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REVIEW

Recent advances in targeted therapy for pancreatic adenocarcinoma

Yu-Ting Fang, Wen-Wei Yang, Ya-Ru Niu, Yong-Kun Sun

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Abstract

Pancreatic adenocarcinoma (PDAC) is a fatal disease with a 5-year survival rate of 8% and a median survival of 6 mo. In PDAC, several mutations in the genes are involved, with Kirsten rat sarcoma oncogene (90%), cyclin-dependent kinase inhibitor 2A (90%), and tumor suppressor 53 (75%–90%) being the most common. Mothers against decapentaplegic homolog 4 represents 50%. In addition, the self-preserving cancer stem cells, dense tumor microenvironment (fibrous accounting for 90% of the tumor volume), and suppressive and relatively depleted immune niche of PDAC are also constitutive and relevant elements of PDAC. Molecular targeted therapy is widely utilized and effective in several solid tumors. In PDAC, targeted therapy has been extensively evaluated; however, survival improvement of this aggressive disease using a targeted strategy has been minimal. There is currently only one United States Food and Drug Administration-approved targeted therapy for PDAC – erlotinib, but the absolute benefit of erlotinib in combination with gemcitabine is also minimal (2 wk). In this review, we summarize current targeted therapies and clinical trials targeting dysregulated signaling pathways and components of the PDAC oncogenic process, analyze possible reasons for the lack of positive results in clinical trials, and suggest ways to improve them. We also discuss emerging trends in targeted therapies for PDAC: combining targeted inhibitors of multiple pathways. The PubMed database and National Center for Biotechnology Information clinical trial website (www.clinicaltrials.gov) were queried to identify completed and published (PubMed) and ongoing (clinicaltrials.gov) clinical trials (from 2003-2022) using the keywords pancreatic cancer and targeted therapy. The PubMed database was also queried to search for information about the pathogenesis and molecular pathways of pancreatic cancer using the keywords pancreatic cancer and molecular pathways.

Key Words: Pancreatic carcinoma; Targeted therapy; Cancer stem cell; Monoclonal antibody; Epigenetic modifier

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Core Tip: Pancreatic adenocarcinoma (PDAC) is a fatal and rare disease with a 5-year survival rate of 8% and a median survival of 6 mo. In PDAC, targeted therapy has been extensively evaluated; however, survival improvement of this aggressive disease using a targeted strategy has been minimal. This manuscript summarizes current targeted therapies and clinical trials targeting dysregulated signaling pathways and components of the PDAC oncogenic process, analyzes possible reasons for the lack of positive results in clinical trials, and suggests ways to improve them. We also discuss emerging trends in targeted therapies for PDAC: combining targeted inhibitors of multiple pathways.

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INTRODUCTION

Pancreatic adenocarcinoma (PDAC) is a fatal disease with a 5-year survival rate of 8% and a median survival of 6 mo[1]. It ranks fourth among cancer-related deaths in the United States, and will become the number two cause within a decade[2]. In PDAC, several mutations in the genes are involved, with Kirsten rat sarcoma oncogene (*KRAS*) (90%), cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (90%), and tumor suppressor 53 (*TP53*) (75%–90%) being the most common. Mothers against decapentaplegic homolog 4 (SMAD4) represents 50% (Table 1). In addition, the self-preserving cancer stem cells (CSCs), dense tumor microenvironment (fibrous accounting for 90% of the tumor volume), and suppressive and relatively depleted immune niche of PDAC are also constitutive and relevant elements of PDAC. They are considered significant clinical barriers to successful therapy development, making PDAC one of the most challenging diseases to treat. At present, only surgical resection is a potentially curative treatment for this refractory disease, which shows improvement in survival rates[3,4].

Conventional cytotoxic treatments, such as chemotherapy and radiation therapy, have not been successful in improving the chances of survival in pancreatic cancer patients. Since 2011, two combination regimens for metastatic pancreatic cancer have become the gold standard: 5-fluorouracil/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX); and nab-paclitaxel with gemcitabine. With these approaches, response rates range from 23% to 31%, progression-free survival (PFS) rates are 5.5–6.6 mo, and overall survival (OS) is between 8.5 and 11 mo. Single-agent gemcitabine, and its combinations, have failed to provide the expected results, only achieving moderate life expectancy prolongation. However, most patients are diagnosed at the unresectable stage. Therefore, the development of novel and effective therapeutic strategies is vital to improving treatments that are both targeted and personalized.

Imatinib ushered the era of targeted therapies for solid tumors in 2001. Since then, targeted therapies have been approved for renal, colorectal, gastroenteropancreatic neuroendocrine tumors, non-small cell lung cancer, and malignant melanoma[5-9]. There is only one United States Food and Drug Administration (FDA)-approved targeted therapy for PDAC-erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, combined with gemcitabine hydrochloride in patients with metastatic, locally advanced, or unresectable PDAC. However, the absolute benefit of gemcitabine plus erlotinib is also minimal (2 wk)[10].

In this review, we summarize current targeted therapies and clinical trials targeting dysregulated signaling pathways and components of the PDAC oncogenic process, analyze possible reasons for the lack of positive results in clinical trials, and suggest ways to improve them. We also discuss emerging trends in targeted therapies for PDAC: combining targeted inhibitors of multiple pathways. The PubMed database and National Center for Biotechnology Information clinical trial website (www.clinicaltrials.gov) were queried to identify completed and published (PubMed) and ongoing (clinicaltrials.gov) clinical trials (from 2003-2022) using the keywords pancreatic cancer and targeted therapy. The PubMed database was also queried to search for information about the pathogenesis and molecular pathways of pancreatic cancer using the keywords pancreatic cancer and molecular pathways.

Table 1 Molecular targets for pancreatic cancer treatment

Target	Frequency of mutation/expression, %
KRAS	95
VEGF	93
Sonic hedgehog	70
Notch3	69-74
TP53	70
NF-kB	70
IGF-1R	64
CDKN2A	60
EGFR	43-69
Akt/mTOR	40
SMAD	40
BRCA1/2	1-3
NRG1 fusion	0.5
NTRK fusion	0.3

Akt: Akt serine/threonine kinase; *BRCA1/2*: Breast cancer susceptibility gene 1/2; *CDKN2A*: Cyclin-dependent kinase inhibitor 2A; EGFR: Epidermal growth factor receptor; *KRAS*: Kirsten rat sarcoma oncogene; IGF-1R: Insulin-like growth factor receptor; mTOR: mammalian target of rapamycin NF-κB: Nuclear factor kappa B; Notch3: Notch receptor 3; NRG1: Neuregulin 1; NTRK: Neurotrophic receptor tyrosine kinase; SMAD: Mothers against decapentaplegic homolog; *TP53*: Tumor suppressor 53; VEGF: Vascular endothelial growth factor.

TARGETED THERAPY

Targeted therapy highlights the association between tumor characteristics and individualized treatment response. Biomarkers and genomic mutations may serve as potential targets or prognostic indicators based on the expression of biomarkers. Overall, targeted therapies are based on three main approaches: inhibition of aberrant activation of oncogenes, interference with inactivation of tumor suppressor genes, and exploitation of biological functional defects in specific genes.

Most pancreatic tumors (about 95%) carry *RAS* mutations, the most common of which are *KRAS* alterations (85%)[11]. Mutations of *KRAS* and other genes, such as inactivation of *CDKN2A* (in about 90% of PDAC cases) and SMAD4/DPC4 (approximately 55%), breast cancer susceptibility gene 2 (*BRCA2*), MutL homolog 1, or protease serine 1 alterations accumulate throughout the development of tumors. Approximately 50%-70% of PDAC cases carry mutations in the *TP53* gene, which occurs in late pancreatic intraepithelial neoplasia and leads to the malignant progression of PDAC[12]. As a result of these mutations, multiple critical processes-related signaling pathways are dysregulated, including apoptosis and cell proliferation. In addition, key molecules and pathways from the tumor and surrounding stroma, such as EGFR-mediated and pro-angiogenic pathways, influence the resistance of PDAC to therapy and are associated with a poor prognosis[13]. A total of 60 mutations in 12 different signaling pathways accompany the occurrence of aberrant ducts in PDAC[14], making targeted therapy a possible way to improve the efficacy of existing therapies (Table 2, Figure 1).

KRAS pathway and downstream signaling pathways

KRAS: *KRAS* oncogenic mutations can be observed in more than 90% of PDAC cases. Unfortunately, in mouse models, the resulting mitogen-activated protein kinase (MAPK) inhibition after *KRAS* inhibition (or direct blockade of downstream MEK) may further lead to the activation of protein kinase B alpha (Akt), EGFR, human epidermal growth factor receptor 2 (HER2), platelet-derived growth factor receptor α (PDGFRα), and AXL, resulting in the ineffectiveness of such drugs[15]. Therefore, the development of clinically effective *KRAS* inhibitors has been challenging. Initially, the strategy to target *KRAS* was to inhibit farnesyltransferase, as farnesylation is critical for RAS activation. A phase II trial (SWOG 9924) evaluated the efficacy of an oral farnesyltransferase inhibitor R115777 as first-line therapy for metastatic PDAC patients, but there was no clinical benefit[16]. A novel alternative strategy for targeting KRAS involves the use of exosomes, or small extracellular vesicles loaded with small interfering RNAs targeting *KRASG12D*, the most common KRAS mutation in PDAC[17], and was studied in a recent phase I trial (NCT03608631) that included patients with metastatic PDAC (mPDAC).

Table 2 Clinical trials evaluating the impact of chemotherapeutic agents against specific targets

Target	Study treatment	Phase	Population	No. of patients	mPFS	mOS	Ref.
EGFR	GEM + Erlotinib	III	Locally advanced or metastatic PDAC	285	3.75	6.24	Moore *et al*[10], 2007
	GEM + Placebo			284	3.55	5.91	
EGFR	GEM + Nimotuzumab	IIb	Locally advanced or metastatic PC	96	5.1	8.6	Schultheis *et al*[50], 2017
	GEM + Placebo			96	3.4	6	
EGFR	GEM + Nimotuzumab	III	K-Ras wild-type, locally advanced or metastatic PC	46	4.2	10.9	Qin *et al*[51], 2022)
	GEM + Placebo			46	3.6	8.5	
ERB2	GEM + Afatinib	II	Metastatic PC	79	3.9	7.3	Haas *et al*[57], 2021
	GEM + Placebo			40	3.9	7.4	
VEGF	GEM + axitinib	II	Advanced PC	69	4.2	6.9	Spano *et al*[67], 2008
	GEM			34	3.7	5.6	
VEGF	GEM + axitinib	III	Advanced PDAC	314	4.4	8.5	Kindler *et al*[68], 2011
	GEM + Placebo			316	4.4	8.3	
VEGF	GEM + aflibercept	III	Metastatic PC	271	3.7	6.5	Rougier *et al*[69], 2013
	GEM + Placebo			275	5.1	7.8	
PARP	Veliparib	II	*BRCA*-mutated PDAC	16	3.1	1.7	Lowery *et al*[101], 2018
PARP	Olaparib	III	*gBRCA1* or *BRCA2* mutation and metastatic PC	92	7.4	18.9	Golan *et al*[103], 2019
	Placebo			62	3.8	18.1	
PARP	Cisplatin and GEM + Veliparib	II	Untreated *gBRCA* /PALB2+ PDAC with measurable stage III to IV PDAC	27	10.1	15.5	Sohal *et al*[40], 2020
	Cisplatin and GEM			23	9.7	16.4	
RET	GEM + Vandetanib	II	Locally advanced or metastatic PC	72	NA	8.83	Middleton *et al* [107], 2017
	GEM + Placebo			70		8.95	
Hedgehog	GEM + Vismodegib	II	Metastatic PC	53	4	6.9	Catenacci *et al*[160], 2015
	GEM + Placebo			53	2.5	6.1	
Hyaluronic acid	mFOLFIRINOX + PEGPH20	II	Metastatic PDAC	55	4.3	7.7	Ramanathan *et al* [169], 2019
	mFOLFIRINOX			59	6.2	14.4	
MMP	GEM + Marimastat	NA	Advanced PC	120	NA	5.51	Bramhall *et al*[172], 2002
	GEM + Placebo			119		5.47	
MMPs	Tanomastat	III	Advanced or Metastatic PDAC	138	1.68	3.74	Moore *et al*[173], 2003
	GEM			139	3.5	6.59	
NOTCH	RO4929097	II	Previously treated metastatic PDAC	18	1.5	4.1	De Jesus-Acosta *et al*[190], 2014
NOTCH	GEM + Tarextumab	II	Untreated metastatic PC	89	3.7	6.4	Hu *et al*[193], 2019
	GEM + Placebo			88	5.5	7.9	
Wnt	GEM and nab-paclitaxel + Ipafricept	Ib	Untreated stage IV PC	26	5.9	9.7	Dotan *et al*[196], 2020
Autophagy	GEM and nab-paclitaxel + Hydroxychloroquine	II	Advanced PC	55	5.7	11.1	Karasic *et al*[209], 2019
	GEM and nab-paclitaxel			57	6.4	12.1	

EGFR: Epidermal growth factor receptor; ERBB2: Erb-B2 receptor tyrosine kinase 2; GEM: Gemcitabine; mFOLFIRINOX: Modified fluorouracil plus leucovorin, oxaliplatin and irinotecan; MMPs: Matrix metalloproteinases; mOS: Median overall survival; mPFS: Median progression-free survival;

NOTCH: Notch receptor; PARP: Poly (ADP-ribose) polymerase; PC: Pancreatic cancer; PDAC: Pancreatic adenocarcinoma; *RET*: Ret proto-oncogene; VEGF: Vascular endothelial growth factor.

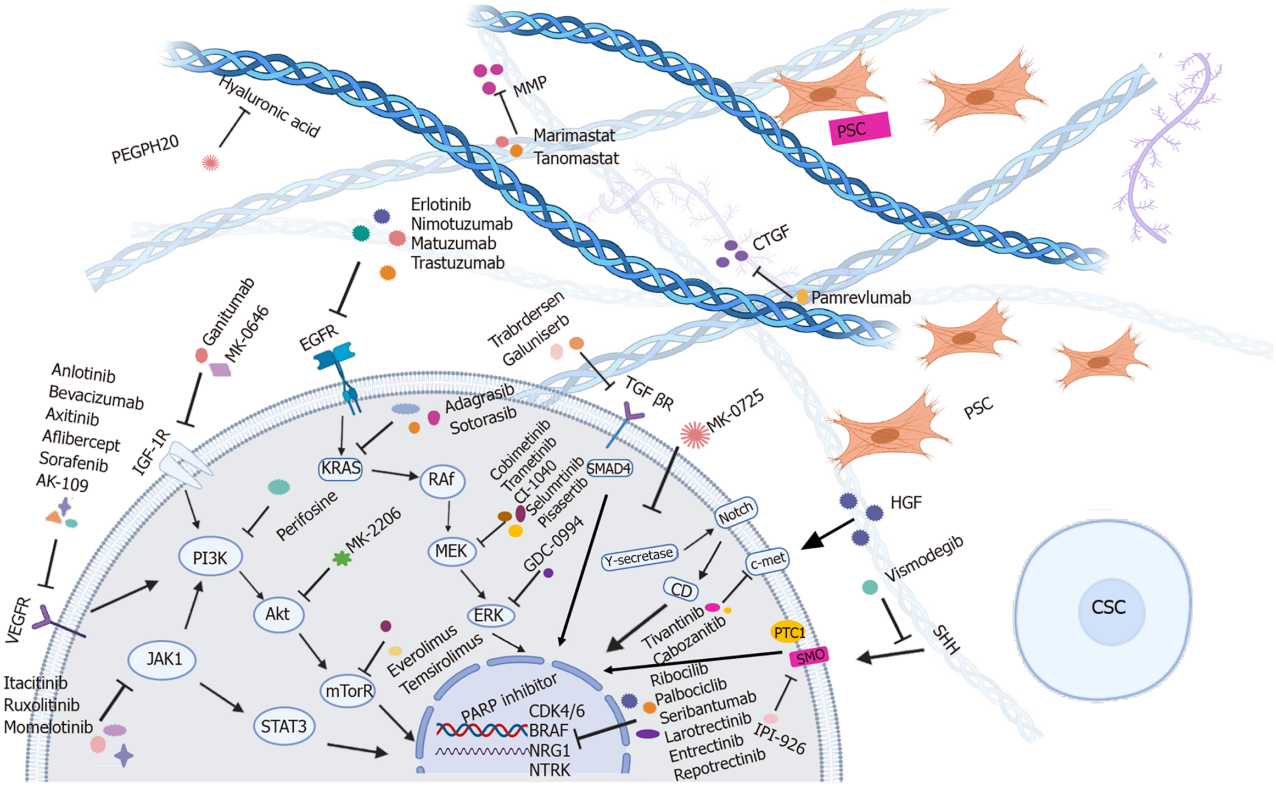

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Figure 1 Overview of targeted therapy strategies for pancreatic adenocarcinoma. The figure summarizes the systemic therapeutic targets and corresponding drugs for pancreatic cancer, including treatment strategies for many aspects such as signaling pathways and gene mutations in tumor cells, and molecules in the extracellular environment, and extracellular matrix. "—|" indicates "targeting"; Akt: Akt serine/threonine kinase; BTK: Bruton's tyrosine kinase; CDK4/6: Cyclin-dependent kinase 4/6; CSC: Cancer stem cell; CTGF: Connective tissue growth factor; DC: Dendritic cell; EGFR: Epidermal growth factor receptor; ERK: Extracellular-regulated protein kinase; HGF: Hepatocyte growth factor; IGF-1R: Insulin-like growth factor receptor; JAK: Activation of the Janus kinase; *KRAS*: Kirsten rat sarcoma oncogene; MEK: Mitogen-activated protein kinase; MMP: Matrix metalloproteinase; mTOR: Mammalian target of rapamycin; Notch: Notch receptor; PARP: Poly (ADP-ribose) polymerase; NRG1: Neuregulin 1; NTRK: Neurotrophic receptor tyrosine kinase; PEGPH20: Pegylated recombinant human hyaluronidase PH20; PI3K: Phosphatidylinositol 3-kinase; PSC: Pancreatic stellate cell; RAF: Rapid accelerated fibrosarcoma; SMAD4: Mothers against decapentaplegic homolog 4; SHH: Sonic hedgehog pathway; SMO: Smoothened; STAT: Signal transducer and transcription; TGF-β: Transforming growth factor-β; VEGFR: Vascular endothelial growth factor receptor.

In addition, the *KRASG12C* mutation was identified in 2% of PDACs[18], and its molecular inhibitors ARS-1620 and sotorasib have shown preliminary antitumor efficacy in preclinical models[19] and patients with advanced solid tumors[20]. To date, only a small subset of patients carrying the *KRASG12C* mutation can be treated with FDA-approved sotorasib or adagrasib. The CRYSTAL-1 phase II clinical trial applied adagrasib to patients with *KRASG12C*-mutated pretreated solid tumors, and 1 PDAC patient achieved a partial response. Phase I/II trials (NCT03785249 and NCT04330664) evaluating the effectiveness of adagrasib are ongoing.

Given the difficulty of directly targeting *KRAS*, therapies targeting its major downstream effector pathways are in development, including the RAS/rapid accelerated fibrosarcoma (RAF)/MEK/extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3 kinase (PI3K)/phosphoinositide-dependent kinase-1/Akt signaling pathways[21].

RAF/MEK/ERK MAPK pathway: Mitogen-activated extracellular kinases are a component of the RAS/RAF/MEK/ERK pathway and play a key role in proliferation, apoptosis, differentiation, and angiogenesis[22]. ERK1/2 MAPK is phosphorylated and activated after RAF serine/threonine kinase phosphorylates and activates MEK1 and MEK2. Activated ERK subsequently modulates the activity of approximately 160 substrates including transcription factors, protein kinases, phosphatases, and regulators of apoptosis[23]. However, several phase II studies of MEK inhibitors did not show efficacy as monotherapy for PDAC including CI-1040[24], selumetinib[25], pimasertib[26], and trametinib[27]. Most likely, the unsatisfactory results were caused by feedback activation and crosstalk between

pathways, resulting in the activation of PI3K/mammalian target of rapamycin (mTOR)/Akt[28].

Mirzoeva *et al*[29] demonstrated the utility of the combinatorial effect of EGFR plus MEK inhibitors in the epithelial molecular subtype of PDAC. In addition, Brauswetter *et al*[30] identified specific molecular isoforms with *KRAS G12C* mutants that responded better to MEK inhibition than the more common *G12D* variant. Therefore, outcomes can be improved by identifying molecular subtypes and appropriate combination therapy to select the right targeted therapy for the right patient.

However, even considering the abovementioned issues, the MEK inhibitors' therapeutic effect is still unsatisfactory. It was shown in a phase I trial that afatinib combined with selumetinib, an inhibitor of MEK, had limited anticancer activity in patients with *KRAS*-mutated solid tumors including pancreatic cancers[31]. Similarly, the results of a phase II trial of selumetinib and MK-2206 (Akt inhibitor) in combination with modified FOLFIRINOX showed that the combination was less effective than FOLFIRONIX in PDAC patients, but had more significant toxicity[32]. The THREAD trial evaluated the efficacy of trametinib and hydroxychloroquine in PDAC patients at different stages of PDAC (NCT03825289).

Currently, approximately 10 clinical trials of MEK1/2 inhibitors targeting PDAC (selumetinib, cobimetinib, and trametinib) are underway, and it is crucial to evaluate the results before considering them for the clinical treatment of PDAC patients.

Furthermore, cobimetinib (MEK inhibitor) or GDC-0994 (ERK1/2 inhibitor) alone only transiently suppresses the MAPK pathway in *KRAS* mutant cancer cell lines[33,34]. Alternatively, co-targeting MEK and ERK with these drugs demonstrates significant antitumor activity in cancer cells and tumor models with dysregulated MAPK pathways. However, in the clinical setting, combining cobimetinib and GDC-0994 in clinical settings is no longer recommended due to overlapping adverse events (AEs)[35]. Overall, developing inhibitors targeting this pathway is promising, but further research is needed to find more appropriate combinations while reducing AEs.

***KRAS* wild-type PDAC:** As mentioned above, most patients with PDAC have *KRAS* mutations. In the small subset of patients with *KRAS* wild-type (WT) PDAC, other mutations, such as neurotrophic receptor tyrosine kinase (NTRK) and neuregulin 1 (NRG1), can initiate PDAC tumorigenesis and be targeted. The incidence of NTRK fusions is 0.3%[36]. Chromosomal rearrangements in the *NTRK* gene family promote the expression of chimeric rearranged promyosin receptor kinases[37]. It is possible that these chimeric proteins signal through the same MAPK and PI3K/Akt pathways as normal TRK proteins and are involved in tyrosine kinase crosstalk[38]. Therefore, a promising approach for targeted therapy is to address fusions of tropomyosin receptor kinase genes 1, 2, or 3 (*NTRK1*, *2*, *3*).

