high in patients with advanced GC/GEJC. In addition, more than 50% of patients experienced intolerable toxicity caused by the reduction of irAEs or discontinuation of their medication. Therefore, it is essential to predict and manage irAEs in cancer immunotherapy.

The findings of the current study showed that incidence of all grade organ-specific irAEs in hematology and hepatology was low. However, the incidence of severe grade irAEs was high. Although hypothyroidism is the most common irAE of the endocrine system, its specific pathophysiological mechanism is still unknown. Furthermore, there was no association between hypothyroidism and cancer outcomes and the strongest associations for hypothyroidism were higher baseline thyroid-stimulating hormone and female sex[49]. Therefore, there is a need for positive clinical tests, such as thyroid function tests (T3, T4, and TSH) which should be performed before and during treatment. Further standardization and improvement are also required for the clinical indicators of other irAEs.

Increasing numbers of drugs targeting immunotherapy and molecular pathways are moving from clinical trials to the clinic. However, the selection of the most appropriate therapy, timing of drug administration, and management of adverse events remain a challenge for severe toxicity and disease progression. Meanwhile, patients are treated with steroids and it has been found that the irAEs either persists or disappears.

Several studies have demonstrated that the use of steroids may inhibit the anti-tumor immune response and hence cause poor prognosis[50-51]. Drug withdrawal and decrease in compliance of patients may also contribute to occurrence of a poor prognosis. On the contrary, a different study has indicated that groups with poor prognoses were more likely to receive steroid treatment and that steroids were less likely to affect the efficacy of immunotherapy[52]. Therefore, there is a need for more research to show the relationship between toxicity and clinical outcomes.

In this systemic review, 14 case reports and 6 case series were included to qualitatively supplement the quantitative findings of the meta-analysis. The statistical analysis is usually constrained because the case studies typically report only novel or rare irAEs. Nonetheless, case studies included in the present review provide an opportunity to assess and study the incidence and nature of irAEs.

Case studies demonstrated that endocrine-related irAEs were the most common and this was in agreement with the findings of another previous meta-analysis[53]. It was evident that the incidence of irAEs was comparable in both case studies and clinical trials of anti-PD-1 therapy. Similar situations apply to deaths caused by irAEs. IrAEs resulted in a 2% mortality rate in case series and a 1% mortality rate in clinical trials, with colitis, myocarditis, and interstitial lung disease being the leading causes of death. This meta-analysis showed a higher mortality rate than a previous one which involved 112 trials and 19,217 patients whereby toxicity-related deaths occurred at 0.36% (anti-PD-1), 0.38% (anti-PD-L1), and 1.08% (anti-CTLA-4)[54].

This study had some advantages. First, it systematically evaluated the incidence of global irAEs and organ-specific irAEs associated with the ICIs monotherapy for advanced GC or GEJC. There are currently very few meta-analyses on irAEs in patients with GC and GEJC. Second, the trials selected for this meta-analysis were RCTs, with large samples, and a high evidence-based value. In addition, a random-effect model and subgroup analysis was used based on different targets, tumor types, drug types, organ specificity, and irAE grade to reduce both variance and bias. Third, the study included both case reports and case series, as well as a comprehensive evaluation of the occurrence, treatment, and prognosis of irAEs. Therefore, these improved the quality of the results and in strengthening the validity of the conclusions made in this study.

This study also had some limitations. First, there were selection, reporting, and publication biases among the included studies. Second, common symptoms such as fatigue, nausea, infusion reactions, and malaise were more likely to be diagnosed as treatment-related adverse events (trAEs) rather than irAEs and hence missed the diagnoses. Therefore, there is urgent need for standardization of the quantifiable standards between irAEs and trAEs, irAEs and non-irAEs. To effectively diagnose and manage irAEs and trAEs, clinicians must also avoid confusing clinical symptoms with test indicators. Third, our study was a meta-analysis of irAEs with a single sample rate. Therefore, odds ratio could not be used for statistical analysis. Lastly, the number of articles included in our analysis is limited. Numerous indicators may be heterogeneous, and the outcome may readily amplify research findings and

inaccuracies. This was because of the limited number of published clinical studies on immunotherapy for GC/GEJC and even fewer studies describing irAEs. Consequently, the results of this study should be interpreted with caution and there is additional research to validate the obtained results.

CONCLUSION

This systematic review shows that there is an increasing number of irAEs associated with ICIs that are being reported in patients with GC or GEJC. This is particularly severe organ-specific irAEs and death because of irAEs, which poses significant challenges for clinical oncologists. Therefore, to help clinicians effectively identify and manage irAEs as well as strike a balance, a comprehensive understanding, systematic prediction, and appropriate management of the adverse events are critical.