In solid tumors with *NTRK* gene fusions, regardless of tumor type, larotrectinib, and other TRK inhibitors have shown significant and durable antitumor activity (overall response rate 75%, 95% confidence interval [CI]: 61%-85%)[39]. The latest American Society of Clinical Oncology-Gastrointestinal data reconfirmed that larotrectinib is recommended for a variety of gastrointestinal tumors (including pancreatic cancer) carrying *NTRK* fusion mutations[40]. A pooled analysis of clinical trials (NCT02122913, NCT02637687, NCT02576431, NCT02097810, NCT02568267, EudraCT, and 2012-000148-88) revealed that the selective TRK inhibitors larotrectinib and entrectinib were effective against solid tumors (including PDAC) harboring *NTRK* gene fusions (79% response rate for larotrectinib; 57% for entrectinib). Larotrectinib and entrectinib have received FDA's breakthrough designation targeting NTRK fusion-positive solid tumors[41,42]. Next-generation TRK inhibitors, such as selitrectinib and repotrectinib, are being developed to address on-target resistance[43]. Among them, second-generation TRK inhibitor LOXO195 achieved efficacy in 2 patients with NTRK fusion-positive solid tumors, who had disease progression after larotrectinib therapy[44].

NRG1 fusions are rare oncogenic drivers, found in approximately 0.2% of all solid tumors[36]. These fusions trigger hyperactivation of ERBB3/HER3, which drives tumor growth and cancer cell survival. Seribantumab is a fully humanized anti-HER3 immunoglobulin G2 (IgG2) monoclonal antibody (mAb) that inhibits tumor growth in NRG1 fusion-driven preclinical models. CRESTONE is a phase II trial of seribantumab in patients with locally advanced or metastatic solid tumors with NRG1 fusions. Preliminary data suggest that seribantumab induces durable responses with a favorable safety profile. These data support the continued evaluation of seribantumab in the CRESTONE study (NCT04383210).

Tyrosine kinase receptor pathway

EGFR: EGFR is highly expressed in 30%-50% of PDACs[45-47]. Interestingly, EGFR signaling input is required for pancreatic carcinogenesis even in the presence of an oncogenic *KRAS* mutation[48,49]. The small molecule erlotinib, a selective inhibitor of EGFR tyrosine kinases, is the first approved targeted therapy in PDAC. In a phase III trial of metastatic PDAC, the combination of gemcitabine and erlotinib improved median OS (mOS) significantly by 0.33 mo (about 10 d) in the entire study population[10].

Nimotuzumab, an anti-EGFR mAb, showed significantly prolonged OS in combination with gemcitabine *vs* gemcitabine monotherapy in a phase II trial (median PFS 3.2 mo *vs* 5.5 mo, hazard ratio [HR] 0.55, P = 0.0096; median OS 5.2 mo *vs* 8.6 mo, HR 0.66, P = 0.034)[50]. A phase III trial (NCT02395016) showed that nimotuzumab in combination with gemcitabine improved OS and PFS in patients harboring *KRAS* WT with locally advanced or metastatic pancreatic cancer, with significantly

longer median OS in the nitrozumab-gemcitabine group (10.9 mo *vs* 8.5 mo, HR = 0.50, 95%CI: 0.06-0.94; *P* = 0.025). In addition, median PFS was 4.2 mo in the trial group compared with 3.6 mo in the control group (HR = 0.56, 95%CI: 0.12-0.99; *P* = 0.013)[51].

Positive trends have been reported for the EGFR inhibitors matuzumab (phase I)[52] and panitumumab in combination with gemcitabine and erlotinib (phase II)[53]. By contrast, the combination of cetuximab and gemcitabine failed to improve OS, with an mOS of 6.3 mo and PFS of 3.4 mo in the combination arm, compared with 5.9 and 3 mo, respectively, in the gemcitabine monotherapy arm[54].

Trastuzumab, a humanized Ab against HER2, has not yet improved the prognosis of pancreatic cancer in clinical trials. The 12-wk PFS rate for trastuzumab in combination with capecitabine was 23.5%, with a median OS of 7.0 mo[55]. Another recombinant humanized mAb against HER2, pertuzumab, has been used to treat solid tumors including pancreatic cancer. Two pancreatic cancer patients showed partial responses with stable disease for 15.3 mo in 1 patient[56]. Afatinib, a second-generation irreversible inhibitor of ERBB receptors (both EGFR and HER2/neu), is approved as monotherapy for the first-line treatment of non-small cell lung cancer (NSCLC) with EGFR mutations and treatment of lung squamous cell carcinoma after failure of platinum-based chemotherapy. A phase II trial conducted by the "Arbeitsgemeinschaft Internistische Onkologie" was designed to evaluate whether the gemcitabine/afatinib combination was more effective than gemcitabine alone in metastatic PDAC. However, adding afatinib to gemcitabine did not improve therapeutic efficacy and was more toxic. Median OS in the combination group was 7.3 and 7.4 mo in the gemcitabine group. The median PFS was identical in both groups (3.9 mo *vs* 3.9 mo). In addition, AEs were more frequent in the combination group, especially diarrhea (71% *vs* 13%) and rash (65% *vs* 5%)[57].

Vascular endothelial growth factor: Overexpression of vascular endothelial growth factor (VEGF) in PDAC is associated with tumor progression and poorer prognosis[58,59]. However, angiogenesis-targeted therapy is clinically ineffective in pancreatic cancer patients. The reason may be that dense stromal tissue with reduced vascular density impedes the delivery of effective drugs. Moreover, the withdrawal of antiangiogenic agents after therapy may be associated with increased tumor aggressiveness and invasion, offsetting the potential therapeutic benefits offered by antiangiogenic agents[60].

Multiple clinical trials of antiangiogenic agents have been conducted to treat PDAC, yet the results have been overwhelmingly disappointing. For PDAC patients, it has shown improvement in PFS in a few clinical trials[61], but no significant prolongation in OS has been observed. Humanized monoclonal antibodies such as bevacizumab have an affinity for circulating VEGF-A, but phase II and III studies have shown no survival advantage for bevacizumab in combination with gemcitabine and erlotinib[61-64]. A meta-analysis concluded that bevacizumab plus gemcitabine treatment elicited only a moderate response rate without survival modifications[65]. Other VEGF inhibitors, such as axitinib and aflibercept, provide no survival advantage[66-69]. Likewise, sorafenib (an inhibitor of VEGFR and RAS/RAF/MAPK signaling) had no additional value for patient survival over gemcitabine[70].

The promising drug in the field is currently anlotinib. Anlotinib is a novel oral tyrosine kinase inhibitor that targets VEGFR, fibroblast growth factor receptor, PDGFR, and c-kit. Compared to the placebo, it improved PFS and OS in a phase III trial in patients with advanced NSCLC[71]. A phase II trial of anlotinib, toripalimab, and nab-paclitaxel in patients with locally advanced/metastatic pancreatic cancer is underway (NCT04718701). A first-in-human phase I study of AK109, an anti-VEGFR2 Ab, in patients with advanced or metastatic solid tumors, including 2 patients with pancreatic cancer (2/40), showed a controlled safety profile and promising antitumor activity (NCT04547205). Two phase II studies of AK109 in combination with AK104 (anti-PD-1/cytotoxic T-lymphocyte-associated protein 4 [CTLA-4] bispecific Ab) are being evaluated in patients with multiple solid tumors (NCT05142423, NCT04982276).

Insulin-like growth factor receptor 1: Insulin-like growth factor receptor 1 (IGF-1R), a transmembrane receptor tyrosine kinase, is overexpressed in pancreatic cancer. Activation of IGF-1R is associated with decreased apoptosis, cancer cell proliferation, and angiogenesis[72,73]. Yet the use of gemcitabine and a single IGF-1R inhibitor alone has not achieved satisfactory clinical results. A phase III clinical trial of the IGF-1R mAb ganitumab showed no improvement in patient survival[74].

A previous study showed that the simultaneous blockade of IGF1R and EGFR/HER2 synergistically inhibited pancreatic tumor growth and eliminated the activation of IRS-1, Akt, and MAPK phosphorylation. Based on this, combining these two inhibitors may prevent drug-resistance reactions caused by monotherapy[75]. A phase I/II study of gemcitabine and erlotinib in combination or not with MK-0646, an IGF1R inhibitor, in advanced pancreatic cancer showed that the combination of MK-0646 with gemcitabine plus erlotinib was tolerable and improved OS but not PFS compared with gemcitabine plus erlotinib[76]. Istiratumab (MM-141), a quadrivalent bispecific Ab recognizing IGF-1R and ERBB3, provided promising results in preclinical studies[77], but its phase II clinical trial was negative[78].

PI3K/Akt/mTOR pathway

The overexpression of Akt is found in more than 40% of PDAC cases[79,80]. PI3K/Akt/mTOR, as a critical pathway in many aspects of cell growth, survival, and apoptosis, plays an essential role in the

occurrence and development of various tumors including PDAC[81]. Dysregulation of this pathway may lead to tumor resistance to chemotherapy[82,83]. It has been documented that activation of Akt is associated with a poor prognosis[84,85]. Inhibition of Akt signaling induces apoptosis and limits tumor growth[86].

Alkyl phospholipid perifosine acts as an inhibitor of Akt and PI3K phosphorylation[87]. Combining perifosine with gemcitabine exhibits synergistic effects on pancreatic cancer cells expressing high levels of phosphorylated Akt, primarily inhibiting tumor migration/invasion and inducing tumor cell apoptosis[88].

Clinical activity of everolimus (mTOR inhibitor) in patients with gemcitabine-refractory pancreatic cancer was limited, with a median PFS of 1.8 mo and median OS of 4.5 mo[89]. Combining everolimus with capecitabine achieved appropriate efficacy, with a mean OS of 8.9 mo (95%CI: 4.6-13.1) and median PFS of 3.6 mo (95%CI: 1.9-5.3)[90]. Temsirolimus is another mTOR inhibitor tested in locally developed or metamorphosic conditions[91,92]. A phase I/II trial evaluating sirolimus, a selective inhibitor of mTOR, enrolls patients with advanced pancreatic cancer (NCT03662412). In addition, other drugs targeting this pathway have been developed such as PI3K inhibitors, BKM120 and BYL179 (NCT02155088); RX-0201 (Akt antisense oligonucleotide inhibitor); and BEZ235 (combined inhibitor of PI3K and mTOR)[93,94].

Poly (ADP-ribose) polymerase pathway

Germline *BRCA* mutation is an autosomal dominant mutation associated with an increased risk of breast, gynecologic, colorectal, and pancreatic cancers. In families with germline *BRCA2* mutations, the relative risk of pancreatic cancer is 3.5% (95%CI: 1.9–6.6)[95]. Mounting evidence has demonstrated that *BRCA1/2* mutant breast and ovarian cancers are susceptible to DNA damage-related therapies, including poly (ADP-ribose) polymerase inhibitors (PARPis) and platinum-based drugs[96].

Clinically, PARPis have shown significant efficacy against other refractory *BRCA*-mutated solid tumors[97-100]. Olaparib is a PARPi that was effective in a single-arm phase II trial[98]. Veliparib, another PARPi, has modest activity in patients with previously platinum-treated germline *BRCA1/2* mutation-positive pancreatic cancer[101]. The RUCAPANC study, which evaluated the PARPi rucaparib, was discontinued during the interim analysis due to a lack of patient response[102].

A phase II trial of niraparib, a highly specific PARP-1 and PARP-2 inhibitor, is currently being conducted in metastatic PDAC patients with somatic or germline defects in multiple DDR genes (NCT05442749). A randomized phase II trial (PARPVAX) of niraparib (nira) *vs* an immune checkpoint inhibitor, nivolumab (nivo, PD-1 mAb) or ipilimumab (ipi, CTLA-4 mAb), has been evaluated in a non-genomic selected, advanced PDAC patient population that has received at least 16 wk of platinum-based therapy without progression (NCT03553004). Another similar trial showed that compared to nira/nivo, nira/ipi prolonged median PFS as maintenance therapy for advanced PDAC patients with no progressive disease after first-line platinum-based chemotherapy, with an mPFS of 1.9 mo (95%CI: 1.8-1.9) for nira/nivo and 7.6 mo (95%CI: 4.0-11.1) for nira/ipi (NCT03404960).

A prospective phase III trial (POLO, NCT02184195) evaluated olaparib in metastatic PDAC patients with *BRCA* mutations[103]. The results indicated that metastatic PDAC patients with germline *BRCA1* or *BRCA2* mutations were significantly less likely to progress after taking olaparib. The trial included 154 patients with germline *BRCA* mutations whose tumors had not progressed after 16 wk of platinum-based induction chemotherapy. They were randomly assigned: 92 to receive olaparib and 62 to placebo. PFS was significantly longer in the olaparib group compared with the placebo group (median PFS: 7.4 mo *vs* 3.8 mo, HR = 0.53; P = 0.004). There was no difference in OS between the placebo and olaparib groups, despite the fact that some patients in the placebo group received PARPis as follow-up therapy. The risk of disease progression was reduced by 47% in the olaparib group, and patients treated with olaparib were at least twice as likely to be disease progression-free at 6, 12, 18, and 24 mo as those receiving a placebo. Based on this, National Comprehensive Cancer Network guidelines included olaparib as recommended maintenance therapy for PDAC patients with germline *BRCA1/2* mutations, good performance status, metastatic disease, and no disease progression after 4-6 mo of first-line chemotherapy. In addition, the safety of olaparib was also validated in the POLO trial, where patients' health-related quality of life was assessed and found to remain unchanged with no clinically meaningful deterioration. Grade ≥ 3 or higher AEs occurred in 39.6% of the olaparib group and 23.3% of the placebo group; 5.5% and 1.7% of patients discontinued treatment due to AEs, respectively[99,104].

Multidrug combination therapy is also a promising strategy. Antiangiogenic agents act synergistically with PARP inhibitors, resulting in increased levels of hypoxia and downregulation of homology-driven repair genes[105]. This combination will be further investigated in a phase II trial, including patients with mPDAC (NCT02498613). In addition, an ongoing phase II trial is evaluating the efficacy of olaparib in combination with pembrolizumab (an immunotherapy cancer drug) in patients with *BRCA*-mutated pancreatic cancer (NCT04548752). A phase II trial evaluating talazoparib in patients with advanced cancer and DNA repair variants is ongoing (NCT04550494).

RET pathway

Genetic abnormalities in the RET proto-oncogene have been reported in PDAC. In phase I trials for pancreatic and biliary tract cancer, vandetanib (a multitargeted tyrosine kinase inhibitor of EGF,

VEGFR, and RET) was evaluated in combination with gemcitabine and capecitabine. A 78% disease control rate (> 2 mo), 3 partial responses, and 15 patients with stable disease were observed in this trial [106]. A subsequent phase II trial of vandetanib in combination with gemcitabine *vs* gemcitabine monotherapy has shown that the combination did not improve OS in advanced PDAC (8.83 mo *vs* 8.95 mo, HR = 1.21, 80.8%CI: 0.95-1.53; *P* = 0.303)[107]. In addition, LOXO-292, a selective RET inhibitor, is being investigated in a phase I study (NCT03157128).

Tumor suppressor pathway

***TP53* tumor suppressor pathway**: Contrary to the role of proto-oncogenes, the role of a tumor suppressor is to suppress tumorigenesis. *TP53* is the most frequently inactivated suppressor in PDAC, and *TP53* gene alterations are found in approximately 70% of PDAC patients[108,109]. *p53* is a transcription factor that regulates the expression of multiple genes. Its biological functions include inhibiting cell proliferation by inducing p21 expression, promoting tumor cell apoptosis, maintaining gene stability, and inhibiting tumor vascularization by stimulating B-cell lymphoma 2-associated X protein expression[110,111]. *TP53* reactivators include Zn2+ chelators such as COTI-2, cys-targeting agents such as APR-246 and CP-31398, and other proteins that assist in *p53* resilience, inhibit abnormal *p53* aggregation, or stabilize *p53*[112].

A clinical trial of COTI-2 is ongoing in patients with *TP53* mutant PDAC (NCT02433626). In addition to reactivation, inhibition of Mouse double minute 2 homolog (MDM2) is another emerging strategy for targeting *TP53*-mutated tumors. The p62-NRF2-MDM2 axis is involved in tumor progression and programming[113], and MDM2 antagonizes *p53* through direct interaction or ubiquitin-dependent degradation[114]. Therefore, inhibition of MDM2 may increase *p53* activity and suppress *p53*-mutant cancers[115]. Recent studies have confirmed the efficacy of MDM2 inhibitors, such as Nutlin, MA242, SP141, and MI-319, *in vitro* and *in vivo*[116-119]. MANTRA-2 is a phase II trial evaluating the clinical benefit of Milademetan, a selective MDM2 inhibitor, in MDM2 amplified (copy ≥ 12) TP53-WT solid tumors and is currently recruiting (NCT05012397).

Transforming growth factor/β SMAD4 pathway: Another tumor suppressor gene associated with the pathogenesis of pancreatic cancer is the *SMAD4* gene, and approximately 40% of PDAC patients carry *SMAD4* mutations[109]. In normal cells, the product of this gene (a 64-kDa protein) plays a role in transforming growth factor beta (TGF-β)-mediated signal transduction, gene transcription, and growth arrest. The TGF-β/SMAD4 signaling pathway mediates tumor-stromal interactions and the epithelial-stromal transition. Evidence suggests that TGF-β inhibitors, including trabedersen and galunisertib, reduce tumor metastasis and invasion in animal models[120,121]. A randomized phase II trial showed that galunisertib in combination with gemcitabine improved OS compared with gemcitabine alone [122]. The combination of galunisertib and durvalumab (programmed death-ligand 1 mAb) has also been studied in metastatic PDAC patients[123]. The sponsor has since terminated further studies of galunisertib due to limited clinical activity. Instead, a new generation of TGF-β pathway inhibitors, such as TGF-βR inhibitors and TGF-β-checkpoint traps, are under development[124,125]. NIS793, a human IgG2 mAb TGF-β antagonist, is in a phase III trial to evaluate the efficacy of NIS793 in treatment-naïve patients with mPDAC (NCT04935359). Furthermore, TGF-β levels are reduced in fibroblasts due to blockade of the angiotensin type III receptor[126,127]. Thus the angiotensin receptor blocker losartan was tested in a preclinical model of pancreatic cancer and subsequently tested in combination with FOLFIRINOX in a phase II trial[128], which enabled R0 resection in 69% (30/49) of patients with locally advanced disease[129]. A randomized phase II trial evaluating losartan in combination with FOLFIRINOX and stereotactic body radiotherapy in neoadjuvant setting is ongoing (NCT03563248).

Dysfunctional CDKN2A and CDK4/6 inhibitors: *CDKN2A* is a multifunctional gene that creates p16 and p19, arrests the cell cycle at the G1/S checkpoint through a CKD4/6-regulated mechanism[130], and the proteins bind to MDM2 to block the reduction in *p53* levels[131]. Approximately 60% of PDAC patients carry *CDKN2A* mutations, with an odds ratio of 12.33, indicating that germline mutations in *CDKN2A* are associated with a high risk of developing PDAC[108,109]. CDK4/6 is a potential target for *CDKN2A*-deficient tumors[132,133]. The CDK4/6 inhibitors ribociclib and palbociclib have shown safety and efficacy in metastatic breast cancer and liposarcoma[134,135]. Additionally, CDK4 inhibitors are efficacious in preclinical models of PDAC[136-139], and a related clinical trial (NCT02501902) is ongoing. Researchers have concluded that CDK4/6 inhibitors alone exert limited antitumor effects and can show greater promise when used in combination with other targeted agents[140]. Mechanistically, CDK4/6 inhibitors block DNA repair mechanisms and increase the sensitivity of PDAC cells to PARPis [141]. PDAC cells are more sensitive to immune checkpoint blockers when CDK4/6 and MEK are inhibited jointly[142]. A phase I clinical trial of palbociclib in combination with the PI3K/mTOR inhibitor gedatolisib in advanced PDAC patients is ongoing (NCT03065062).

Nuclear factor kappa B pathway

Nuclear factor kappa B (NF-κB) is a protein complex involved in cell proliferation, cell adhesion, apoptosis, and inflammatory responses[143]. Overexpression of the NF-κB pathway is reported in approximately 70% of pancreatic cancers[144,145]. Curcumin is a potent inhibitor of this pathway, and

its effects have been demonstrated in several *in vitro* and *in vivo* pancreatic cancer models[146,147].

Nafamostat mesilate (NM) is a synthetic serine protease inhibitor that inhibits NF-kB activation[148]. NM infusion with gemcitabine for inoperable advanced pancreatic cancer was evaluated in a phase I/II study. The median OS and 1-year survival rates were 10 mo and 40%, respectively[149]. Subsequently, a phase II study of NM/gemcitabine adjuvant chemotherapy showed that gemcitabine combined with local arterial perfusion adjuvant chemotherapy with NM is safe and may be an option in the adjuvant setting after curative surgery for pancreatic cancer[150].

STROMA TARGETS

PDAC is characterized by dense fibrous stroma representing up to 90% of the tumor volume. Desmoplasia means excessive proliferation of fibrotic tissue with a modified extracellular matrix providing a protumorigenic environment[151,152]. Pancreatic stellate cells play a major role in stromal responses, and they are closely associated with pancreatic cancer cells[153,154], controlling matrix synthesis, cell growth, migration, and invasion through a diverse set of signaling cascades. In addition, hepatocyte growth factor (HGF) from stromal cells was associated with the growth, angiogenesis, and invasiveness of pancreatic cancer[155]. The pro-fibroproliferative response is accompanied by a relatively avascular tumor microenvironment, followed by hypoperfusion and hypoxia in the cancerous tissue, which leads to the generation of more aggressive tumor subclones[156], altered tumor metabolism, increased glycolysis[157], and decreased chemotherapeutic drug concentrations. Therefore, stroma-specific therapeutic strategies can be developed. One way is to directly target specific components of the extracellular matrix, such as matrix metalloproteinases (MMPs), and the other is to target specific signaling pathways that promote the development of the tumor stroma, such as the Sonic Hedgehog (SHH) pathway.

SHH pathway

Hedgehog signaling is an essential pathway for proliferation and survival in embryonic development. In response to hedgehog ligand binding to PATCHED 1 receptor protein in target cells, a signaling cascade is triggered, eliminating the inhibitory effect of Smoothened (SMO), which then enhance tumor progression, metastasis, and tumorigenesis[158].

Combined with gemcitabine, cyclopamine, an SMO antagonist, was shown to reduce metastatic potential in the GEMM (KPC) model of PDAC[159]. A phase II trial of vismodegib (a second-generation SMO inhibitor) combined with gemcitabine had a PFS benefit (4 mo *vs* 2.5 mo; $P = 0.30$) but did not improve OS (6.9 mo *vs* 6.1 mo; $P = 0.84$)[160]. These results are consistent with another clinical trial (NCT01088815)[161]. In addition, a phase I trial (NCT00878163) enrolled metastatic PDAC patients to evaluate the combination of vismodegib and erlotinib. Although the combination was well tolerated and 20% of patients exhibited stable disease, there was no significant tumor shrinkage effect[162]. Overall, the clinical trials with vismodegib did not meet expectations. Thus, the clinical development of this drug has been discontinued. In another phase II trial, saridegib (an SMO inhibitor) plus gemcitabine had a survival disadvantage (NCT01130142). Nevertheless, when combined with FOLFIRINOX, there was clinical activity with an objective response rate of 67%[163]. The clinical development of this drug was also halted.

The reasons for the disappointing results of hedgehog inhibition could be arising SMO mutations under therapy and compensatory feedback loops leading to a (hyper) activation of the PI3K pathway or downstream targets of the hedgehog pathway (*e.g.*, Gli2)[164,165]. This suggests that targeting both the Hedgehog pathway and PI3K pathway could be used for treating pancreatic cancer, as shown in medulloblastoma[166].

Hyaluronic acid

Hyaluronic acid (HA) is a glycosaminoglycan that is abundantly present in the extracellular matrix and contributes to the dense desmoplastic stroma surrounding the tumor. The degradation of HA by hyaluronidase may help disrupt the stroma and enhance drug delivery to the tumor[167]. Recombinant human hyaluronidase (PEGPH20) has been studied in mouse models of pancreatic cancer and was found to degrade HA, reduce interstitial fluid pressure, increase vascular permeability, and enhance doxorubicin delivery to tumors. In combination with gemcitabine, PEGPH20 inhibits tumor growth and prolongs survival[167].

The HALO 202 trial examined improvements in PFS in patients with untreated metastatic PDAC. In this phase II trial, 269 patients were randomized to treatment with PEGPH20 plus nab-paclitaxel/gemcitabine (PAG) *vs* nab-paclitaxel/gemcitabine (AG). The mPFS was significantly improved in the PAG arm for 6 mo *vs* 5.3 mo in the AG arm (HR = 0.73; $P = 0.045$). In patients with > 50% of HA staining, the PAG group had a higher objective response rate (45% *vs* 31%) and a longer mOS (11.5 mo *vs* 8.5 mo, HR = 0.96, 95%CI: 0.57-1.61)[168]. The HALO109-301 phase III clinical trial evaluating PEGPH 20 (NCT02715804) was terminated due to unsatisfactory results. In a phase II trial (SWOG S1313) of modified FOLFIRINOX (mFOLFIRINOX) plus PEGPH20 compared with mFOLFIRINOX monotherapy.

Ramanathan *et al*[169] reported an inferior OS when PEGPH20 added to mFOLFIRINOX (7.7 mo [95%CI: 4.6-9.3 mo] *vs* 14.4 mo [95%CI: 10.1-15.7 mo]). Several phase I/II trials of PEGPH20 combined with programmed cell death protein 1 mAbs and other drugs are currently recruiting patients (NCT03634332, NCT03193190). There may soon be new treatment paradigms for this disease based on the randomized phase III trials of PEGPH20.

MMPs

MMPs can disrupt the extracellular matrix and basement membrane, thus contributing to tumor invasion, angiogenesis, and metastasis[170]. Marimastat is an MMP inhibitor demonstrating single-agent activity and safety in PDAC patients[171]. However, when combined with gemcitabine, marimastat did not show any clinical benefit or survival advantage, with mOS of 165.5 d in the combination group compared with 164 d in the gemcitabine monotherapy group and 1-year survival rates of 18% and 17%, respectively[172]. Similarly, tanomastat, an MMP inhibitor, did not show any clinical benefit in PDAC compared with gemcitabine[173]. The study ended after a second interim analysis (median OS of 3.74 mo for tanomastat *vs* 6.59 mo for gemcitabine). Andecaliximab, an mAb targeting MMP9, demonstrated favorable safety and clinical activity in a phase I trial in combination with gemcitabine and nab-paclitaxel in advanced PDAC patients, with an mPFS of 7.8 mo (90%CI: 6.9-11.0), an objective response rate of 44.4% and a median duration of response of 7.6 mo[174].

Connective tissue growth factor

Connective tissue growth factor (CTGF) is overexpressed in PDAC and is a profibrotic mediator. In a preclinical study, FG-3019, an mAb against CTGF, increased the effectiveness of gemcitabine, resulting in a significant tumor response[175]. A phase II clinical trial for advanced PDAC showed that FG-3019 in combination with gemcitabine and erlotinib was well tolerated, with median PFS and OS of 3.7 and 7.4 mo, respectively[176]. Based on the results of a phase II trial, gemcitabine plus nab-paclitaxel, in combination with FG-3019 or placebo, showed significant improvement in median PFS in the group using FG-3109 (18.4 mo *vs* 10.4 mo) (NCT02210559). In early 2018, FDA granted a fast-track designation to FG-3019 (pamrevlumab) for treating patients with locally advanced, unresectable PDAC. An ongoing phase III, randomized, double-blind trial is enrolling patients with locally advanced, unresectable PDAC to evaluate the efficacy of receiving gemcitabine in combination with pamrevlumab (NCT03941093).

HGF/c-MET pathway

HGF and its receptor c-MET are vital to the onset and progression of pancreatic cancer. HGF, present on pancreatic stellate cells, increases stromal production and interacts with its ligand, c-MET, on pancreatic cancer cells. This process is vital to the proliferation and migration of pancreatic cancer cells[177].

Among c-MET-targeted therapies, the most advanced clinical development is tivantinib, a c-MET inhibitor in phase III development for various malignancies[178]. A randomized phase II study has been conducted to evaluate the efficacy of tivantinib in combination with gemcitabine in patients with unresectable locally advanced or metastatic untreated pancreatic cancer (NCT00558207). Recently, an HGF-neutralizing Ab, YYB101, has been developed with encouraging preclinical results and has been tested in clinical trials in patients with refractory solid tumors[179]. In addition, NK4, an intramolecular fragment of HGF that targets the HGF/c-MET axis, has demonstrated promising results *in vitro* and *in vivo*[180,181].

Cabozantinib, a small molecule inhibitor targeting c-MET and VEGFR-2, is evaluated in a randomized phase II study in several solid tumors, including metastatic pancreatic cancer (NCT01466036). In addition, anti-MET antibodies (emibetuzumab and onartuzumab) have been successfully used in preclinical models of pancreatic cancer[182,183].

CSCs

CSCs are a unique subset of cells with the potential for self-renewal and differentiation, which can lead to carcinogenesis, progression, metastasis, and drug resistance. Pancreatic CSCs were first described by Li *et al*[184]; they identified a subpopulation of pancreatic cancer cells expressing CD44, CD24, and epithelial surface antigen (ESA) (CD44+ CD24+ ESA+). CSCs with this phenotype form pancreatic tumors when injected into the tail of orthotopic immunocompromised mice[185]. Wnt/β-catenin, Notch, and activation of the Janus kinase/signal transducer and transcription (JAK/STAT) pathways play a central role in developing pancreatic CSCs[186].

Notch pathway

The Notch pathway is an evolutionarily conserved pathway important in mammalian pancreas organogenesis. Upregulation of Notch has been found in PDAC and increases tumorigenesis. Evidence suggests that crosstalk between phytochemicals, microRNAs, and Notch signaling regulates the self-renewal division of CSCs[187]. The intracellular domain of Notch induces proliferative signaling and

differentiation by altering gene transcription. The Notch pathway interacts with the Hedgehog, *KRAS*, and NF-κB pathways[93,188,189].

Since Notch signaling is activated by γ-secretase, γ-secretase inhibitors have been developed as therapeutic agents for the treatment of PDAC. A single-arm phase II trial of the γ-secretase inhibitor RO4929097 was discontinued due to intolerable toxic effects. The 6-mo survival rate is 27.8%, the mOS is 4.1 mo, and median PFS is 1.5 mo[190]. A phase I trial of MK-0725 (a γ-secretase inhibitor) and gemcitabine for PDAC patients, achieved 13 stable disease and one partial response of 19 evaluable patients[191]. Tarextumab is a fully human IgG2 Ab targeting Notch2 and Notch3 receptors[192]. The results of a randomized phase II study evaluating tarextumab in combination with gemcitabine and nab-paclitaxel in patients with untreated metastatic PDAC were suboptimal, without improvement in OS, PFS, or ORR[193].

WNT pathway

The WNT pathway is important in cell differentiation and proliferation. In preclinical mouse models, abnormal WNT signaling leads to pancreatic cancer[194].

Vantictumab is an mAb that blocks WNT signaling. Preclinical studies have shown that this Ab reduces cancer stem cell frequency and increases the activity of chemotherapy[195]. The safety and tolerability of vantictumab combined with nab-paclitaxel and gemcitabine are being investigated in a phase Ib dose-escalation study (NCT02005315).

Ipafricept inhibits WNT signaling by acting as a decoy receptor while binding and sequestering WNT ligands. The combination of ipafricept and gemcitabine and nab-paclitaxel was well tolerated in a phase Ib study for patients with untreated stage IV pancreatic cancer, with a median PFS of 5.9 mo and a median OS of 9.7 mo[196].

JAK/STAT pathway

JAK/STAT pathway has been found in pancreatic cancer[197,198]. Abnormalities in the JAK/STAT pathway directly leads to increased cell transformation, cell proliferation, apoptosis, and angiogenesis. Additionally, STAT3 inhibition results in increased sensitivity to chemotherapy (mainly gemcitabine) and delays tumor progression in PDAC patients[199]. PDAC cell death and proliferation increases when STAT3 inhibitors are administered with chemotherapeutic agents. A phase III trial of evaluating STAT3 inhibitors on PDAC when co-administered with standard chemotherapy regimens has been completed (NCT02231723), but results have not yet been uploaded.

Itacitinib, a selective JAK1 inhibitor, combined with nab-paclitaxel and gemcitabine was evaluated in a phase Ib/II study in patients with advanced solid tumors including locally advanced/metastatic pancreatic cancer patients[200]. The combination therapy demonstrated acceptable safety and clinical activity[201]. However, after an interim analysis of the phase III JANUS 1 and 2 trials of ruxolitinib (JAK1/2 inhibitor) in combination with capecitabine showed no additional clinical benefit of ruxolitinib compared to capecitabine (NCT02117479, NCT02119663), the sponsor prematurely terminated this study on itacitinib on February 11, 2016.

Napabucasin is an investigational, oral agent hypothesized to inhibit multiple oncogenic pathways. Several clinical trials have been initiated to evaluate the safety and efficacy of the drug in various gastrointestinal malignancies[202]. Single-arm phase Ib/II study with napabucasin and nab-paclitaxel plus gemcitabine recruited 59 patients with mPDAC. According to published abstracts, the combination regimen was well tolerated. Among the 50 patients evaluated, the disease control rate was 92%, with 2 complete remissions (4%) and 26 partial responses (52%)[203]. Of all 59 patients enrolled, the 1- and 2-year OS rates were 46% and 13%, respectively. These results led to the further investigation of this treatment combination in the ongoing phase III CanStem111P trial (NCT02993731).

Momelotinib is a JAK1/2 inhibitor with additional activity against TANK-binding kinase 1[204]. Momelotinib was safe and well tolerated in a phase I dose-escalation trial of momelotinib combined with gemcitabine and nab-paclitaxel in patients with previously untreated metastatic PDAC (NCT02101021). However, there was no OS or PFS benefit *vs* gemcitabine plus nab-paclitaxel in the context of suboptimal engagement of the target. This study does not support momelotinib as a first-line treatment for pancreatic cancer[205].

CSC may be an important target for treatment, but there is still a question of whether targeting them is the best way to counteract their ability to progress, expand and resist treatment in the host environment[206]. Future studies should focus on clonal evolution, especially on monitoring CSC during cancer progression and after treatment.

AUTOPHAGY

An autophagy process primarily involves degrading damaged organelles or proteins[207] and enables cells to recycle cellular contents as an internal fuel source during cellular recycling. This process is necessary for pancreatic cancer cells to overcome nutritional deficiencies.

Hydroxychloroquine (HCQ) was one of the first autophagy inhibitors to enter clinical trials. However, HCQ alone did not show significant antitumor effects[208]. According to a randomized phase II study, gemcitabine/nab-paclitaxel with or without HCQ did not improve OS (11.1 mo *vs* 12.1 mo; P = 0.44) or PFS (5.7 mo *vs* 6.4 mo; P = 0.25)[209]. In a randomized phase II trial, there was a significant improvement in Evans Grade histopathology and carbohydrate antigen 19-9 response after adding HCQ in the preoperative setting. OS and DFS were not different between groups in this study, nor were AEs or R0 resections[210].

In a recent study, *KRAS* inhibition and ERK inhibition increased autophagic flux in PDAC[211]. Thus, autophagy inhibitors synergistically act with ERK inhibitors in inhibiting PDAC driven by *KRAS* mutations[211]. A synergistic antiproliferative effect was observed when autophagy inhibition was combined with MEK1/2 inhibition in PDAC cells as well as patient-derived xenograft models[212]. According to these studies, inhibiting autophagy genetically or pharmacologically may enhance the antitumor effects of other antitumor drugs such as ERK inhibitors and MEK inhibitors in PDAC. Several promising studies have evaluated the combination of autophagy inhibitors and MEK (NCT04132505, NCT03825289) or ERK inhibitors (NCT04386057) in patients with locally advanced or metastatic cancer [213-215].

OTHER TARGETS

Adenosine has been identified as an essential regulator of tumor proliferation, survival, and migration. Inhibition of adenosine receptors has been shown to modulate immune responses within the tumor microenvironment, thereby enhancing antitumor effects[216]. Several clinical trials evaluate the safety and efficacy of adenosine A2 receptor antagonists in combination with immunotherapy or cytotoxic therapy in patients with advanced solid tumors including PDAC, Ciforadenant (NCT03454451), and NIR178 (NCT03207867).

Accumulating clinical evidence suggests that overexpression of urokinase-type plasminogen activator (uPA) or its cell surface receptor is closely associated with worse clinicopathological features and poor prognosis in PDAC patients[217]. RHB-107, the only known agent targeting the uPA pathway, was effective in a phase II clinical trial in patients with locally advanced unresectable pancreatic cancer (NCT00499265). RHB-107, combined with gemcitabine, significantly improved 1-year survival by 17% in patients with unresectable PC. In 2017, RHB-107 received an Orphan Drug Designation from the FDA for PDAC adjuvant therapy.

CONCLUSION

Despite the advances in the last 20 years, pancreatic cancer remains a devastating malignancy with limited options for effective treatment. As mentioned above, the self-preserving CSCs, dense tumor microenvironment, and suppressive and relatively depleted immune niche of PDAC are considered significant clinical barriers to successful therapy development, making it one of the most challenging diseases to target.

Targeting individual molecules is not a good approach. In the currently known studies on the mechanisms of ineffectiveness or resistance of targeted therapies, it is suggested that inhibition of one pathway may lead to activation or compensatory upregulation of others, *e.g.*, inhibition of the PI3K/Akt/mTOR pathway may lead to tumor escape *via* the MAPK pathway. This suggests to us that, in fact, most clinical trials have also demonstrated that monotherapy of targeted drugs is not feasible. Therefore, combining targeted inhibitors of multiple pathways may be the future targeted therapy research's primary direction. At the same time, in addition to considering drug efficacy, we must consider that a multidrug combination implies a superposition of AEs and toxicity.

Based on the characteristics of pancreatic cancer - dense fibrous stroma, accounting for 90% of the tumor volume, and excessive proliferation of fibrous histochemistry, drugs are not easy to reach the tumor interior. Investigating targeted or cytotoxic drugs that are more accessible to the tumor, or using more efficient delivery methods, such as local arterial delivery, may improve efficacy.

Most of the studies conducted to date have been designed based on gemcitabine activity. Given that gemcitabine is no longer the reference drug, future studies should focus on targeted therapy with either nab-paclitaxel or FOLFIRINOX as the control group, which may improve the results achieved. Furthermore, most studies showed promising results in preclinical evaluations, but the vast majority failed to proceed to more advanced clinical studies due to the lack of positive results. This suggests that better preclinical models should be developed to accurately reflect the tumor characteristics and environment in humans, thereby making clinical trials more relevant to preclinical studies.

PDAC is a very complex entity, joining different molecular particularities and in a dynamic manner, not in a static one. As some guidelines already stated and can be concluded from de data shown here, is very important to spread the genetic and transcriptomic profiling of every PDAC to capture the vulnerabilities of the tumor as far as possible as the way to improve therapeutic results. In conclusion,

developing the targeted drug for pancreatic cancer has a long way to go. The complex interactions within targeted biological pathways, the pharmacokinetics of targeted drugs, predictive markers of the targeted drug benefit, and the combined application of targeted drugs still require extensive and in-depth studies.

FOOTNOTES

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REVIEW

Role of tumor-associated macrophages in common digestive system malignant tumors

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Abstract

Many digestive system malignant tumors are characterized by high incidence and mortality rate. Increasing evidence has revealed that the tumor microenvironment (TME) is involved in cancer initiation and tumor progression. Tumor-associated macrophages (TAMs) are a predominant constituent of the TME, and participate in the regulation of various biological behaviors and influence the prognosis of digestive system cancer. TAMs can be mainly classified into the antitumor M1 phenotype and protumor M2 phenotype. The latter especially are crucial drivers of tumor invasion, growth, angiogenesis, metastasis, immunosuppression, and resistance to therapy. TAMs are of importance in the occurrence, development, diagnosis, prognosis, and treatment of common digestive system malignant tumors. In this review, we summarize the role of TAMs in common digestive system malignant tumors, including esophageal, gastric, colorectal, pancreatic and liver cancers. How TAMs promote the development of tumors, and how they act as potential therapeutic targets and their clinical applications are also described.

Key Words: Tumor-associated macrophages; Digestive system malignant tumors; Tumor development; Therapeutic targets; Clinical applications

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Core Tip: This review summarizes the role of tumor-associated macrophages (TAMs) in common digestive system malignant tumors, including esophageal, gastric, colorectal, pancreatic and liver cancers. How TAMs promote the development of tumors, and how they act as potential therapeutic targets and their clinical applications are also described.

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INTRODUCTION

Many digestive system malignant tumors have high incidence and mortality rate, including esophageal cancer (EC), gastric cancer (GC), colorectal cancer (CRC), pancreatic cancer (PC), and liver cancer (LC). There is increasing evidence that the tumor microenvironment (TME), which encompasses the tumor tissue structure comprising stromal cells, is involved in cancer initiation and tumor progression[1-4]. Tumor-associated macrophages (TAMs) as a predominant constituent of the TME, are a special type of macrophages generated by circulating monocytes and recruited into the TME[5]. TAMs are categorized into two functionally contrasting subtypes: Classically activated M1 macrophages and alternatively activated M2 macrophages. TAMs are extensively present in various tumors[6,7], which can participate in the regulation of various biological behaviors and influence the prognosis of digestive system cancers. In this review, we summarize the role of TAMs in EC, GC, CRC, PC and LC. More specifically, we also described how TAMs promote the development of tumors (Figure 1), and how they act as potential therapeutic targets (Figure 2) and their clinical applications.

CHARACTERISTICS OF TAMS

Origin of TAMs

It was originally believed that macrophages in the TME originated from circulating monocyte precursors in the bone marrow (BM), under the influence of tissue microenvironmental signals. However, other studies suggested a minor splenic[8] and early embryonic[9] contribution to the main proportion of TAMs derived from the BM, validating the coexistence of macrophages with different origins.

TAM polarization

In accordance with the commonly accepted theory[10], TAMs can be primarily categorized into the antitumor M1 phenotype (classically activated state) and the protumor M2 phenotype (alternatively activated state), which have contrasting functions. The former has the capacity to remove tumor cells [11] and facilitate tumor cell destruction *via* initiating cytokine[12] production within the TME and recruitment of immunostimulating leukocytes and tumor cells phagocytosis. On the contrary, M2 macrophages have a central role in propagating tumorigenesis. The function of M2 macrophages includes the removal of debris, promotion of angiogenesis, tissue reconstruction, and injury repair, as well as facilitation of tumorigenesis and progression[6].

TAM plasticity

Upon recruited to the TME by tumor-secreted stimuli, TAMs undergo M1- or M2-like activation in response[13]. However, as a result of their remarkable plasticity, TAMs can reversibly respond to specific stimuli in the TME and switch from one phenotype to another[14], transition between antitumor M1-like and protumor M2-like phenotypes amidst the immune response. Colegio *et al*[15] have reported that the hypoxic TME can induce M2-type polarization through the production of tumor-derived lactic acid and hypoxia-inducible factor (HIF)-1α[15]. Many other cytokines can govern M2 polarization, including interleukin (IL)-21[16] and IL-33[17]. TAM plasticity highlights that the reprogramming of TAMs is an attractive potential therapeutic target to inhibit tumor progression.