ARTICLE HIGHLIGHTS

Research background

In recent years, there has been a steep rise in the development and implementation of anti-cancer immunotherapies. Although there has been a large amount of research focusing on adverse events associated with immune checkpoint inhibitors (ICIs), few studies have focused specifically on advanced gastric cancer (GC) and gastroesophageal junction cancer (GEJC).

Research motivation

By unbalancing the immune system, these new immunotherapies also generate dysimmune toxicities, called immune-related adverse events (irAEs) that mainly involve the gut, skin, endocrine glands, liver, and lung but can potentially affect any tissue. Although steroids can be used to treat these IRAEs, the associated immunosuppression may compromise the antitumor response. To help clinicians effectively identify and manage irAEs as well as strike a balance are critical.

Research objectives

This study focuses on the mechanisms of irAEs generation, putative relationship between dysimmune toxicity and antitumor efficacy.

Research methods

In the study, we systematically evaluated the incidence of global irAEs and organ-specific irAEs and proposed a random-effect model and subgroup analysis based on different targets, tumor types, drug types, organ specificity, and irAE grade to reduce variance and bias.

Research results

It was found that the overall incidence of irAEs was 16% (95%CI: 11-20) for all grades and 3% (95%CI: 2-4) for the severe grade. It was evident that the incidence of irAEs varied with the type of inhibitor and organs. In clinical trials, it was found that the incidence of death related to irAEs was 1% (95%CI: 0-2.0) whereby colitis and interstitial lung diseases were the leading causes of death.

Research conclusions

This systematic review shows that there is an increasing number of irAEs associated with ICIs that are being reported in patients with GC or GEJC. This is particularly severe for organ-specific irAEs and death because of irAEs, which poses significant challenges for clinical oncologists. Therefore, to help clinicians effectively identify and manage irAEs as well as strike a balance, a comprehensive understanding, systematic prediction, and appropriate management of the adverse events are critical.

Research perspectives

In the study, we systematically evaluated the incidence of global irAEs and organ-specific irAEs and proposed a random-effect model and subgroup analysis based on different targets, tumor types, drug types, organ specificity, and irAE grade to reduce variance and bias. Another strength of our study is that both case reports and case series were included, as well as a comprehensive evaluation of the occurrence, treatment, and prognosis of irAEs. The study would be of great interest to a broad range of readers including oncologists, clinical researchers, patients, and other researchers in related fields.

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LETTER TO THE EDITOR

Comment on "Crosstalk between gut microbiota and COVID-19 impacts pancreatic cancer progression"

Jian Yang, Ying Liu, Shi Liu

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has become a global burden, further exacerbating the occurrence of risk events in cancer patients. The high risk of death from pancreatic cancer makes it one of the most lethal malignancies. Recently, it was reported in the *World Journal of Gastrointestinal Oncology* that COVID-19 influences pancreatic cancer progression *via* the lung–gut–pancreatic axis, and the authors provided insights into the intrinsic crosstalk mechanisms in which the gut microbiota is involved, the characteristics and effects of inflammatory factors, and immunotherapeutic strategies for treating both diseases. Here, we review the latest cutting-edge researches in the field of the lung-gut-pancreatic axis and discuss future perspectives to address the severe survival challenges posed by the COVID-19 pandemic in patients with pancreatic cancer.

Key Words: COVID-19; Pancreatic cancer; Lung–gut–pancreatic axis; Gut microbiota; Inflammatory factors; Immunotherapeutic

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has become a global burden, further exacerbating the occurrence of mortality risk events in patients with pancreatic cancer. The aim of this new article is to highlight the need for lung-gut-pancreatic axis-based studies with a focus on intra-axis microbiota crosstalk and potential mechanisms of association to address the severe survival challenges posed by the COVID-19 pandemic in patients with pancreatic cancer.