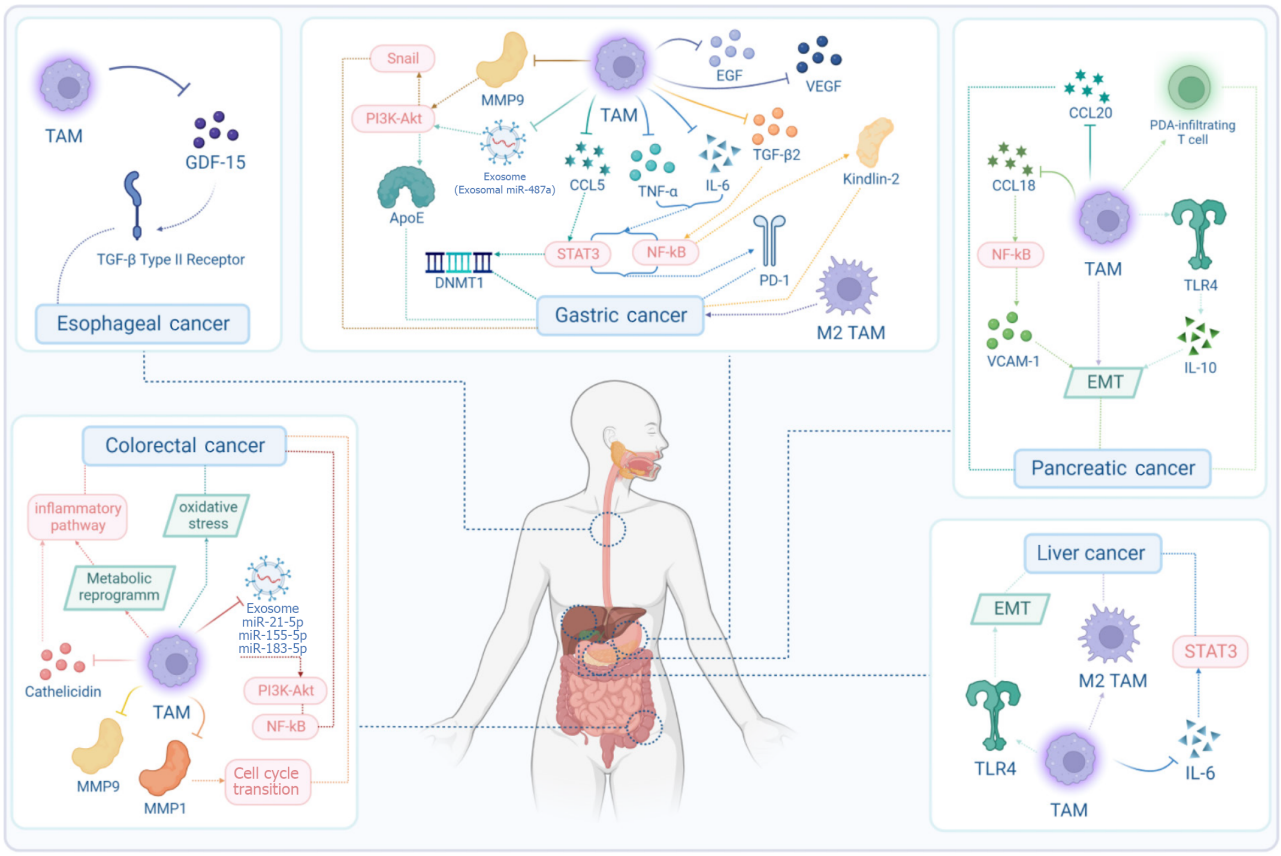

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Figure 1 Tumor-associated macrophages can promote the development of tumors. Tumor-associated macrophages (TAMs) can affect cancer progression through multiple mechanisms, which are varying in esophageal cancer (EC), gastric cancer (GC), colorectal cancer (CRC), pancreatic cancer (PC), liver cancer (LC). Color differences indicate various strategies the TAMs use on their targets, the arrows represent secretory or regulatory behaviors, and braces represent combined action of the factors. Moreover, the pink icons stand for common signaling pathways and the green icons, biological processes. In EC, growth/differentiation factor-15 and transforming growth factor-beta receptor are involved in regulations. In GC, stimulation with anti-inflammatory triggers, growth factors, chemokine, exosomes and enzymes, leads to expression of transcription factors. In CRC, TAMs work with exosomes, matrix metalloproteinases and cathelicidin, concerning signaling pathways, cell cycle transition, metabolic reprogram, inflammatory pathways and oxidative stress. In PC and LC, TAMs regulate their development similarly through interleukins and Toll like receptor 4, leading to activation of transcription factors and epithelial mesenchymal transition of tumors. Thus, TAMs can regulate digestive system malignant tumors by diverse direct and indirect mechanisms. TAM: Tumor-associated macrophages; GDF-15: Growth/differentiation factor-15; TGF-β: Transforming growth factor-β; PI3K: Phosphoinositide 3-kinase; MMP9: Matrix metalloproteinases 9; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; CCL5: CC ligand 5; TNF-α: Tumor necrosis factor-α; IL: Interleukin; STAT3: Signal transducers and activator of transcription 3; NF-kB: Nuclear factor kB; PD-1: Programmed death 1; PDA: Pancreatic ductal adenocarcinoma; TLR4: Toll like receptor 4; VCAM: Vascular cellular adhesion molecule-1; EMT: Epithelial mesenchymal transition; DNMT1: DNA methyltransferase 1.

TAM STATUS IN TUMORS

TAMs can influence tumor progression

M2 TAMs are crucial drivers of tumor invasion, growth, angiogenesis, metastasis, immunosuppression, and resistance to therapy[18]. TAMs can propagate tumor progression through upregulation of proteolytic enzymes[19] and in a manner dependent on tumor necrosis factor (TNF)-α and matrix metalloproteinases (MMPs)[20]. TAMs can also express a number of soluble factors[13] and major inflammatory mediators[21], stimulating tumor cell proliferation and survival.

TAMs act in various microenvironments, such as invasive regions where they facilitate cancer cell movement, stromal and perivascular regions where they promote metastasis, and avascular and perivascular regions where hypoxic TAMs induce angiogenesis[18].

Clinical implication of TAMs

Research advances in cancer immunology have led to multifarious strategies for modulation of TAMs for therapeutic applications[22], including strategies to deplete TAMs, inhibit TAM recruitment, influence TAM polarization, and target TAM receptors. M2 TAMs can also contribute to evaluating prognosis, which has been proven to be correlated with poorer outcomes in almost all digestive system malignant tumors[23]. On the contrary, increasing levels of M1 TAMs indicate better prognosis[24], resulting in emerging therapeutic strategies to remove M2 TAMs or alter TAM phenotypes, which can facilitate promising therapeutic benefits.

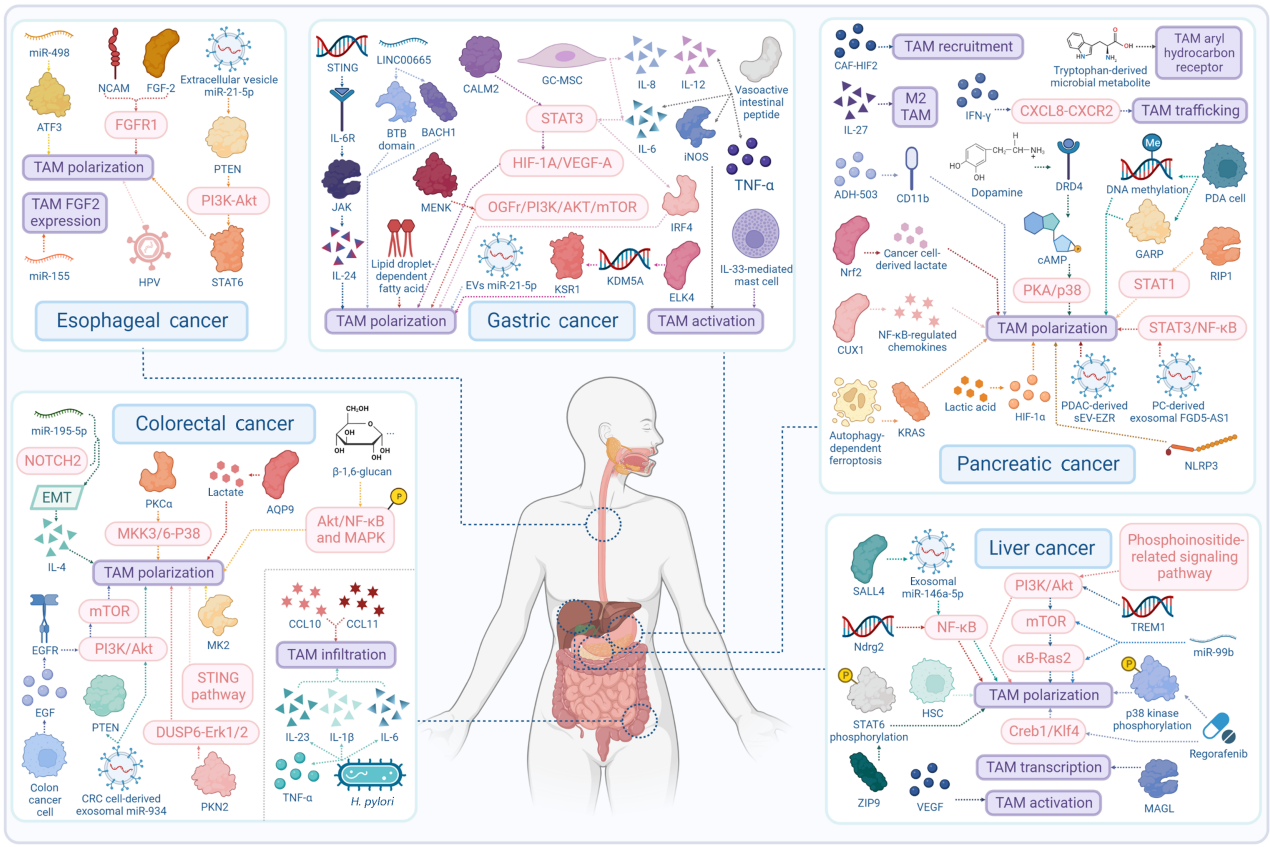

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Figure 2 Tumor-associated macrophages act as potential therapeutic targets for tumors. Multifarious strategies for modulation of tumor-associated macrophages (TAMs) are unveiled for therapeutic applications, which are varying in different digestive system malignant tumors. Color differences indicate various approaches to regulate TAMs' behaviors, the arrows represent secretory or regulatory behaviors, and braces represent combined action of the factors. Moreover, the pink icons stand for common signaling pathways, the green icons stand for biological processes, and the purple icons stand for different reactions of TAMs, including TAMs' polarization, activation, recruitment, trafficking, infiltration, transcription, and so on. Tumor and immune cells secrete growth factors, cytokines, chemokines, metabolites and extracellular vesicles that promote TAM protumor polarization. Besides, RNA, virus and specific cells also exert influence on TAM plasticity and activation. Several key signaling pathways are involved in these regulation processes, including phosphoinositide 3-kinase-Akt-mammalian target of rapamycin, nuclear factor kB, stimulator of interferon genes, and so on. Thus, TAMs can act as a promising potential therapeutic target for digestive system malignant tumors. NCAM: Neural cell adhesion molecule; FGF-2: Fibroblast growth factor 2; ATF3: Activation transcription factor 3; TAM: Tumor-associated macrophages; PI3K: Phosphoinositide 3-kinase; HPV: Human papillomavirus; STAT3: Signal transducers and activator of transcription 3; STING: Stimulator of interferon genes; IL: Interleukin; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor; mTOR: Mammalian target of rapamycin; iNOS: Inducible nitric oxide synthase; NF-kB: Nuclear factor kB; MAGL: Monoacylglycerol lipase; TREM: Triggering receptors expressed on myeloid cells; EMT: Epithelial mesenchymal transition; EGF: Epidermal growth factor; HSC: Hematopoietic stem cell; GARP: Glycoprotein A repetitions predominant; IFN-γ: Interferon-γ; EVs: Extracellular vesicles; CCL: CC ligand; PTEN: Phosphatase and tensin homolog.

Interaction of TAMs and T cells

Numerous studies have shown that TAMs can directly and indirectly dampen the antitumor activity of cytotoxic T lymphocytes (CTLs)[25] and tumor-infiltrating T cells[26] in various tumors[27,28]. Underlying this functional role are molecular mechanisms that initially involve immune checkpoint engagement, which is initially mediated through the expression of molecules like programmed cell death 1 (PD-1) ligand 1 (PD-L1)[29]. In addition, the production of inhibitory cytokines and transcription factors are also implicated in the suppression progress, which mainly include IL-10[28], interferon (IFN)-γ[30], transforming growth factor (TGF)-β[31,32] and HIF-1α[26]. Metabolic activities of TAMs, concerning the consumption of metabolites such as L-arginine[33] and generation of reactive oxygen species, also contribute to suppression of T-cell responses that is either specific to or independent of antigens. Finally, TAMs inhibit T-cell responses indirectly by controlling the immune microenvironment, including regulation of the vascular structure, extracellular matrix[34] and the chemokine milieu, such as TAM-derived chemokine CXC ligand (CXCL)9 and CXCL10[35]. Conversely, T regulatory (Treg) cells maintain metabolic adaptability, mitochondrial integrity, and survival rate of M2-like TAMs in an indirect but selective manner. This is achieved through the inhibition of IFN-γ secretion by CD8+ T cells, which subsequently hinders the activation of fatty acid synthesis intervened by sterol regulatory element binding protein 1 in immunosuppressive M2-like TAMs[36].

Specifically in digestive system cancers, TAMs are similarly thought to have mutual modulation with T cells, including but not limited to blocking the recruitment and priming of T cells and resulting in T-cell exclusion within the TME. In GC, TAMs and LAMP3$^+$ dendritic cells (DCs) are involved in mediating T-cell activity and form intercellular interaction hubs with tumor-associated stromal cells [37]. IL-10$^+$ TAM infiltration yielded an immunoevasive TME featured by Treg cell infiltration and CD8$^+$ T-cell dysfunction[38]. In CRC, C1q$^+$ TAMs modulate tumor-infiltrating CD8$^+$ T cells by expressing multiple immunomodulatory ligands in an RNA N6-methyladenosine (m6A)-dependent manner. There is evidence that compensation between TAMs and Forkhead box (Fox)p3$^+$ Treg cells promote tumor progression by limiting antitumor immunity. Decreasing colony-stimulating factor (CSF)1-dependent TAMs led to heightened CD8$^+$ T-cell against tumors, although the impact on tumor growth was restricted by a compensatory rise in Foxp3$^+$ Treg cells[39]. In pancreatic ductal adenocarcinoma (PDAC), receptor-interacting serine/threonine protein kinase (RIP)1 inhibition in TAMs resulted in CTL activation and T helper (Th) cell differentiation toward a mixed Th1/Th17 phenotype[40]. By targeting proliferating tumor-infiltrating macrophages, the infiltration of CD8$^+$ CTL and the spatial redistribution of CD8$^+$ T cells within tumors could be escalated[41]. TAMs are critical regulators in orchestrating epigenetic profile of PDAC-infiltrating T cells towards a protumoral phenotype[42]. In hepatocellular carcinoma (HCC), HCC-derived exosomes instigate macrophages to heighten IFN-γ and TNF-α expression in T cells, while upregulating the expression of inhibitory receptors PD-1 and cytotoxic T-lymphocyte-associated antigen-4[43]. These findings collectively demonstrate that TAMs are central drivers of immunosuppressive TME within digestive system tumors by suppressing T cell mobilization and performance.

TAMS AND TARGETED THERAPIES OF DIGESTIVE SYSTEM MALIGNANT TUMORS

EC

TAMs can promote development of EC: TAMs can facilitate a variety of protumorigenic mechanisms in EC (Figure 1 and Table 1). In esophageal squamous cell carcinoma (ESCC), growth differentiation factor 15 derived from TAMs promoted cancer progression *via* TGF-β type II receptor activation[44].

TAMs act as potential therapeutic targets for EC: TAMs might be potential therapeutic targets to prevent EC progression (Table 2). There is evidence supporting that miR-498 inhibits autophagy and M2-like polarization of TAMs in EC *via* inhibiting murine double minute 2-mediated degradation of activated transcription factor-3[45]. miR-155-regulated fibroblast growth factor (FGF)-2 expression from TAMs inhibited EC cell invasion, migration and proliferation, and blocked vasculature formation[46]. EC-derived extracellular vesicle miR-21-5p upregulated ESCC-derived EVs-miR-21-5p through the phosphatase and tensin homolog (PTEN)/AKT/signal transducers and activator of transcription (STAT)6 pathway, thus disorganizing macrophage polarization through, and contributing to epithelial mesenchymal transition (EMT) of ESCC cells *via* TGF-β/Smad2 signaling[47]. PTEN induced M2 TAM polarization through the phosphoinositide 3-kinase (PI3K)/AKT cascade, thus enhancing the malignant behavior of tumor-associated vascular endothelial cells and promoting ESCC angiogenesis[48]. Neural-cell-adhesion-molecule- and FGF2-mediated FGFR1 signaling in the TME of EC regulated the survival and migration of TAMs and cancer cells[49]. Human papillomavirus 16 infection can promote an M2 macrophage phenotype, contributing to the invasion and metastasis of ESCC[50].

Clinical significance of TAMs in EC: Clinically, TAMs are associated with the response of EC to chemotherapy. In patients undergoing neoadjuvant chemotherapy, high infiltration of CD68$^+$/CD163$^-$ macrophages can serve as an adverse prognostic factor in esophageal and gastric adenocarcinoma[51, 52].

GC

TAMs can promote development of GC: In GC, peritoneal dissemination transpires through an invasive mechanism in which cancer cells directly penetrate the gastric wall and exfoliate into the peritoneal cavity (Table 1). Stimulation with anti-inflammatory triggers (such as TNF-α and IL-6), growth factors [such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and TGF-β2], chemokines [such as chemokine CC ligand (CCL)5], exosomes and enzymes (such as MMP and PI3K/Akt), leads to expression of transcription factors [such as STAT6, nuclear factor (NF)-κB and Snail) (Figure 1). Intraperitoneal TAMs are involved in promoting peritoneal dissemination of GC *via* secreted IL-6[53] and polarization to the M2 phenotype[54].

Numerous studies have demonstrated that TAMs are capable of express multifarious cytokines and chemokines that promote tumor cell proliferation and viability, including EGF[55], VEGF[55], TNF-α [56], TGF-β2[57], IL-6[56], and CCL5[58]. TAMs can facilitate the development of GC through multiple signal pathways, such as cyclooxygenase-2/prostaglandin E2/TGF-β/VEGF[59], and CCL5/chemokine CC receptor 5/STAT3[60].

Table 1 Tumor-associated macrophages can promote the development of tumors

Diseases	Factors	Functions	Mechanism	Ref.
EC	GDF-15 derived from TAMs	Promoting progression of ESCC	Activating TGF-β type II receptor	[44]
GC	TAMs	Promoting peritoneal dissemination of GC	Secreting IL-6	[53]
	TAMs	Promoting progression in GC	Polarizing to the M2 phenotype	[54]
	TAMs	Supporting peritoneal metastasis	Producing EGF and VEGF	[55]
	TNF-alpha and IL-6 secreted by TAMs	Promoting proliferation of GC cells	Activating the NF-κB and STAT3 signaling pathway to regulate PD-L1 expression	[56]
	TGFβ2 secreted by TAMs	Promoting the invasion of GC cells	Regulating Kindlin-2 through NF-κB	[57]
	CCL5 secreted by TAMs	Promoting the proliferation, invasion and metastasis of GC cells	Stat3 signaling pathway	[58]
	TAMs	Influencing omental milky spots and lymph nodes micrometastasis	COX-2/PGE-2/TGF-β/VEGF signal pathways	[59]
	TAMs	Promoting epigenetic silencing of tumor suppressor gelsolin, and silence GSN	Upregulation of DNMT1 by CCL5/CCR5/STAT3 signaling	[60]
	TAMs	Inducing invasion and poor prognosis in GC	Promoting MMP9 expression	[63]
	MMP-9 secreted by TAMs	Suppressing distant metastasis in GC	PI3K/AKT/Snail dependent pathway	[64]
	Exosomal miR-487a derived from TAMs	Promoting the proliferation and tumorigenesis in GC	-	[65]
	M2 macrophage-derived exosomes	Remodeling the cytoskeleton-supporting migration in recipient GC cells	Mediating an intercellular transfer of ApoE-activating PI3K-Akt signaling pathway	[66]
CRC	TAMs	Potentiating the angiogenic capacity of the TME	Oxidative stress-dependent manner	[91]
	Metabolic reprogramming in TAMs	Building a bridge between metabolic dysfunction and the onset and progression of CRC	Inflammatory pathways	[92]
	M2 macrophage-derived exosomes	Promoting CRC cells' migration and invasion	MiR-21-5p and miR-155-5p	[93]
	Exosomal miR-183-5p Shuttled by M2 TAMs	Promoting the development of colon cancer	THEM4 mediated PI3K/AKT and NF-κB pathways	[94]
	MMP1 derived from TAMs	Facilitating colon cancer cell proliferation	Accelerating cell cycle transition from G0/G1 to S and G2/M phase	[95]
	M2 TAMs	Inducing colon cancer cell invasion	MMP-9	[96]
	Cathelicidin secreted by TAMs	Promoting the growth of CRC	Recruiting inflammatory cells	[97]
PC	Intraperitoneal TAMs	Promoting peritoneal dissemination and chemoresistance	Inducing EMT	[123]
	M2 TAMs	Promoting EMT	TLR4/IL-10 signaling	[124]
	TAMs	Promoting progression and the Warburg effect	CCL18/NF-Kb/VCAM-1 pathway	[125]
	CCL20 secreted by M2 TAMs	Promoting the migration, epithelial-mesenchymal transition, and invasion of pancreatic cancer cells	-	[126]
	TAMs	Orchestrating functions PDA-infiltrating T cells	Odulating PDA-infiltrating T cells epigenetic profile towards a pro-tumoral phenotype	[42]
LC	TAMs	Promoting LCSLC self-renewal capability and carcinogenicity	M2 polarization	[151]

TAMs	Promoting EMT of Hep3B hepatoma cells	TLR4	[153]
IL-6 secreted by TAMs	Promoting expansion of these CSCs and tumorigenesis	STAT3 signaling	[154]

Tumor-associated macrophages (TAMs) contribute to the development of esophageal cancer, gastric cancer, colorectal cancer, pancreatic cancer and liver cancer. The effective factors are TAMs and their derivants or secretions. The function indicates that how these factors exert influence on tumor progression, concerning proliferation, invasion, metastasis, migration and so on. In addition, the mechanism indicates the corresponding signaling pathways or regulatory intermediates, through which TAMs and their derivants promote or suppress development of the cancers. The last column indicates the corresponding reference of the entry. EC: Esophageal cancer; GC: Gastric cancer; CRC: Colorectal cancer; PC: Pancreatic cancer; LC: Liver cancer; GDF-15: Growth/differentiation factor-15; TAM: Tumor-associated macrophages; ESCC: Esophageal squamous cell carcinoma; TGF-β: Transforming growth factor-β; IL: Interleukin; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; TNF-α: Tumor necrosis factor-α; NF-kB: Nuclear factor kB; STAT3: Signal transducers and activator of transcription 3; PD-L1: Programmed Cell Death Ligand 1; PGE-2: Prostaglandin E2; PI3K: Phosphoinositide 3-kinase; MMP9: Matrix metalloproteinases 9; CCL5: CC ligand 5; TLR4: Toll like receptor 4; LCSLC: Liver cancer stem-like cell; CSC: Cancer stem cell.

It is also reported that TAMs may promote the invasion, metastasis and poor prognosis of GC cells by increasing expression of MMP9 and MMP2[61-63], mechanistically involving the PI3K/AKT/Snail-dependent pathway[64].

As for exosomes like exosomal miR-487a[65] derived from M2 macrophages, they can promote the proliferation and tumorigenesis, and remodel cytoskeleton-supporting migration in GC, through the ApoE-activating PI3K/Akt signaling pathway[66].

TAMs act as potential therapeutic targets for GC: As a potential therapeutic target in GC, TAMs can be reprogrammed into a proinflammatory subtype by targeting many pathways (Table 2), such as the stimulator of interferon genes (STING) pathway[67]. At the RNA level, LINC00665 interfaces with transcription factor BTB domain and CNC homology 1 to activate Wnt1 and mediates M2 polarization of TAMs in GC[68]. Many proteins can also mediate TAM polarization (calmodulin 2[69], methionine enkephalin[70], ETS-like transcription factor 4[71], IL-6[72], and IL-8[73]) and repress TAM activation (vasoactive intestinal peptide[74]), *via* signaling pathways such as STAT3/HIF-1A/VEGF-A axis[69], opioid growth factor receptor/PI3K/AKT/mammalian target of rapamycin (mTOR) axis[70], and IL-6/STAT3/interferon regulatory factor 4 axis[72], and so on. Lipid-droplet-dependent fatty acid metabolism[75] and miR-151-3p derived from GC exosomes[76] can also control the immunosuppressive phenotype of TAMs.

TAMs can be regulated by other cells, such as tumor-promoting GC-derived mesenchymal stromal cells[74] and IL-33-mediated mast cells[77].

Clinical significance of TAMs in GC: More clinically, TAMs can be used to potentiate localized immunotherapy of GC. For instance, researchers created an injectable hydrogel that can shear-thin and is loaded with polyphyllin II and resiquimod, which can help potentiate localized immunotherapy of GC by repolarizing TAMs[78]. Polyclonal antibody stimulator monotherapy or combined with PD-1 antibody[79], as well as using a natural alkaloid product isolated from sophora alopecuroides. L, sophoridine[80], may decrease the number of immunosuppressive M2-polarized TAMs.

When it comes to chemotherapy, exosomes and other factors could represses the chemosensitivity of gastric tumor cells in a TAM-dependent manner. Exosomal transfer of TAM-derived miR-21 confers cisplatin resistance in GC cells[81]. Yu *et al*[82] discovered that macrophages can be stimulated into a tumor-protective M2-like phenotype by tumor-derived leukemia inhibitory factor through activation of the STAT3 signaling pathway[82]. 5-Fluorouracil (5-FU) treatment activates HIF-1α in GC cells, leading to the accumulation of M2 TAMs that shield tumor cells from the effects of chemotherapeutic agents [83]. By generating growth differentiation factor 15 to exacerbate fatty acid β-oxidation in tumor cells, the recruited TAMs display the tumor-supporting M2 phenotype and enhance the chemoresistance of GC cells. And inversely polarized M2 macrophages can potentiate 5-FU resistance in tumors *via* CCL8 and phosphorylation of the Janus kinase 1/STAT3 signaling pathway[84].

The positive correlation between high level of TAMs in tumors and low overall survival of patients has been demonstrated. High density of M2 TAMs was associated with larger tumor size, diffuse Lauren type, poor histological differentiation, deeper tumor invasion, lymph node metastasis, and advanced TNM stage[85]. Abundance of CD163-positive TAMs in early GC[86] as well as $CD206^+$ myeloid-derived TAMs[87] predict te recurrence after curative resection. $CD8^+$ tumor-infiltrating lymphocytes and $CD68^+$ TAMs[88], and high expression of HIF-1α combined with TAM infiltration[89] and coexistence of osteopontin and infiltrating M2 TAMs[90] can serve as a prognostic marker in GC.

CRC

TAMs can promote the development of CRC: The protumor role of TAMs in the development of colon carcinoma has been confirmed (Table 1). TAMs work with exosomes, MMP and cathelicidin, concerning signaling pathways (such as PI3K/Akt and NF-κB), cell cycle transition, metabolic reprogramming, inflammatory pathways, and oxidative stress (Figure 1). TAMs potentiate the angiogenic capacity of the

Table 2 Tumor-associated macrophages act as potential therapeutic targets for tumors

Diseases	Factors	Types	Targets	Functions	Mechanism	Ref.
EC	MiR-498	MiRNA	Inhibiting autophagy and M2-like polarization of TAMs in esophageal cancer	-	Inhibiting MDM2-mediated ATF3 degradation	[45]
	MiR-155	MiRNA	Regulating TAMs FGF2 expression	Suppressing EC cell proliferation, migration, invasion and inhibiting vasculature formation	-	[46]
	EC-Derived Extracellular Vesicle miR-21-5p	MiRNA	Disorganizing macrophages polarization	Contributing to EMT of ESCC cells *via* TGF-β/Smad2 signaling	PTEN/AKT/STAT6 pathway	[47]
	PTEN	Protein	Inducing M2 TAMs polarization	Enhancing the malignant behavior of TECs, promoting ESCC angiogenesis	Activating the PI3K/AKT signaling pathway	[48]
	NCAM- and FGF-2-mediated FGFR1 signaling	Signaling	Regulating the survival and migration of TAMs and cancer cells	-	NCAM knockdown *via* a suppression of PI3K-Akt and FGFR1 signaling, and rhFGF-2 -through FGFR1 signaling	[49]
	HR-HPV; HPV16 infection	Virus	Promoting M2 macrophages phenotype	Promoting the invasion and metastasis of esophageal squamous cell carcinoma	-	[50]
GC	STING	Gene	Promoting TAMs polarizing into pro-inflammatory subtype	Inducing apoptosis of GC cells	IL6R-JAK-L24pathway	[67]
	LINC00665	LncRNA	Activating Wnt1 and mediating TAMs M2 polarization	-	Interacting with BTB domain and BACH1	[68]
	CALM2	Protein	Polarizing TAMs	Facilitating angiogenesis and metastasis of GC	STAT3/HIF-1A/VEGF-A	[69]
	MENK	Protein	Skewing macrophages toward M2 phenotype from M1 phenotype	Inducing cells apoptosis	OGFr/PI3K/AKT/Mtor signaling pathway	[70]
	ELK4	Transcription factor	Promoting M2 polarization	Promoting the development of GC	Reducing the PJA2-dependent inhibition of KSR1 by transcriptional activation of KDM5A	[71]
	IL-6	Cytokine	Polarizing the Mφs	Promoting tumor invasion	IL-6/STAT3/IRF4 signaling pathway	[72]
	GC-MSCs	Cell	Promoting M2 polarization	Promoting metastasis and EMT in GC	Secreting IL-6 and IL-8	[73]
	Vasoactive intestinal peptide	Protein	Repressing activation of TAMs	-	Regulating TNFα, IL-6, IL-12 and Inos	[74]
	Lipid droplet-dependent fatty acid	Fatty acid	Controlling the immune suppressive phenotype of TAMs	-	-	[75]
	MiR-151-3p derived from GC exosomes	Exosome	Inducing M2-phenotype polarization	Promoting tumor growth	-	[76]
	IL-33-mediated mast cell	Cell	Mobilizing macrophages	Promoting GC	-	[77]
CRC	PKN2	Protein	Inhibiting M2 phenotype polarization	-	DUSP6-Erk1/2 pathway	[98]
	AQP9	Protein	Stimulating M2-like polarization	Promoting colon cancer progression	Transporting lactate	[99]
	PKCα	Tumor suppressor	Promoting M1 macrophages polarization	-	MKK3/6-P38 signaling pathway	[100]
	MK2	Protein	Promoting polarization	-	-	[101]

			of protumorigenic TAMs			
	MiR-195-5p/NOTCH2-mediated EMT	-	Affecting M2-like TAMs polarization	-	Modulating IL-4 secretion	[102]
	CRC cell-derived exosomal miR-934	Exosome	Inducing M2 macrophages polarization	-	Downregulating PTEN expression and activate the PI3K/AKT signaling pathway	[103]
	Stimulator of STING pathway	Signaling pathway	Activating reprogramed TAMs toward the M1 phenotype	-	-	[104]
	Colon cancer cell	Cell	Promoting M2 polarization of TAMs	-	Secreting EGF; EGFR/PI3K/AKT/Mtor pathway	[105]
	CXCL10 and CXCL11	Chemokine	Inducing the infiltration of TAMs	Leading to the poor prognosis of CRC	-	[106]
	β-1, 6-glucan	Organic compound	Reseting TAMs from M2-like to M1-like phenotype	Inhibiting the viability of colon cancer cells	Increasing the phosphorylation of Akt/NF-κB and MAPK	[107]
	H. pylori infection	Becteria	Reducing the infiltration of M2-like TAMs	-	Downregulating TNF-α, IL-1β, IL-6 and IL-23	[108]
PC	Autophagy-dependent ferroptosis	-	Driving TAMs polarization	-	Releasing and uptaking of oncogenic KRAS protein	[127]
	RIP1	Kinase	Reprogramming TAMs	-	STAT1-dependent manner	[40]
	Deletion of CAF-HIF2	Protein	Decreasing the intratumoral recruitment of immunosuppressive M2 macrophages	-	-	[128]
	ADH-503	Small-molecule agonist	Leading to the repolarization of TAMs	-	Partial activation of CD11b	[129]
	NLRP3	Inflammasome	Regulating TAMs polarization	Enhancing lung metastasis of PDAC	-	[130]
	IL-27	Cytokine	Targeting M2 TAMs	Dampening the proliferation, migration and metastasis of PC cells	-	[131]
	IFN-γ	Chemokines	Preventing trafficking of TAMs	Improving the efficacy of PD1 blockade therapy in PC	Blocking the CXCL8-CXCR2 axis	[132]
	PC-derived exosomal FGD5-AS1	Exosome	Stimulating M2 macrophages polarization	Promoting proliferation and migration of PC cell	Activating STAT3/NF-κB pathway	[133]
	PDAC-derived Sev-EZR	Exosome	Modulating TAMs polarization	Promoting PDAC metastasis	-	[134]
	CUX1	Transcription factor	Mediating M1 polarization	Inhibiting angiogenesis and tumor progression	Downregulating several NF-κB -regulated chemokines	[135]
	Tryptophan-derived microbial metabolite	Metabolite	Activating the aryl hydrocarbon receptor in TAMs	Suppressing anti-tumor immunity	-	[136]
	Nrf2	Transcription factor	Stimulating M2 macrophages polarization	Promoting EMT	Activating cancer cell-derived lactate	[137]
	Lactic acid	Organic compound	Redistributing M2TAMs subsets	Upregulating PDL1 to assist tumor immune escape	HIF1α signaling pathway	[138]
	Activation of DRD4 by DA	Protein	Suppressing the tumor-promoting inflammation of TAMs	-	Decreasing Camp; inhibit the activation of PKA/p38 signal pathway	[139]
	PDA cells	Cell	Reprogramming M1-like macrophages	-	GARP-dependent and DNA methylation-mediated mechanism	[140]

LC	Ndrg2	Gene	Influencing TAMs polarization	-	NF-κB pathway	[155]
	TREM1knockdown	Gene	Shifting M2 macrophages towards a M1 phenotype	-	Inhibiting PI3K/AKT/Mtor activation	[156]
	MiR-99b	MiRNA	Promoting M1 while suppressing M2 macrophages polarization	-	Targeting κB -Ras2 and/or mTOR	[157]
	MAGL	Kinase	Promoting the transcription and secretion of inflammatory factors in TAMs	-	-	[158]
	-	-	Blocking triggering receptor expressed on myeloid cells-1-positive TAMs	Reversing immunosuppression and anti-PD-L1 resistance in LC	-	[159]
	Regorafenib	Multikinase inhibitors	Reversing M2 polarization	-	Suppressing p38 kinase phosphorylation and downregulating Creb1/Klf4 activity in BMDMs	[160]
	ZIP9	Protein	Promoting M2 macrophages polarization	-	Enhancing phosphorylated STAT6	[161]
	Phosphoinositide-related signaling pathway	Signaling pathway	Reprogramming TAMs	-	Enhancing activation of the PI3K/Akt pathway	[162]
	Inhibite VEGF signaling pathway	Signaling pathway	Attenuating TAMs activity in liver cancer	-	-	[163]
	SALL4-mediated upregulation of exosomal miR-146a-5p	Exosome	Leading to M2-polarized TAMs	-	Activating NF-κB signaling and inducing pro-inflammatory factors	[43]
	Activated HSCs	Cell	Converting macrophages to TAMs	-	-	[164]

In esophageal cancer, gastric cancer, colorectal cancer, pancreatic cancer and liver cancer, there are multifarious approaches to regulate tumor-associated macrophages (TAMs), the effective factors of which, and corresponding types, are presented in the second and third column. The targets indicate which behaviors of TAMs that are modulated. In addition, the functions, mechanism and reference section are similar as Table 1. EC: Esophageal cancer; GC: Gastric cancer; CRC: Colorectal cancer; PC: Pancreatic cancer; LC: Liver cancer; TAM: Tumor-associated macrophages; PTEN: Phosphatase and tensin homolog; TEC: Tumor endothelial cells; STAT3: Signal transducers and activator of transcription 3; ESCC: Esophageal squamous cell carcinoma; NCAM: Neural cell adhesion molecule; FGF-2: Fibroblast growth factor 2; HPV: Human papillomavirus; VEGF: Vascular endothelial growth factor; STING: Stimulator of interferon genes; MSC: Mesenchymal stem cell; TNF-α: Tumor necrosis factor-α; IL: Interleukin; PI3K: Phosphoinositide 3-kinase; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; STAT1: Signal transducers and activator of transcription 1; IFN-γ: Interferon-γ; GARP: Glycoprotein A repetitions predominant; HIF-1α: Hypoxia-inducible factor-1α; PD-L1: Programmed Cell Death Ligand 1; EMT: Epithelial mesenchymal transition; mTOR: Mammalian target of rapamycin; HSC: Hematopoietic stem cell; OGFR: Opioid growth factor receptor; Nrf2: Nuclear factor erythroid 2-related factor 2.

TME in an oxidative-stress-dependent manner[91] or by metabolic reprogramming[92].

M2-macrophage-mediated regulation of CRC cell migration and invasion relies on M2-macrophage-derived exosomes, such as miR-21-5p and miR-155-5p[93], which may take effect through downregulating expression of *BRG1*. Exosomal miR-183-5p transferred by M2 polarized TAMs facilitate colon cancer through targeting thioesterase superfamily member 4-mediated PI3K/AKT and NF-kB pathways [94].

Multiple studies indicated that MMPs, such as MMP1 and MMP9, derived from TAMs may induce colon cancer cell invasion and proliferation[95,96]. It has been demonstrated that cathelicidin secreted by TAMs can promote the growth of CRC in mice by recruiting inflammatory cells such as macrophages into the TME[97].

TAMs act as potential therapeutic targets for CRC: In colon carcinoma, TAM M2 phenotype polarization can be regulated by diverse proteins (Table 2), such as protein kinase N2[98], aquaporin 9 [99], tumor suppressor protein kinase (PK)Cα[100], and MAPKAP kinase 2[101]. miR-195-5p/NOTCH2-mediated EMT also affects M2-like TAM polarization by modulating IL-4 secretion in CRC[102], as does CRC-cell-derived exosomal miR-934 by downregulating PTEN expression and activating the PI3K/AKT signaling pathway[103].

The activation of pathways like the STING[104] and EGFR/PI3K/AKT/mTOR axis[105] has some of this same functionality. As chemokines, neuroendocrine-like-cell-derived CXCL10 and CXCL11 expand the infiltration of TAMs, accounting of the poor prognosis of CRC[106].

From the metabolic perspective, β-1,6-glucan resets tumor-supporting M2-like macrophages to tumor-inhibiting M1-like phenotype by activating the phosphorylation of Akt/NF-kB) and mitogen-activated protein kinase[107]. In mice with colitis-associated colorectal tumors, *Helicobacter pylori* infection quenched infiltration of TAMs, especially M2-like TAMs, while downregulating proinflammatory and protumorigenic factors TNF-α, IL-1β, IL-6, and IL-23[108].

Clinical significance of TAMs in CRC: TAMs with an M2-like phenotype have been associated with immunosuppression and resistance to chemotherapy of CRC. CD206/CD68 ratio[109] functions as a potent prognostic biomarker for predicting postoperative adjuvant chemotherapy in stage II colon cancer. In CRC, high infiltration of $CD68^+$ TAMs[110], as well as type and number of intratumoral macrophages and clever-$1^{(+)}$ vessel density[111] could both function as a favorable prognostic marker.

Several TAM-targeting immunotherapies have been shown to promote antitumor immunity in CRC. A ketogenic diet restrains colon tumors *via* inducing intratumor oxidative stress through downregulation of MMP9 expression, and facilitating the polarization of TAM towards an M1-like proinflammatory phenotype[112]. By re-educating TAMs in CRC, piceatannol is an effective TGF-β1/TGF-Br1 pathway inhibitor and TME modulator that inhibits tumor progression and metastasis[113]. Licorne-mediated immunogenic photodynamic therapy synergizes with myeloid-derived suppressor cell (MDSC)-targeting immunotherapy[114], Bte-Pd-Au-R-combined radiophotothermal therapy[115], as well as combination of foretinib and anti-PD-1 antibody immunotherapy[116] significantly inhibited tumor growth *via* decreasing tumor infiltration or the percentage of M2-like TAMs. Numerous studies have demonstrated that triptolide decreased TAM infiltration and M2 polarization[117] to remodel the colon cancer immune microenvironment through suppressing the sphingosine kinase–sphingosine-1-phosphate signaling pathway[118], or inhibiting tumor-derived CXCL12 *via* NF-kB and the extracellular signal-regulated protein kinases 1 and 2 axis[119]. Plinabulin[120], a distinct microtubule-targeting chemotherapy, as well as short-course radiotherapy[121], promoted a shift in M2 to M1 TAM polarization.

PC

TAMs can promote development of PC: Pancreatic tumors are characterized by a desmoplastic stroma consisting of fibroblasts, immune cells, and a dense network of collagen fibers. Within this stroma, TAMs are among the most numerous immune cell populations[122]. Their protumorigenic function is predominantly attributed to their capacity to facilitate immune evasion and metastasis (Figure 1 and Table 1).

In PC, intraperitoneal TAMs potentially play a crucial role in promoting peritoneal dissemination and chemoresistance by inducing EMT[123]. Similarly, M2-polarized TAMs enhanced EMT in PC cells partially *via* Toll like receptor (TLR)-4/IL-10 signaling[124]. TAMs promote progression and the Warburg effect *via* CCL18/NF-kB/vascular cellular adhesion molecule 1 pathway in PDAC[125]. In addition, CCL20 secreted by M2 macrophages promoted the migration, EMT, and invasion of PC cells [126]. The study indicated a decisive role of TAMs in orchestrating functions of PDAC-infiltrating T cells by modifying their epigenetic profile towards a pro-tumoral phenotype[42].

TAMs act as potential therapeutic targets for PC: TAMs can also act as potential therapeutic targets for PC (Table 2). Autophagy-dependent ferroptosis accelerates TAM polarization *via* secretion and absorption of oncogenic KRAS protein[127]. Researchers discovered upregulation of RIP-1 in TAMs in PDAC[40]. Deletion of cancer-associated fibroblast HIF-2 significantly decreased the intratumoral recruitment of immunosuppressive M2 macrophages[128]. Fractional activation of CD11b by a small-molecule agonist contributes to TAM repolarization[129]. NLRP3 activation in TAMs enhanced lung metastasis of PDAC through regulation of TAM polarization[130].

By targeting M2-like TAMs, IL-27 dampened the proliferation, migration and metastasis of PC cells and boosted the potency of gemcitabine[131]. IFN-γ is a potential translational strategy to optimize performance of PD-1 blockade therapy in PC by preventing migration of $CXCR2^+CD68^+$ macrophages by blocking the CXCL8/CXCR2 axis[132]. PC-derived exosomal FGD5-AS1 induced M2 macrophage polarization *via* STAT3/NF-κB pathway[133]. The PDAC-derived small extracellular vesicle Ezrin can modulate macrophage polarization and promote PDAC metastasis[134]. Cut like homeobox 1 suppresses handful NF-κB-regulated chemokines like CXCL10, which are linked with M1 polarization and hindrance of angiogenesis and tumor development[135].

In addition, tryptophan-derived microbial metabolites stimulate the aryl hydrocarbon receptor in TAMs to inhibit antitumor immunity[136]. Cancer-cell-derived lactate activates macrophage nuclear factor erythroid 2-related factor 2 (Nrf2), skewing macrophages polarization towards an M2-like phenotype. These educated macrophages then trigger Nrf2 activation in cancer cells, ultimately promoting EMT[137]. Modulation of lactic acid level can redistribute M2 TAMs and upregulate PD-L1 to assist tumor immune escape, possibly through the HIF-1α signaling pathway[138]. Activation of dopamine receptor D4 by dopamine is instrumental in a depletion of cAMP, thereby hindering the

activation of the PKA/p38 signaling pathway, ultimately leading to the suppression of tumor-promoting inflammation of TAMs[139].

For PC cells themselves, they render TAMs metabolically reprogrammed through a glycoprotein A repetitions predominant (GARP)-dependent and DNA-methylation-mediated mechanism to adopt a precancerous fate[140].

Clinical significance of TAMs in PC: The association between TAMs and immune response has primarily been observed as a reduction in the immunostimulatory function of TAMs.

An exosome-based dual delivery biosystem was created to improve immunotherapy for PDAC and reverse immunosuppression of M2 TAMs upon disruption of the galectin-9/dectin 1 axis[141]. A TME-responsive micellar system co-loaded with gemcitabine and PI3K inhibitor wortmannin was employed to achieve dual targeting of TAMs and tumor cells, aimed at repolarizing TAMs and improving the chemoimmunotherapy efficacy against PC[142].

Hyaluronic acid nanoparticle-encapsulated miRNA-125b reprogrammed TAMs to an antitumor phenotype in PDAC[143]. M2-TAM-targeting nanomicelles were created to simultaneously deliver PI3K-γ inhibitor NVP-BEZ 235 and CSF-1R-siRNA, leading to specific TAM reprogramming and antitumor immune response activation[144]. A customized nanocomplex through the self-assembling synthetic 4-(phosphonooxy)phenyl-2,4-dinitrobenzenesulfonate and Fe^{3+}, subsequently decorated with hyaluronic acid, jointly repolarized TAMs to deactivate stromal cells and therefore weaken stroma[145]. A reduction-responsive RNAi nanoplatform utilized its reduction-responsive characteristic to rapidly release siRNA, inducing depolarization of TAMs into tumor-inhibiting M1-like phenotype[146].