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TO THE EDITOR

The coronavirus disease 2019 (COVID-19) pandemic has become a global burden, further exacerbating the occurrence of risk events in patients with cancer[1,2]. Contracting COVID-19 significantly increases the risk of morbidity, mortality, and ICU admission in patients with cancer[3]. Additionally, cancer patients have a 60% increased risk of contracting COVID-19 compared with patients without cancer[4]. Owing to the worsening disease and poorer prognosis resulting from COVID-19 in patients with cancer, this patient group is considered a high-risk vulnerable population[5]. The high risk of death from pancreatic cancer makes it one of the most lethal malignancies[6], and the COVID-19 pandemic not only poses a survival challenge for patients with pancreatic cancer but also seriously threatens the execution of pancreatic cancer research[7]. We are very interested in the review by Zhang *et al*[8] published in the August 2022 issue of the *World Journal of Gastrointestinal Oncology*. We consider it to be a good quality review because the authors included in their article many articles from international high-quality journals, such as *Lancet, JAMA, Nature*, and *Cell*, and the article conclusions accurately and clearly summarize the findings of the included literature. From the 98 literature reviews included by the authors, they identified a key connector between COVID-19 and pancreatic cancer; that is, the gut microbiota regulates the host systemic immune response. The question highlighted by Zhang *et al*[8] is how COVID-19 affects pancreatic cancer progression, *i.e.*, *via* the lung–gut–pancreatic axis, and the authors explained the physiological basis, relevance, and potential biological mechanisms of targeting this axis. The novelty of the article is that, the authors highlight therapeutic perspectives in response to COVID-19 and pancreatic cancer based on the intrinsically linked mechanisms of the lung-gut-pancreatic axis, including dietary interventions to stabilize the endostasis of the intestinal flora, the therapeutic efficacy of pharmacological interventions, and strategies to manage inflammatory storms. We thank Zhang *et al*[8] for their review, which has been instrumental in exploring pancreatic cancer treatment options and the development of risk event prevention programs in the context of the severe challenges of the COVID-19 pandemic.

Regional citrate anticoagulation (RCA) is an artificial intelligence technology-based open multidisciplinary citation analysis database. We searched the RCA database for articles in cutting-edge fields in the last 2 years using the search terms "COVID-19", "pancreatic cancer", and "gut microbiota". In addition to highlighting that the gut microbiota regulates immune and inflammatory responses to influence disease severity in COVID-19 and pancreatic cancer[9,10], recent studies have revealed a complex intrinsic association between the three. Current studies indicate that the microbiota alters the malignant phenotype and prognosis of pancreatic cancer in ways that include stimulating persistent inflammation, altering the tumor microenvironment, modulating the anti-tumor immune system, and affecting cellular metabolism[11]. The emerging link between the gut microbiota and pancreatic cancer has recently highlighted the concept of local (direct pancreatic effects) and remote (non-pancreatic) effects of bacteria on organ physiology, which offers potential therapeutic options for pancreatic cancer[12]. However, research on the microbiota influencing pancreatic cancer progression has focused mainly on bacteria, and studies involving intestinal fungi and viruses are just starting to be published[12]. Future work on how these gut microbes are intrinsically linked and on the exact mechanisms by which they influence pancreatic cancer progression is needed. The latest cutting-edge research has bridged the gap between COVID-19 and the gut microbiota, discovering mechanisms that link the gut microbiota to the expression of the viral entry receptor angiotensin-converting enzyme 2 (ACE2)[13], the inflammatory response[14], the immune homeostasis[15], the microbiota metabolism[16], and the "gut–lung axis"[17]. In COVID-19, the main factor associated with disease severity is the involvement of a cytokine storm in the immune response, *i.e.*, tissue damage and systemic inflammation[13]. The gut microbiota may influence the severity of COVID-19 by regulating the host immune response[18]. However, it is unclear whether the reported gut microbial changes are directly responsible for the inflammatory storm in patients with COVID-19 or if they represent the result of severe disease[19], and future studies investigating these possibilities are pending. Zhang *et al*[8] reported that the inflammation-induced immune response is an intrinsic mechanism through which the lung–gut–pancreatic axis produces crosstalk between COVID-19 and pancreatic cancer. On the basis of this mechanism, the authors proposed some strategies on how to manage COVID-19 and pancreatic cancer, including the regulation of microbiota homeostasis to improve patient immunity and the application of anti-inflammatory drugs to reduce the amount of inflammatory damage[8]. However, the survival outcomes of applying these strategies for treating COVID-19 and pancreatic cancer co-morbidity and the effectiveness of such strategies during radiotherapy are not yet known. Future studies could focus on these issues. In conclusion, COVID-19 impacts pancreatic cancer progression based on lung–gut–pancreatic axis, nevertheless, more studies

investigating the potential mechanisms of the crosstalk between COVID-19, pancreatic cancer and gut microbiota are needed in patients with COVID-19 and pancreatic cancer co-morbidity to achieve a better management. Focusing on the lung-gut-pancreatic axis is expected to move us into a new paradigm of treatment for COVID-19 in patients with pancreatic cancer.

FOOTNOTES

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