To aid diagnosis, metabolizable near-infrared-II nanoprobes were applied to dynamic imaging of deep-seated TAMs in PC[147]. DN-ICG nanoprobes were qualified to discern dynamic variation of TAMs stimulated by low-dose radiotherapy and zoledronic acid.

By activating M2-like TAM polarization, atorvastatin mitigates the effect of aspirin on PC development and the chemotherapeutic potency of gemcitabine in PC[148]. Combined blockade of TGF-β1 and granulocyte-macrophage CSF improves chemotherapeutic effects in PC by modulating the TME [149]. In tumor-bearing *Klebsiella pneumoniae* carbapenemase mice, pharmacological TAM depletion enhanced therapeutic response to gemcitabine[150].

LC

TAMs can promote development of LC: TAMs have been proved to promote the development of LC (Table 1). M2 polarization of TAMs in the TME promotes LC stem-like cell self-renewal capability and carcinogenicity[151,152]. Since TAMs can hasten EMT of Hep3B hepatoma cells, reduction of TLR4 expression in TAMs may attenuate that[153]. TAMs produce IL-6, which promotes expansion of these cancer stem cells and tumorigenesis. Restraint of TAM-stimulated CD44+ cell activity can be attainable by obstructing IL-6 signaling using tocilizumab, a drug approved by the United States Food and Drug Administration (FDA) for the treatment of rheumatoid arthritis[154].

TAMs act as potential therapeutic targets for LC: TAMs have also been found to serve as potential therapeutic targets for LC (Table 2). Researchers demonstrated that loss of *Ndrg-2* influenced TAM polarization *via* the NF-κB pathway[155]. Knocking down triggering receptors expressed on myeloid cells (TREM1) in macrophages quenched the activation of the PI3K/AKT/mTOR pathway in M2 macrophages polarization[156]. Targeted delivery of miR-99b and/or miR-125a into TAMs substantially decelerated the progression of HCC and Lewis lung cancer, particularly following miR-99b delivery [157].

The mechanistic study illustrated that the high expression of monoacylglycerol lipase promoted the transcription and excretion of inflammatory factors such as IL-1β, IL-6 and TNF-α in M2-type TAMs cells[158]. Blocking TREM1-positive TAMs induced by hypoxia reverses immunosuppression and anti-PD-L1 resistance in LC[159]. Regorafenib, a multikinase inhibitor, reversed M2 polarization by suppressing p38 kinase phosphorylation and downstream Creb1/Klf4 activity in BM-derived macrophages[160]. The zinc-regulated transporters, iron-regulated transporter-like protein 9 upregulates phosphorylated STAT6 to facilitate polarization of M2 macrophages while downregulating the phosphorylation of IκBα/β to hinder M1 macrophage polarization[161].

TNF-α-induced protein 8-like 1 redounded arousal of the PI3K/Akt pathway in macrophages by directly attaching to and modulating the metabolism of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate[162]. Inhibiting the VEGF signaling pathway was shown to attenuate TAM activity in LC[163]. Sal-like protein-4-mediated upregulation of exosomal miR-146a-5p remodeled macrophages by triggering NF-κB signaling and proinflammatory factors, contributing to M2-type polarization in TAMs[43].

In the TME, activated hematopoietic stem cells transform macrophages to TAMs and respectively stimulate the differentiation of DCs and monocytes into regulatory DCs and MDSCs[164].

Clinical significance of TAMs in LC: To reverse immunosuppressive process, a BisCCL2/5i mRNA nanoplatform was directly evolved, which appreciably ignited the antitumoral M1-type polarization in TAMs and reduced immunosuppression in the TME[165]. Researchers developed a nanoliposome-loaded C6-ceremide (LipC6) to reduce the number of TAMs and their production of reactive oxygen

species[166]. LipC6 animated TAM differentiation into M1 phenotype, which engendering a decrease in immunosuppression and an increase in CD8+ T cell activity.

By interference with insulin-like growth factor (IGF)-1 secretion, sorafenib altered macrophage polarization, reduced IGF-1-driven cancer growth *in vitro* and partially inhibited macrophage activation *in vivo*[167]. Elevated serum levels of taurocholic acid were associated with reduced sirtuin (SIRT)5 expression and an increase in M2-like TAMs in HCC patient samples. Treatment with cholestyramine, a bile acid sequestrant and FDA-approved medication for hyperlipemia, reversed the implication of SIRT5 deficiency in impelling M2-like polarized TAMs and LC progression[168]. The novel glycyrrhetinic acid-tetramethylpyrazine conjugate TOGA exerted an anti-hepatocarcinogenic effect by attenuating effectiveness of TAMs on tumor cells through a mechanism related to the NF-κB pathway [169].

In the HCC microenvironment, M2 TAMs secreted considerable amounts of IL-17, which suppressed oxaliplatin-induced tumor cell apoptosis by triggering chaperone-mediated autophagy and curtailing cyclin D1 expression[170]. Radiofrequency ablation suppressed protumoral activation of local TAMs [171]. The combination of zwitterionic chito-oligosaccharides (COSs) with a photothermal material impaired the undesirable tumor promotion of TAMs, thus enhancing the outcome of photothermal therapy. Zwitterionic COSs acted as potent immune activators to re-educate TAMs to M1[172].

CONCLUSION

TAMs play a significant role in digestive system malignant tumors; therefore, TAM modulation is an attractive potential therapeutic target to enhance antitumor immune response and inhibit tumor progression. So far, diverse clinical therapies targeting TAMs have proven to be effective, highlighting the clinical significance of TAMs in digestive system malignant tumors. However, there are still many questions about the characteristics and functions of TAMs in digestive system malignant tumors. Continuous basic, transformation and clinical research may reveal some new prospects, such as how to use TAMs to improve cancer outcomes. Therefore, this is a promising field of cancer treatment, which may provide fruitful results.

FOOTNOTES

Author contributions: Wu J and Wang Y designed study, revised the manuscript, reviewed the results and made critical comments on the manuscript; Shen Y, and Chen JX analyzed data and performed manuscript drafting; Li M and Xiang Z searched the literature and collected data; All authors reviewed and approved the final version; Shen Y and Chen JX contributed equally to this work; Wu J and Wang Y contributed equally to this work.

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REVIEW

Lipid metabolism of hepatocellular carcinoma impacts targeted therapy and immunotherapy

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Abstract

Hepatocellular carcinoma (HCC) is a common malignant tumor that affecting many people's lives globally. The common risk factors for HCC include being overweight and obese. The liver is the center of lipid metabolism, synthesizing most cholesterol and fatty acids. Abnormal lipid metabolism is a significant feature of metabolic reprogramming in HCC and affects the prognosis of HCC patients by regulating inflammatory responses and changing the immune microenvironment. Targeted therapy and immunotherapy are being explored as the primary treatment strategies for HCC patients with unresectable tumors. Here, we detail the specific changes of lipid metabolism in HCC and its impact on both these therapies for HCC. HCC treatment strategies aimed at targeting lipid metabolism and how to integrate them with targeted therapy or immunotherapy rationally are also presented.

Key Words: Hepatocellular carcinoma; Lipid metabolism; Targeted therapy; Immunotherapy; Drug resistance; Therapeutic efficacy

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Core Tip: This review systematically summarizes the aberrant changes of lipid metabolism in hepatocellular carcinoma (HCC), and for the first time expounds the impact of lipid metabolism on HCC targeted therapy and immunotherapy. It vividly displayed the changes of lipid metabolism in HCC and the targets of some reagents by drawing figures, and summarized the impact of lipid metabolism related reagents on HCC targeted therapy and immunotherapy through table.

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INTRODUCTION

Liver cancer, a common malignancy, has documented an increasing incidence over the years. In 2020, there were 910000 new cases of liver cancer worldwide and 830000 people died of liver cancer with the global number of patients and deaths ranking sixth and third positions, respectively[1]. Hepatocellular carcinoma (HCC), accounting for nearly 90% of total cases, is the most frequently occurring type of liver cancer[2]. HCC has many risk factors related to its occurrence, including virus hepatitis, nonalcoholic fatty liver disease (NAFLD), alcoholic hepatitis, and some genetic metabolic diseases like Wilson disease, alpha1-antitrypsin deficiency, and hereditary tyrosinemia type I[3,4].

HCC has a poor prognosis, and many patients have no obvious early symptoms, leading to untimely diagnosis. To date, radical resection and liver transplantation are still the only curative treatment for HCC patients. However, many patients are already in the advanced stage (Barcelona Clinic Liver Cancer B-D stage) when they are initially diagnosed with HCC, making them unable to undergo radical surgery[5]. The increasing shortage of liver donors and resources also makes liver transplantation a treatment strategy that only a few HCC patients can receive. It is worth noting that systemic treatment, like molecular targeted therapy and immunotherapy has brought fresh hope to HCC patients. Phase I/II/III clinical trial reports provide strong evidence for these above drugs in treating HCC[6]. Since its approval in 2007, sorafenib has been the only targeted therapy drug for advanced HCC patients for a long time. In recent years, the introduction of lenvatinib and immunotherapy such as the anti-programmed cell death protein 1/programmed death ligand 1 (anti-PD-1/PD-L1), belonging to immune checkpoint inhibitors (ICIs), has enriched the therapeutic strategy for HCC patients with unresectable tumors. Although these treatments have documented certain achievements, they are far from satisfactory. Current targeted therapies aim to inhibit tumor blood supply or directly inhibit tumor growth by affecting proliferation related signal pathways However, these targets do not have a significant and direct impact on tumor metabolism[7].

Metabolic reprogramming is an important malignancy feature that significantly impacts tumor's occurrence, proliferation, and invasion[8]. Compared with normal liver cells, the metabolism of HCC cells also documents many changes. Among these alterations, abnormal lipid metabolism is a significant aspect. On the one hand, aberrant lipid metabolism helps HCC cells obtain more energy to meet their rapid growth, proliferation, and metastasis needs. On the other hand, such altered lipid metabolism products and intermediates impact cell signal transmission and the formation of cell structures[9]. This review mainly discusses the abnormal changes in lipid metabolism in HCC and their impacts on targeted therapy and immunotherapy of HCC to explore the potential clinical application value of targeting lipid metabolism in treating HCC.

ABNORMAL LIPID METABOLISM IN HCC

In HCC, abnormal lipid metabolism mainly manifests as changes in lipid uptake and efflux, upregulation of endogenous lipid synthesis, enhanced cholesterol esterification, and changes in lipid oxidation (Figures 1 and 2). These above changes closely correlate with tumor survival, growth, proliferation, and metastasis.

Changes in lipid uptake and efflux

HCC cells can promote their growth and proliferation by increasing the uptake of extracellular fatty acids (FAs). FA uptake by cancer cells is an active transport process dependent on the fatty acid translocase (FAT, also called CD36) on the cell membrane surface[10]. CD36 is abnormally expressed in HCC and promotes tumor metastasis and epithelial-mesenchymal transformation (EMT) by increasing FA uptake, thereby promoting tumor progression[11]. There is a mixed opinion on the exogenous cholesterol uptake of HCC. In many malignant tumors, cholesterol uptake is significantly increased, related to the high expression of low-density lipoprotein receptor (LDLR)[12]. However, some studies have found that the expression of LDLR in HCC cells is significantly lower than that in peri-tumorous normal cells around the tumor, thus leading to a decrease in LDL uptake of HCC cells. The upregulation of intracellular cholesterol biosynthesis may cause this phenomenon. Interestingly, the low expression of LDLR can increase cholesterol synthesis in HCC cells by activating MEK/ERK signaling pathway [13]. Cholesterol efflux, as opposed to extracellular uptake, is also downregulated in HCC. Further, the low expression of ATP binding cassette subfamily A member 8, transporters responsible for cholesterol

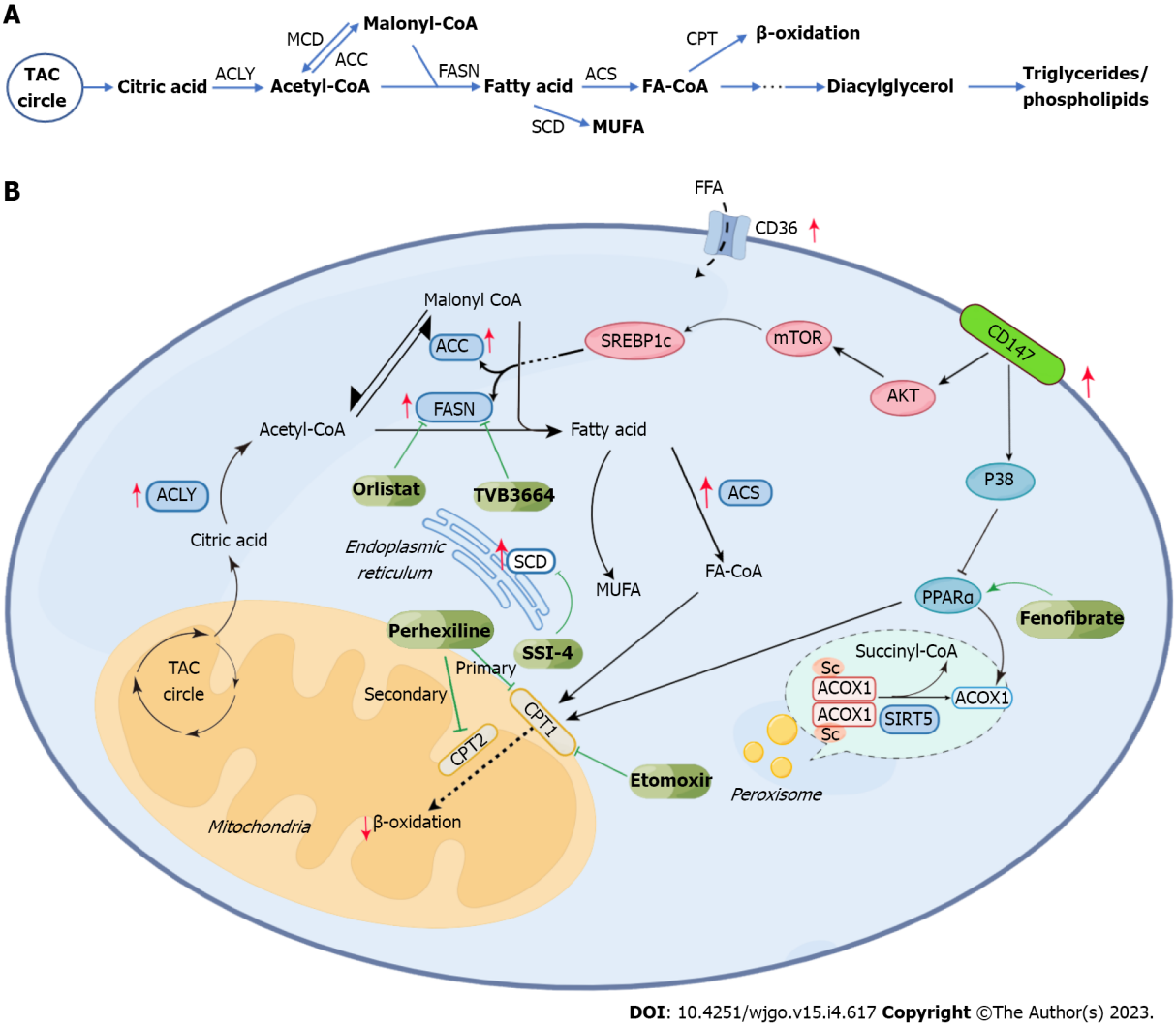

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Figure 1 Fatty acid metabolism in hepatocellular carcinoma. A: Fatty acid metabolic pathway diagram; B: Changes of fatty acid metabolism in hepatocellular carcinoma and related targets. ACC: Acetyl- CoA carboxylase; ACLY: ATP citrate lyase; ACOX: Acyl-CoA oxidase 1; ACS: Acyl-CoA synthetases; CPT: Carnitine palmitoyl transfer; FASN: Fatty acid synthase; FA-CoA: Fatty Acyl-CoA; FFA: Free fatty acids; MCD: Malonyl-CoA decarboxylase; MUFA: Monounsaturated fatty acid; PPARα: Peroxisome proliferator-activated receptor α; TAC circle: Tricarboxylic acid cycle; SCD: Stearoyl-CoA desaturase; SIRT5: Sirtuin5; SREBP1c: Sterol-regulatory element binding protein 1c.

efflux on cell membranes, in HCC is correlated with poor prognosis[14]. P53, a tumor suppressor gene, upregulates the expression of ABCA1, inhibiting sterol regulatory element binding protein (SREBP) maturation by affecting cholesterol transport[15].

Upregulation of endogenous lipid synthesis

The synthesis of FAs in HCC cells is abnormally higher, closely related to the aberrant expression of some key enzymes in this process. Synthesis of FA occurs in the cytoplasm. To begin with, citric acid generated by the tricarboxylic acid cycle forms acetyl coenzyme A (acetyl-CoA) upon the catalysis by ATP citrate lyase (ACLY). It has been reported that ACLY is highly expressed in HCC. Inhibition of ACLY can improve drug resistance to sorafenib[16]. Acetyl-CoA then forms malonyl coenzyme A (malonyl-CoA) by the action of acetyl-CoA carboxylase (ACC). Upregulation of ACC expression is significantly related to the poor prognosis of HCC. The survival of ACC-expressing rats was improved by an ACC inhibitor alone or combined with sorafenib[17]. Malonyl-CoA and acetyl-CoA form FA upon catalysis by fatty acid synthase (FASN). Overexpression of FASN promotes the carcinogenesis of HCC. TVB compounds, FASN inhibitors, can potentially treat HCC[18]. Stearoyl-CoA desaturase (SCD) then catalyzes the conversion of saturated fatty acids into monounsaturated fatty acids. HCC patients with high expression of SCD1 often have a worse prognosis[19].

In HCC, the synthesis of cholesterol is also upregulated. High cholesterol levels in the liver give rise to nonalcoholic steatohepatitis (NASH) and promote its further development into HCC[20]. Such abnormal upregulation of cholesterol synthesis is linked to some key pathways and molecules. Blocking the SREBP pathway can inhibit cholesterol synthesis and slow down the progress of HCC by inhibiting

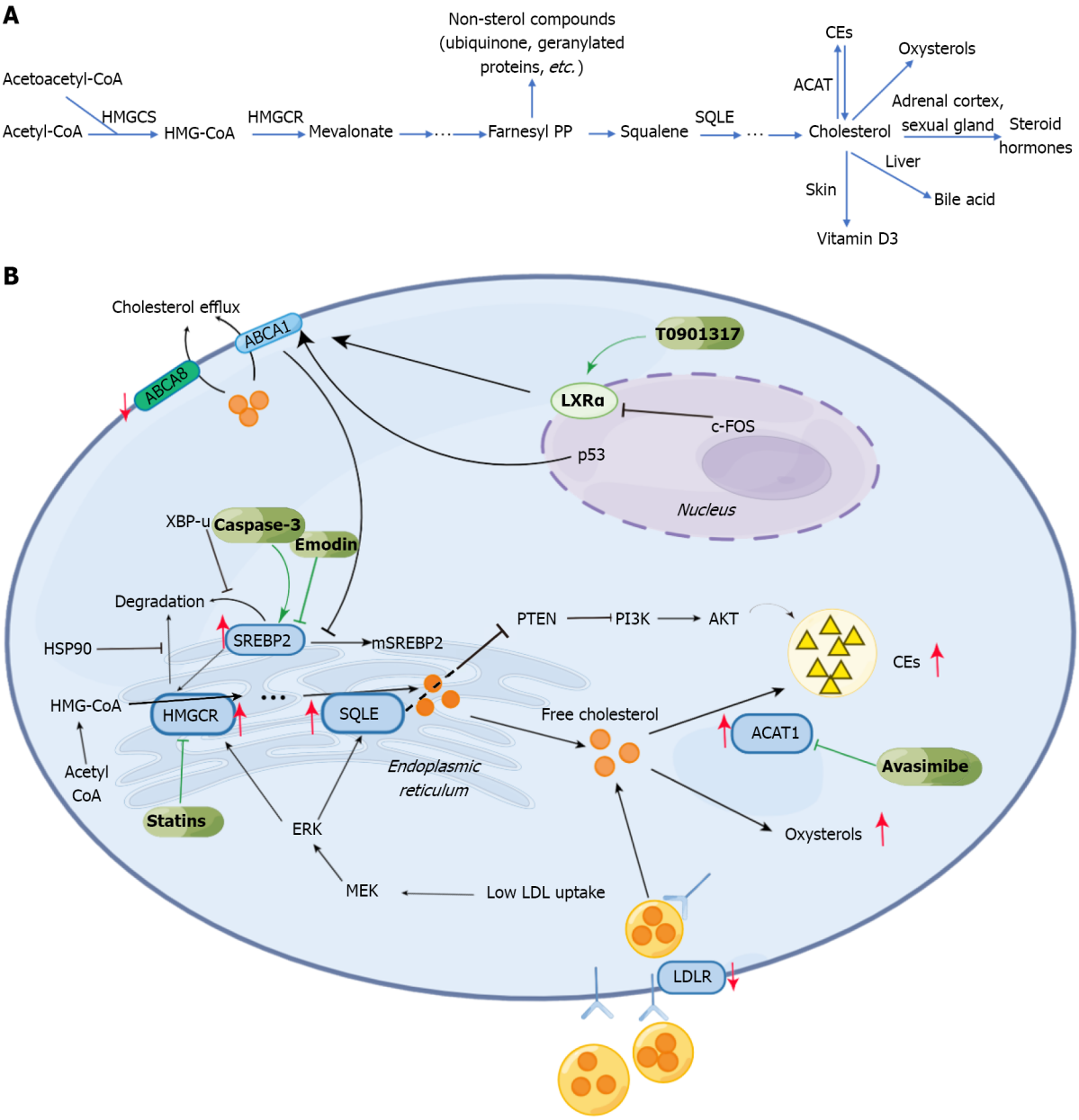

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Figure 2 Cholesterol metabolism in hepatocellular carcinoma. A: Cholesterol metabolic pathway diagram; B: Changes of cholesterol metabolism in hepatocellular carcinoma and related targets. ABCA: ATP binding cassette subfamily A; ACAT1: Acyl-CoA cholesterol acyltransferase 1; CEs: Cholesterol esters; Farnesyl PP: Farnesyl diphosphate; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS: 3-hydroxy-3-methylglutaryl-CoA synthase; HSP90: Heat shock protein 90; LDLR: Low-density lipoprotein receptor; LXR: Liver X receptor; mSREBP2: Mature sterol regulatory element binding protein 2; PI3K: Phosphatidylinositol-3-kinase; PTEN: Phosphatase and tensin homolog deleted on Chromosome 10; SQLE: Squalene epoxidase; XBP-u: The unspliced X-box binding protein 1.

inflammation[21]. 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a key enzyme in cholesterol biosynthesis, in the ER, is subjected to precise regulation by SREBP2[22]. The unspliced X-box binding protein 1 was shown to inhibit the degradation of SREBP2, which can activate the transcription of HMGCR. This pathway promotes cholesterol biosynthesis in HCC cells, contributing to tumorigenesis and progression[23]. In addition, Heat shock protein 90 can directly interact with HMGCR and inhibit its degradation from promoting the pathway of cholesterol biosynthesis and, thereby, HCC[24]. A report showed an overexpression of squalene epoxidase (SQLE), another rate-limiting enzyme in cholesterol biosynthesis, which promoted tumor cell proliferation in NAFLD-HCC[25].

Enhanced cholesterol esterification

Increased cholesterol esterification is another crucial aspect of abnormal cholesterol metabolism documented in HCC. Cholesterol esters (CEs) are a storage form of cholesterol occurring in cells and are of especially great significance for the energy supply of tumor cells[26]. Reports show the overexpression of sterol-O-acyltransferase 1 (SOAT1; also called ACAT1), a key enzyme of cholesterol esteri-

fication, as a characteristic feature that promotes tumor proliferation[27]. Interestingly, such a high expression of ACAT1 in $CD8^+$T cells inhibits their cellular functions and thereby helps tumor cell survival indirectly. The ACAT1 inhibitor, avasimibe, was found to directly inhibit HCC incidence and its progress by interfering with energy metabolism. Furthermore, it was found to boost tumor immunity by enhancing the function of cytotoxic lymphocytes[28]. Moreover, studies have shown that the loss of expression of Phosphatase and tensin homolog deleted on Chromosome 10 (PTEN) increases CE accumulation, which leads to the progress of viral hepatitis and HCC[29]. The SQLE inhibits PTEN, and its influence on CEs is closely related to the AKT pathway[25,30].

Changes in lipid oxidation

β-oxidation of FAs (FAO), occurring in the mitochondria, is significantly downregulated in HCC. Before β-oxidation, FAs must be converted into bioactive Fatty Acyl-CoA (FA-CoA) upon activation by acyl-CoA synthetases (ACS). HCCs have documented a significant overexpression of ACS, especially long-chain acyl-CoA synthetase 4 (ACSL4) and this overexpression promotes the progress of HCC[31]. The carnitine palmitoyl transfer (CPT) system is essential for FA-CoA to enter mitochondria from the cytoplasm. There are two kinds of CPT, CPT1, which is located in the outer mitochondrial membrane, and CPT2, which is located in the inner mitochondrial membrane. Overexpression of CPT1 in NASH leads to extensive activation of oncogenic signals, thereby promoting tumorigenesis and proliferation [32]. In addition, low expression of CPT2 observed in HCC leads to its escape from lipotoxicity by inhibiting the activation of JNK mediated by Src. The subsequent accumulation of acylcarnitine can promote the development of HCC by activating STAT3[33]. Acyl-CoA oxidase 1 (ACOX1) is a key rate limiting enzyme that catalyzes the oxidation of long-chain FAs. Studies have shown that loss of ACOX1 expression can promote the occurrence of HCC[34]. Medium chain acyl-CoA dehydrogenase (ACADM) is another enzyme regulating FAO. Studies have shown the significant downregulation of ACADM in HCC, which is related to the abnormal accumulation of SREBP1 caused by the increased expression of caveolin-1[35]. In addition, deacetylase sirtuin 5 (SIRT5) can inhibit the activity of ACOX1 through desuccinylation. A loss of SIRT5 expression in HCC leads to the increased activity of ACOX1 and oxidative damage of DNA[36]. CD147 is a transmembrane glycoprotein with high expression in HCC and reduces the expression of ACOX1 and CTP1A by inhibiting the MAPK pathway, thereby suppressing β-oxidation. In addition, it can also promote the expression of ACC and FASN through the AKT mTOR pathway and promote FA synthesis[37].

Besides being converted into CE for further storage, the cholesterol in cells can also be oxidized into oxysterols. Some studies have shown that c-FOS can downregulate the nuclear Liver X receptor α (LXRα), which regulates the expression level of genes related to cholesterol transport ABCA1 and hence promote the accumulation of oxysterols and HCC occurrence[38,39]. Oxysterols significantly impact the tumor microenvironment (TME), and their high level promotes immunosuppression and assists tumor metastasis[40]. For example, the accumulation of 22OHC was shown to recruit $CD11b^+Gr1^{high}$ neutrophils with immunosuppressive functions[41]. In addition, 27OHC is closely related to the depletion of cytotoxic $CD8^+$T cells[42]. 27OHC has also been found to have an apparent cancer-promoting effect in HCC, and its adverse effects could be reversed by targeting glucose-regulated protein 75[43]. 25OHC was found to induce HCC cell metastasis by upregulating fatty acid binding protein 4 (FABP4)[44].

IMPACTS OF LIPID METABOLISM ON TARGETED THERAPIES OF HCC

Effect of fatty acid metabolism on targeted therapies

The commonly employed HCC targeting drugs used for clinical treatment include sorafenib, Lenvatinib, and cabozantinib. The peroxisome proliferator-activated receptor (PPAR) signaling pathway is closely related to FA metabolism and controls FAO, synthesis, and desaturation, among other processes[45-47]. The PPAR signaling pathway can raise the stemness of tumor cells to increase the drug resistance of tumor cells to sorafenib and reduce the drug's efficacy[48]. The drug resistance of tumor-initiating cells (TIC) and sorafenib can be regulated by SCD1, which is overexpressed in HCC to regulate desaturating FAs through the PPAR pathway and regulation of endoplasmic reticulum stress. Reports show that the combined efficacy of SSI-4 (a new SCD1 inhibitor) and sorafenib was significantly better than that of sorafenib alone[49]. Sorafenib resistance is also linked to the genes cytochrome P450 family 8 subfamily B member 1 and nuclear receptor subfamily 1 group H member 3 (NR1H3, LXRα), which can induce the increased expression of SCD while retinoid X receptor β can induce the transcription of NR1H3[39,48,50]. Orlistat, a FASN inhibitor, can regulate fat metabolism by inhibiting FA synthesis, lowering the drug resistance of HCC to sorafenib, and improving drug efficacy[51]. The expression of ACSL4 can predict the sensitivity of sorafenib treatment. A higher level of ACSL4 expression was documented in a sorafenib-sensitive sample patient set than in the non-sensitive group [52]. Fatty acid transport protein-5 (FATP5/SLC27A5) is closely related to FA transport, which can inhibit the invasion, metastasis and EMT of HCC. However, this effect is the opposite as FATP5 has a low expression in HCC. The anticancer mechanism of FATP5 depends on inhibiting AMP-activated

protein kinase (AMPK); targeting the FATP5-AMPK axis is promising for individualized treatment of HCC[53]. Furthermore, some studies have shown that the combined use of sorafenib with nuclear factor E2-related factor 2 and thioredoxin reductase 1 inhibitors (brusatol and auranofin) could significantly improve the therapeutic effect in FATP5 deficient HCC cells[54].

In TICs, Toll-like receiver 4 induces NANOG (a unique homeobox transcription factor), which inhibits the mitochondrial oxidative phosphorylation (OXPHS) and activates FAO. This thereby inhibits the oxygen consumption rate and the production of reactive oxygen species, which promotes tumor proliferation and induces chemotherapy resistance in the tumors. Specifically, NANOG has a synergistic effect with PPAR in activating FAO. The drug resistance of HCC to sorafenib is significantly improved when the expression of OXPHS is restored by repressing NANOG or using the FAO inhibitor, etomoxirs [55,56]. Coiled-coil domain containing protein 25 (CCDC25) can potentially mediate liver metastasis of extrahepatic tumors[57], The low expression of CCDC25 in HCC leads to metabolic disorders, including FA alterations, and is significantly related to poor prognosis. However, a contradiction here is that HCCs with low expression of CCDC25 are more sensitive to sorafenib, lapatinib, and gefitinib. The specific mechanism here may involve the process of ferroptosis[58]. Sorafenib also influences lipid metabolism; for example, it can increase the level of erythropoietic acid (EETs) in the blood, and these EETs, in turn, affect the efficacy of sorafenib. According to a study, simultaneous docosahexaenoic acid supplements were recommended for HCC patients undergoing sorafenib treatment to augment their 19,20-erythropoietic acid levels, which can improve the therapeutic effect of sorafenib[59]. Sorafenib can induce tumor cells to upregulate glycolysis during treatment, reducing efficacy. However, the glycolysis upregulation can be inhibited by sodium butyrate (NaBu), a salt of FAs, which regulates the expression of hexokinase 2 and improve the therapeutic effect of sorafenib[60].

In addition to the efficacy as discussed above, lipid metabolism is also closely related to the adverse reactions caused by sorafenib is also related to lipid metabolism. For example, sorafenib-related adverse reactions like the hand-foot skin reaction are significantly related to the low levels of FAA and acylcarnitine in the plasma[61]. Further, patients with significant adverse reactions to sorafenib had higher levels of SREBP-1 in their tumors. The reactions to sorafenib were improved by using Betulin, the inhibitor of SREBP-1, and the curative effect of sorafenib was improved[62].

Lenvatinib significantly affects the metabolism of FAs when treating diseases. The metalloenzyme carbonic anhydrase (CA) is involved in FA biosynthesis and other metabolic processes. It is reported that lenvatinib has the potential to inhibit CA, which can be used to treat obesity[63]. Inhibition of aberrant lipid metabolism may be another potential mechanism of lenvatinib treatment. The efficacy of TVB3664, another FASN inhibitor is limited as a single drug, but it significantly improves the efficacy of cabozantinib and sorafenib in HCC treatment[18]. Tumor necrosis factor-α- induced protein 8 (TNFAIP8) is another molecule related to the metabolism of FAs. When the level of TNFAIP8 increases, it blocks the apoptosis of HCC cells to increase the tumor survival rate and increase the drug resistance of HCC to regorafenib and sorafenib[64].

The combination of atezolizumab and bevacizumab is a first-line clinical treatment for advanced HCC, and its efficacy in treating the unresectable tumor is better than sorafenib[65]. Bevacizumab is a targeted therapy drug that inhibits tumor angiogenesis by regulating vascular endothelial growth factor (VEGF). Studies have shown that the hypoxic environment induced by bevacizumab upregulates FA uptake and transport-related genes such as FABP, thereby promoting the accumulation of lipid droplets in TME, which promotes the survival of tumor cells. When lipid droplet accumulation is blocked by inhibiting FABP, the tumor growth rate decreases significantly[66]. In addition, Pan *et al*[67] found that FABP5 plays an essential role in the angiogenesis of HCC, and its mechanism is closely related to the activation of VEGF-related pathways. Regulation of lipid metabolism may improve the efficacy of anti-angiogenesis drugs such as bevacizumab[67].

Impacts of cholesterol metabolism on targeted therapy

Research has documented that cholesterol metabolism also impacts the targeted treatment of HCC. Simvastatin is a commonly used drug to reduce cholesterol, which itself has the effect of inhibiting tumor growth[68]. A report showed that simvastatin, in combination with sorafenib, significantly increases the sensitivity of HCC to sorafenib[69]. Another study showed that HCC resistance to sorafenib could be significantly addressed by cholesterol-modified agomiR-30a-5p (a miR-30a-5p mimic)[70]. The role of high levels of p90 Ribosomal S6 kinase 2 (RSK2) in promoting tumor invasion and metastasis is known. HCC is closely associated with RSK2 mutations, which activate the MAPK pathway to enhance cholesterol biosynthesis and improve tumor sensitivity to sorafenib[71]. Steroidogenic acute regulatory protein-related lipid transfer domain containing 4 (STARD4) is a key molecule mediating cholesterol transport, which promotes tumor cell proliferation and weakens the response of HCC to sorafenib. SREBP2 upregulates the expression level of STARD4. Knockdown of STARD4 or SREBP2 could increase the drug sensitivity of sorafenib-resistant HCC models to sorafenib[72].

Further, maprotiline, a noradrenergic reuptake blocker, could significantly reduce the phosphorylation level of SREBP2 through the ERK signaling pathway and decrease cholesterol biosynthesis. Hence, when combined, maprotiline increases the sensitivity of HCC to sorafenib[73]. Emodin, an active component of natural herbs, documented a significant sensitization effect on HCC treatment with sorafenib. The specific mechanism of emodin was also related to the inhibition of SREBP2 activity to

inhibit cholesterol synthesis and carcinogenic signal transduction[74]. The SREBP-cleaning activating protein (SCAP) can sense and regulate the intracellular cholesterol level[75]. Abnormal expression of SCAP will cause serum hypercholesterolemia[76], which mediates the drug resistance of the tumor to sorafenib. Hence, the therapeutic effect of sorafenib would be improved when combined with a SCAP inhibitor, lycorine[77]. The regulation of intracellular cholesterol levels also entails the vital functioning of Niemann Pick type C2 (NPC2), a type of secretory glycoprotein. Down-regulation of NPC2 leads to the accumulation of intracellular free cholesterol that activates the MAPK/AKT signal pathway to then reduce the efficacy of sorafenib on HCC. On the contrary, NPC2 overexpression would induce a stronger cytotoxicity effect of sorafenib. Sorafenib also increases NPC2 and free cholesterol levels by blocking the Raf signaling pathway[78]. Previous studies have reported lowering serum cholesterol levels during the treatment of HCC with sorafenib[79]. Using agonists of the LXR, T0901317 can improve the performance of sorafenib in HCC cells by adjusting the cholesterol efflux[80]. High-density lipoprotein binding protein (HDLBP) is a transfer protein that can avoid the excessive accumulation of intracellular cholesterol. Generally speaking, HDLBP is expected to improve the prognosis of HCC. However, the results are the opposite, as HDLBP could stabilize RAF1, which activates the MAPK signaling pathway to promote HCC progression and drug resistance to sorafenib[81].

In addition to directly impacting the efficacy of sorafenib, cholesterol can be used as a drug carrier. For instance, a study showed that the lipopolymers formed by polyethylene and cholesterol could be used to deliver sorafenib and other insoluble drugs[82]. Abnormal cholesterol metabolism is also linked to drug resistance to lenvatinib. Such aberrant metabolism often changes the activity of lipid rafts on the cell surface to influence the activity of ATP-binding cassette transporter B1 and subsequently impact lenvatinib drug resistance by enhancing exocytosis[83]. Caspase-3 can regulate the cleavage of SREBP2 to promote the synthesis of cholesterol and subsequently activate the sonic hedgehog signaling pathway to increase the drug resistance of HCC to lenvatinib[84]. Not only does cholesterol play a role in activating this signaling pathway, but so does its metabolite, 25-OHC[85].

Impacts of other aspects of lipid metabolism on targeted therapy

A study showed that small lipid nanoparticles (usLNPs) composed of several phospholipids and a highly-selective targeting peptide could efficiently deliver sorafenib to HCC in mice[86]. HCC tumorigenesis and its drug resistance have a close correlation with Sphingolipid metabolism. Sphingomyelin synthase1 (SMS1) was reported to be significantly upregulated in HCC after sorafenib treatment. This SMS1 upregulation reduces the cytotoxicity of sorafenib; hence, SMS1 inhibitor D609 significantly improves the therapeutic effect of sorafenib by lowering Ras activity[87]. Sphingosine kinase 2 (SK2) forms pro-survival sphingosine 1-phosphate (S1P). A study showed that the efficacy of sorafenib could be augmented when used with the SK2 inhibitor ABC294640[88]. Bavituximab, which targets phosphatidylserine, inhibits tumor growth by blocking tumor angiogenesis and activating antitumor immunity. The therapeutic effect of a combination of bavituximab with sorafenib was found to be significantly better than that of sorafenib alone[89]. It has been reported that the level of phosphatidylcholine in tumors changes significantly with the increase in drug resistance of tumor cells to sorafenib. This is suggestive of the potential of phosphatidylcholine to be used as a biomarker in sorafenib-resistant HCC[90]. Mitochondria can control the apoptosis of HCC cells by regulating cardiolipin (CL) oxidation. CL and its metabolites significantly increase when sorafenib is used for HCC therapy. A novel study showed the potential of combining mitochondrial CL oxidation control with targeted therapy as worth exploring for HCC treatment[91]. Sphingosine-1-phosphate receptor 1 (S1PR1) has high expression in HCC and promotes angiogenesis by down-regulating the ceramide level. As the expression of S1PR1 was shown to be lowered when lenvatinib was used to treat patients with advanced HCC, S1PR1 may have a close correlation with the antiangiogenic effect of lenvatinib[92].

Lipid metabolism is closely associated with targeted therapy (Table 1). On the one hand, altered lipid profiles and metabolism significantly impact the efficacy of current targeted therapies. On the other hand, long-term exposure to targeted therapy drugs such as sorafenib may also change the expression level of lipid metabolism related genes. Further, many molecules associated with lipid metabolism have the potential to become therapeutic targets.

IMPACTS OF LIPID METABOLISM ON HCC IMMUNOTHERAPY

Impacts of fatty acid metabolism on HCC immunotherapy

In TME, molecules related to FA metabolism and their metabolites change the state of tumor immune responses. For instance, CCDC25, which regulates FA metabolism as described above, affects HCC sensitivity to targeted therapy, the infiltration of immune cells, and the expression level of immune checkpoints. A study showed that CCDC25 is positively correlated with the infiltration of $CD8^+$T cells, macrophages, and dendritic cells, while negatively correlated with regulatory T cells (Treg) infiltration and the expression level of immune checkpoints such as PDCD1, CTLA4, and TIGIT. CCDC25 also blocked the immune escape of tumors by upregulating tumor killer cells, downregulating immunosuppressive cells, and inhibiting immune checkpoints[58].

Table 1 Impacts of lipid metabolism related reagents on the treatment of hepatocellular carcinoma

	Type of lipid metabolism	Reagents	Specific impact	Ref.
Targeted therapy	Fatty acid	SSI-4	Blocking SCD1, then enhancing the efficacy of sorafenib by regulating endoplasmic reticulum stress	[49]
		Orlistat	Improving sorafenib resistance by inhibiting FASN	[51]
		Brusatol	Inhibiting NRF2, then improving the efficacy of sorafenib by improving lipid metabolism disorder and promoting redox homeostasis	[54]
		Auranofin	Inhibiting TXNRD1, then improving the efficacy of sorafenib by improving lipid metabolism disorder and promoting redox homeostasis	[54]
		Etomoxir	Enhancing the efficacy of sorafenib by inhibiting mitochondrial fatty acid oxidation	[55]
		DHA	Enhancing the efficacy of sorafenib by improving 19,20-erythropoietic acid (19,20-EDP) level	[59]
		Sodium butyrate (NaBu)	Improving the curative effect of sorafenib by regulating the expression of hexokinase 2	[60]
		Betulin	Alleviating the adverse reaction of sorafenib and improving its efficacy by blocking SREBP-1	[62]
		TVB3664	Enhancing the efficacy of cabozantinib and sorafenib by inhibiting FASN	[18]
	Cholesterol	Simvastatin	Enhancing the efficacy of sorafenib by inhibiting cholesterol synthesis	[68-69]
		Maprotiline	Enhancing the efficacy of sorafenib by inhibiting cholesterol synthesis through reducing the phosphorylation level of SREBP2	[73]
		Emodin	Enhancing the efficacy of sorafenib by inhibiting cholesterol synthesis through inhibiting SREBP2	[74]
		Lycorine	Lowering the level of intracellular cholesterol by inhibiting SCAP, then enhancing the efficacy of sorafenib	[76-77]
		T0901317	Regulating cholesterol efflux by activating LXR, then enhancing the efficacy of sorafenib	[80]
		Caspase-3	Improve the drug resistance of HCC to lenvatinib by promoting the synthesis of cholesterol	[84]
	Other lipid metabolism	D609	Inhibiting SMS1, and then enhancing sorafenib efficacy by lowering Ras activity	[87]
		ABC294640	Reducing the formation of S1P by inhibiting SK2, then enhancing the efficacy of sorafenib	[88]
		Bavituximab	Enhancing the efficacy of sorafenib targeting tumor angiogenesis and reactivating antitumor immunity	[89]
Immunotherapy	Fatty acid	Perhexiline	Prolonging the survival of CD4+ T cell through inhibiting CPT	[103]
		Fenofibrate	Improving the efficacy of cancer vaccine through activating PPARα in other tumors (worth exploring in HCC)	[104]
	Cholesterol	Avasimibe	Enhancing the function of CD8+T cells by inhibiting ACAT1, thereby improving the therapeutic effect of PD-1 inhibitors (worth exploring in HCC)	[107]
		Simvastatin	Its combination with PD-L1 antibody effectively inhibits the proliferation of HCC	[111]

CPT: Carnitine palmitoyl transfer; FASN: Fatty acid synthase; LXRα: Liver X receptor α; PD-L1: Programmed death-ligand 1; PD-1: Programmed cell death protein 1; PPARα: Peroxisome proliferator-activated receptor α; NRF2: Nuclear factor E2 related factor 2; TXNRD1: Thioredoxin reductase 1; SCAP: The SREBP cleaning activating protein; SCD1: Stearoyl-CoA desaturase 1; SK2: Sphingosine kinase 2; SMS1: Sphingomyelin synthase1; SREBP: Sterol-regulatory element binding protein; S1: Sphingosine 1-phosphate; HCC: Hepatocellular carcinoma; DHA: Docosahexaenoic acid.

It was reported that the FASN inhibitor TVB3664 could improve the therapeutic effect of cabozantinib and sorafenib; however, its combination with PD-L1 treatment could not effectively inhibit the growth of HCC. This may be due to the specific immunosuppression of HCC[18]. As mentioned above, the combination of atezolizumab and bevacizumab is commonly used in HCC patients, and lipid metabolism impacts the efficacy of bevacizumab. Atezolizumab is a kind of ICIs, which reverses the immunosuppression of T cells by targeting PD-L1 to prevent its interaction with PD-1[93]. Liu *et al*[94] found that the expression of FABP5 on tumor-derived monocytes is negatively related to the prognosis of HCC patients because it activates the expression of PD-L1 on Treg cells through the JNK-STAT3 pathway, thereby inhibiting tumor immunity[94]. Studies have shown that the use of atezolizumab plus bevacizumab in HCC patients with hepatic steatosis has an excellent therapeutic effect, which is related to the upregulation of PD-L1 induced by high palmitic acid levels[95]. FAO is also the primary pathway by which Treg and M2 macrophages obtain energy, making it an aspect that can enhance the immunosuppressive function of Treg[96,97]. Short-chain fatty acids (SCFAs) can enhance the function of Treg and impair the functions of $CD8^+$T cells. The $CD8^+$T cells/Treg ratio could predict the therapeutic effect of PD-1 inhibitor immunotherapy. It can be inferred that SCFAs can impact the therapeutic effect of PD-1 inhibitors, such as pembrolizumab[98,99]. High levels of FAs also alter the distribution of FAs in tumors. Specifically, the uptake of FAs by cancer cells increases their uptake of FAs while $CD8^+$T cells have no increasing uptake. In addition, the expression of PD-1 on $CD8^+$T cells was significantly reduced, which affects the function of $CD8^+$T cells in TME. Hence, metabolic reprogramming by blocking FA related genes can significantly improve antitumor immunity[100]. For instance, Zhu *et al*[101] used the TCGA database to assess differential expression genes related to FA metabolism. They built a risk prediction model, which could predict not only patient prognosis, but also the therapeutic effect of anti-PD-1 immunotherapy. When patients' risk score was lower in this model, the efficacy of anti-PD-1 immunotherapy was found to be better[101]. Cheng *et al*[102] described two new HCC cell lines. On the one hand, these two cell lines have different lipid metabolism. On the other hand, they have different responses to the immune system. This research result provides a new practical tool for studying the correlation between lipid metabolism and immunotherapy in HCC[102]. The upregulation of the PPARα -mediated CPT gene could promote the apoptosis of $CD4^+$T cells by influencing FAO. The use of the CPT inhibitor, perhexiline, significantly prolonged the survival of $CD4^+$T and inhibited HCC. Therefore, targeting the CPT family may emerge as a new approach for the immunotherapy of HCC[103]. Interestingly, using fenofibrate, an agonist of PPARα, could improve the efficacy of a cancer vaccine in treating tumors, as it increased the metabolism of FAs and reduced the use of glucose in tumors. This accumulated glucose could provide energy for inducing the generation of $CD8^+$T cells by the vaccine. However, this interesting result has not yet been reported in any HCC cell line making this line of research worthy of future exploration[104].

Impacts of cholesterol metabolism on immunotherapy

Antitumor immunity mainly depends on cell-mediated immunity that mainly involves T-cell functions. The cellular signal transduction and functions of T cells depend on their membrane lipid structure[105]. Cholesterol is the critical component of membrane lipids with an involvement in the formation of vital T-cell immune synapses and the functions of the T cell receptor[106]. While the cholesterol biosynthesis or its uptake by T cells can enhance their antitumor functions, the upregulation of oxysterols in TME can significantly inhibit the function of T cells through the LXR[22]. Further, combining immunotherapies with cholesterol-esterification inhibitors is a prospective therapeutic strategy. Bioenergy utilization of $CD8^+$T cells was optimized by avasimibe, by inhibiting the cholesterol esterase of T cells from enhancing their effector functions and promoting their proliferation. A study showed that the combination of avasimibe and PD-1 inhibitor documented better efficacy than individual drug use[107]. Furthermore, avasimibe, in combination with a tumor vaccine, had an excellent therapeutic effect on other tumors [108]. Also, avasimibe can be used to optimize the adoptive cell therapies for tumors or hepatitis B virus. For example, combining avasimibe with chimeric antigen receptor-T cell therapy showed better curative efficacy[107,109]. These results make similar research also worth exploring for HCC. Statins have the effect of improving metabolism and are promising anticancer agents. Therefore, combining statins and immunotherapy, such as PD-1 inhibitors, may have surprising therapeutic effects, especially for HCC [110]. Previous research has shown that statins play a significant role in remodeling the immune microenvironment of HCC. For instance, simvastatin can inhibit the capillarization of liver sinusoidal endothelial cells to degrade the matrix environment and inhibit tumor progression by recruiting natural killer T cells. According to a recent study, simvastatin and PD-L1 antibody combination demonstrated a considerable therapeutic effect in HCC[111].

To summarize, the aberrant lipid metabolism of HCC provides energy for tumor growth, and regulates different immune cells to change their functional status, thus jointly affecting the progression of the tumor. Targeting HCC lipid metabolism to enhance tumor immunotherapy is a promising anticancer strategy (Table 1).

CONCLUSION

HCC has a high incidence rate and poor prognosis, making it a substantial medical burden globally. The search for efficient treatment strategies is ongoing. Researchers should focus their efforts on developing treatments targeting HCC's metabolic pathways. This is because many studies have shown significantly reprogrammed lipid metabolism in HCC cells compared with normal cells. Such metabolic abnormalities are related to the aberrant activation of key enzymes or related pathways of lipid metabolism. Drugs targeting several essential molecules involved in lipid metabolism have been widely studied for further treatment in HCC and have demonstrated promising therapeutic impacts. Sorafenib and lenvatinib are tyrosine kinase inhibitors widely studied and applied to clinical use. While their combination with ICIs such as PD-1 inhibitors has shown better therapeutic effects, it has not yet demonstrated satisfactory results[112]. Whether it is lipid metabolism, cholesterol metabolism or other lipid metabolism types, all these influence the efficacy of targeted therapy or immunotherapy for HCC; for example, blocking PPAR signal pathway-related molecules could improve the therapeutic effect of sorafenib[48], improving immune cell profiles and inhibiting HCC[103]. It will hence be an effective therapeutic approach to combine biological therapies targeting lipid metabolism with the existing targeted therapy and immunotherapy for HCC.

FOOTNOTES

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MINIREVIEWS

Clinical implications and perspectives of portal venous circulating tumor cells in pancreatic cancer

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Abstract

Despite recent improvements in the diagnosis and treatment of pancreatic cancer (PC), clinical outcomes remain dismal. Moreover, there are no effective prognostic or predictive biomarkers or options beyond carbohydrate antigen 19-9 for personalized and precise treatment. Circulating tumor cells (CTCs), as a member of the liquid biopsy family, could be a promising biomarker; however, the rarity of CTCs in peripheral venous blood limits their clinical use. Because the first venous drainage of PC is portal circulation, the portal vein can be a more suitable location for the detection of CTCs. Endoscopic ultrasound-guided portal venous sampling of CTCs is both feasible and safe. Several studies have suggested that the detection rate and number of CTCs may be higher in the portal blood than in the peripheral blood. CTC counts in the portal blood are highly associated with hepatic metastasis, recurrence after surgery, and survival. The phenotypic and genotypic properties measured in the captured portal CTCs can help us to understand tumor heterogeneity and predict the prognosis of PC. Small sample sizes and heterogeneous CTC detection methods limit the studies to date. Therefore, a large number of prospective studies are needed to corroborate portal CTCs as a valid biomarker in PC.

Key Words: Circulating tumor cell; Pancreatic cancer; Portal vein; Outcomes; Prognosis; Survival

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Core Tip: Circulating tumor cells (CTCs) are emerging minimally invasive biomarkers for evaluating tumor characteristics; however, limited CTCs are detected in the peripheral blood. Portal venous blood, which does not undergo hepatic filtration, can theoretically harbor a large number of CTCs and can be safely assessed using endoscopic ultrasound. The efficacy of CTCs in portal venous blood have shown encouraging results (*i.e.*, higher detection rate and better prediction of prognosis). Here, we provide an overview of CTCs in portal venous blood in the clinical context and future perspectives to enhance the role of portal CTCs as a valid biomarker in pancreatic cancer.

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INTRODUCTION

In recent decades, improvements in screening methods, surgical techniques, and the development of novel chemotherapeutic drugs have improved the prognosis of various cancers. However, clinical outcomes of pancreatic cancer (PC) remain dismal, with a 5-year survival rate of approximately 8% in the United States[1]. Several factors, including the absence of symptoms in the early stages and notoriously aggressive cancer biology, act as hurdles for the early diagnosis of PC[2,3]. Therefore, at the time of diagnosis, only 20% or fewer patients with PC are eligible for surgical resection[4]. Furthermore, the lack of a standardized assessment of perioperative recurrence risk and treatment strategies also contributes to the decreased survival rate. In a retrospective study of 957 patients with PC undergoing curative resection, 51.5% reported recurrence within one year after surgery[5]. This result suggests the presence of undetectable micrometastases in resectable PC before surgery despite extensive evaluation. Several randomized controlled trials have evaluated the effectiveness of neoadjuvant chemotherapy in patients with resectable PC; however, its efficacy has been inconsistent between studies[4]. Therefore, there is a need to discover biomarkers that may enable more precise stratification for recurrence after surgery or to determine which patients will benefit from neoadjuvant chemotherapy. However, biomarkers to identify these patients are not currently available.

Serum carbohydrate antigen 19-9 is the most commonly utilized biomarker of PC. Its value for PC is usually confined to treatment response rather than early detection or prognosis prediction because the sensitivity (80%) and specificity (75%) are not sufficient to meet the needs of clinicians[6]. Circulating tumor cells (CTCs), as part of the liquid biopsy family, are regarded as precursors of metastases[7]. CTCs have been evaluated as minimally invasive biomarkers for assessing prognostic indicators, such as progression-free survival (PFS) and overall survival (OS) in various solid tumors[8-11]. However, detecting CTCs in peripheral blood is challenging because approximately one CTC exists per billion blood cells in patients with PC[12]. Particularly in the non-metastatic status, peripheral blood specimens may have a yield too low for clinical value. A previous study revealed that CTCs have dynamic, spatiotemporal localization according to the location of the tumor; therefore, specific targeting of vascular compartments may increase the yield of CTCs[13]. Because blood drainage bypasses the liver first *via* the portal system in PC, the portal vein may be the most suitable blood vessel for CTC evaluation. The aim of this review was to describe the clinical implications and perspectives of portal venous CTCs in patients with PC.

EFFICACY AND LIMITATIONS OF CTCS IN PERIPHERAL BLOOD

CTCs are shed from the primary tumor site and can enter the vascular system, ultimately leading to metastasis in distant organs. Various methods and technologies have been introduced for CTC enrichment, isolation, and identification[2,14,15]. These techniques use the unique properties of CTCs, which have different sizes, densities, and electrical charges compared with normal blood cells[16]. Among them, the CellSearch® system (Menarini Silicon Biosystems, Huntingdon Valley, PA, United States) is the only Food and Drug Administration (FDA) approved assay method for CTC detection[2]. It relies on capturing CTCs immunomagnetically using antibodies against epithelial cell adhesion molecules, which are commonly expressed in malignant epithelial cells.

Detecting CTCs in cancerous diseases allows for the identification of high-risk patients who may require more intensive surveillance and treatment. Specifically, CTCs could be a potential prognostic indicator of chemoradiotherapy in gastrointestinal malignancies[17,18]. As with many other solid tumors, the clinical usefulness of a prognostic predictor in PC has been demonstrated in previous studies. In a recent meta-analysis of 19 studies of over 1300 patients with PC, the presence of CTCs in

the peripheral venous blood was associated with worse PFS and OS[19]. However, the paucity of CTCs in the peripheral blood considerably limits their use in various clinical settings. PC is one of the malignancies with the least number of CTCs detected by the CellSearch® method in comparison with other tumor entities[20]. Its detection rate was as low as 7%-48% at various stages of PC[2]. A recent study showed that the median number of CTCs was only 4 per milliliter in the peripheral blood of 46 patients with PC[21]. This low value has been attributed to the biophysical characteristics of CTCs and the venous drainage system of the pancreas. The average diameter of CTCs is approximately 25 μm, which is too large to allow them to enter capillary beds (8 μm in diameter)[20]. Furthermore, CTCs shed from the pancreas flow *via* the portal vein into the liver, and subsequent hepatic filtration could make the detection of CTCs in the peripheral blood very challenging[22,23]. Therefore, there is a need to discover blood sources that are abundant in CTCs theoretically.

ENDOSCOPIC ULTRASOUND-GUIDED PORTAL VEIN BLOOD SAMPLING

During surgery, portal venous blood can be collected by direct puncture of the extrahepatic portal vein with a syringe. However, non-surgical access to the portal vein is needed for individual risk stratification decision-making before neoadjuvant chemotherapy or surgery. Endoscopic ultrasonography (EUS) was initially introduced as a diagnostic imaging modality. However, the development of a linear echoendoscope in the early 1990s changed the landscape of EUS from a diagnostic to a therapeutic tool [24]. With the development of devices and accessories fit for echoendoscopes, various techniques for abdominal organs were introduced with minimal invasiveness, *e.g.*, EUS-guided fine needle aspiration (FNA), transmural drainage of pancreatic pseudocysts, EUS-guided bile duct and gallbladder drainage, EUS-guided gastrojejunostomy, and EUS-guided celiac plexus/neurolysis[25]. Furthermore, applications of EUS are not limited to visceral organs, but have also been extended to the field of vascular interventions[26]. Owing to its unique proximity and accessibility to the portal vein, various applications for EUS-guided portal vein interventions have been introduced, including EUS-guided FNA of portal vein thrombosis in hepatocellular carcinoma, portal injection chemotherapy, and measurement of portal vein pressure[27]. Importantly, the role of EUS-guided portal venous blood sampling in the detection of CTCs has recently drawn attention. Catenacci *et al*[22] first reported the feasibility and safety of EUS-guided acquisition of portal venous CTCs in patients with pancreaticobiliary cancer. After verifying the blood flow signal using Doppler ultrasound, the EUS-FNA needle was advanced transhepatically into the portal vein and blood was aspirated safely (Figure 1). It is necessary to pay close attention to the hepatic artery and bile ducts because these structures course together and can potentially lead to complications or inaccurate sampling. It is recommended to use a wide bore needle, such as a 19-G needle, for EUS-guided portal venous sampling to prevent blood clotting and CTC damage[28]. The amount of blood required for CTC isolation and identification is generally between 5 and 10 mL.

CLINICAL UTILITY OF PORTAL VENOUS CTCS

Higher detection rate and number compared to peripheral blood

The portal vein is the main drainage vessel of the pancreas, and the blood in the portal vein does not undergo hepatic filtration. Therefore, sampling from the portal vein might yield higher concentrations of CTCs than sampling from peripheral blood. Catenacci *et al*[22] first reported the feasibility and safety of EUS-guided acquisition of portal venous CTCs in 18 patients with pancreaticobiliary cancer. CTCs were detected in all portal vein samples (100%) but only 22% of peripheral blood samples. The median number of CTCs was significantly higher in samples from the portal vein than the peripheral blood (118.4 CTCs/7.5 mL *vs* 0.8 CTCs/7.5 mL, $P < 0.01$). These findings were validated in subsequent studies. In 41 patients with PC, the detection rate (58.5% *vs* 39.0%, $P = 0.02$) and number of CTCs (mean count, 313.4/3 mL *vs* 92.9/mL, $P < 0.01$) were significantly higher in samples from the portal vein than the peripheral blood[29]. Liu *et al*[23] also evaluated the detection rate and number of CTCs in the portal vein and peripheral blood of 29 patients with advanced or metastatic PC. CTCs were detected in all portal vein blood samples (100%), whereas CTCs were found in only 54% of peripheral blood samples. Furthermore, the mean count of CTCs in the portal venous blood was approximately 10 times higher than that in the peripheral blood (282.0/7.5 mL *vs* 21.0/7.5 mL, $P < 0.01$). Similar results were reported by Chapman *et al*[30]; portal venous blood demonstrated superior outcomes in both the detection rate (100% *vs* 23.5%) and enumeration (mean count, 118/7.5 mL *vs* 0.67/7.5 mL) in 17 patients with pancreaticobiliary cancers. Zhang *et al*[31] and Choi *et al*[32] reported that the number of CTCs in the portal venous blood was higher than that in the peripheral blood, whereas the detection rates were comparable between the portal and peripheral blood. In a meta-analysis by Pang *et al*[33] which included five studies that indicated patient-level data[22,23,34-36], the yield of CTCs was 7.7-fold (95%CI: 1.35-43.9) higher in the portal venous blood than in the peripheral blood.

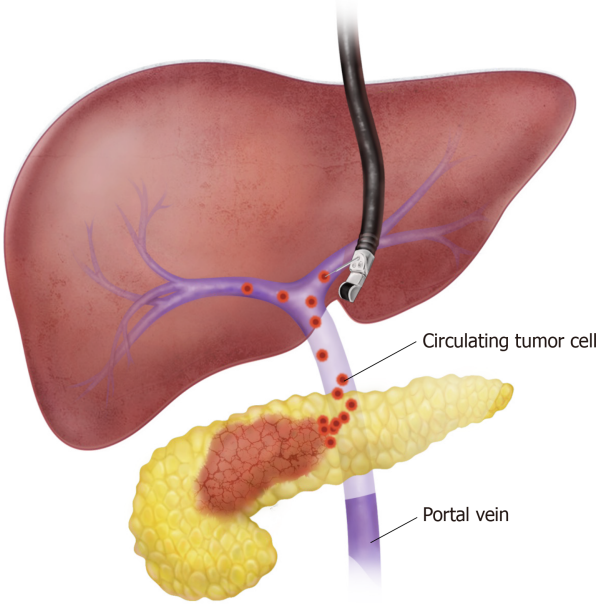

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Figure 1 Illustrated view of portal vein sampling with an endoscopic ultrasonography-fine needle aspiration needle. Circulating tumor cells (CTCs) from pancreatic cancer are released in the portal vein; portal blood sampling before passage in the liver can allow for improvement of the CTC recovery rate. The endoscopic ultrasonography-fine needle aspiration needle is advanced transhepatically into the portal vein and portal venous blood can be aspirated safely.

Some studies, however, have presented contradictory results to those of previous studies. In a study of 32 patients with resectable PC, there was no difference in the detection rate between the portal and peripheral veins (62.5% *vs* 68.8%)[37]. The number of CTCs also did not differ between the two sampling sites in this study. A study by Padillo-Ruiz *et al*[38] also reported no differences between the portal and peripheral blood in terms of the detection rate (100% *vs* 100%) and number of CTCs (median 310 *vs* 405.7, $P = 0.239$). A comparison of the CTC detection rate and number between the portal vein and peripheral blood in patients with PC is summarized in Table 1.

Correlation with tumor staging and histology

The correlation between the number of CTCs in the peripheral blood and tumor burden has been well established in various solid cancers[39]. However, only limited data are available on the correlation between portal CTCs and PC stages. Zhang *et al*[31] reported that the number of portal venous CTCs, especially the mesenchymal subtype, is positively correlated with advanced PC stages, including stages III and IV. In another study by Choi *et al*[32], a higher number of CTCs in the portal vein (≥ 3/7.5 mL) is positively correlated with advanced stages and lymph node metastasis. The association between CTCs and tumor differentiation was evaluated by Padillo-Ruiz *et al*[38] in 35 patients with resectable PC. Patients with poorly differentiated carcinoma had a larger number of CTC clusters than those with well or moderately differentiated types (median 41.0 *vs* 14.0), although the difference was not significant ($P = 0.107$).

Association with liver metastasis

Several studies have investigated the association between portal venous CTCs and liver metastasis in patients with PC. In a study by Bissolati *et al*[36] which included 20 patients who underwent surgery for PC, a greater risk of liver metastasis was observed in patients with CTCs in the portal venous blood than in those without CTCs (53% *vs* 8%, $P = 0.038$). Tien *et al*[29] reported a similar result; identification of CTCs in the portal venous blood was the only significant factor for the development of liver metastasis within 6 mo after surgery in 41 patients with resectable PC. A comparable result was also reported in a study that included locally advanced or metastatic PC[23]; patients with liver metastases demonstrated a higher mean number of CTCs than those without metastases (449.0/7.5 mL *vs* 126.0/7.5 mL, $P < 0.01$).

Correlation with PFS and OS

The role of portal venous CTCs as prognostic markers for PC has also been evaluated. According to a study by Liu *et al*[23] which included 29 patients with locally advanced or metastatic PC, OS was significantly shorter in patients with a CTC count ≥ 150/7.5 mL in portal venous blood compared to those with a CTC count ≤ 150/7.5 mL (median OS 9.2 wk *vs* 19.8 wk, $P < 0.01$). A similar result was reported by Chapman *et al*[30]; specifically, patients with portal venous CTCs ≥ 185/7.5 mL had significantly shorter PFS than patients with CTCs < 185/7.5 mL (mean PFS, 12.8 wk *vs* 43.3 wk, $P <$

Table 1 Comparison of the circulating tumor cell detection rate and number between peripheral and portal venous blood in patients with pancreatic cancer

Ref.	Patients, *N*	Cancer stage	Blood source	PoV sample	CTC isolation method	Detection rate, % (*n*/*N*)	Number of CTCs (mean ± SD)	Main findings
Catenacci *et al*[22], 2015	18	All	PoV, PV	EUS-guided	CellSearch	PoV: 100 (18/18), PV: 22.2 (4/18)	PoV: 118.4 ± 36.8/7.5 mL, PV: 0.8 ± 0.4/7.5 mL	Both the detection rate and number of CTCs were higher in the PoV than in the PV
Tien *et al* [29], 2016	41	Resectable	PoV, PV	Intraoperative	CMx platform	PoV: 58.5 (24/41), PV: 39.0 (16/41)	PoV: 313.4/3 mL, PV: 92.9/3 mL	Both the detection rate and number of CTCs were higher in the PoV than in the PV
Liu *et al*[23], 2018	29	Locally advanced, metastatic	PoV, PV	Transabdominal US-guided	ClearCell FX system	PoV: 100 (29/29), PV: 54 (8/14)	PoV: 282.0/7.5 mL, PV: 21.0/7.5 mL	Both the detection rate and number of CTCs were higher in the PoV than in the PV
Chapman *et al*[30], 2020	17[1]	All	PoV, PV	EUS-guided	CellSearch	PoV: 100 (17/17), PV: 23.5 (4/17)	PoV: 118.4 (1-516)/7.5 mL[2], PV: 0.67 (0-7)/7.5 mL[2]	Both the detection rate and number of CTCs were higher in the PoV than in the PV
Song *et al* [37], 2020	32	Resectable	PoV, PV	Intraoperative	Microfabricated Filter	PoV: 62.5 (20/32), PV: 68.8 (22/32)	Not shown	No differences in detection rate and number of CTCs between the PoV and PV
Padillo-Ruiz *et al*[38], 2021	35	Resectable	PoV, CV	Intraoperative	IsoFlux™	PoV: 100 (35/35), CV: 100 (35/35)	PoV: 310 (132.1-446.0)/mL[3], CV: 405.7 (130.7-533.8)/mL[3]	No differences in detection rate and number of CTCs between the PoV and CV
White *et al* [40], 2021	34	Resectable	PoV, PV	Intraoperative	CellSearch	PoV: 71 (22/31), PV: 50 (11/22)	Not shown	No differences in detection rate and number of CTCs between the PoV and PV
Zhang *et al* [31], 2021	31	All	PoV, PV	EUS-guided	Cyttel detection kit	PoV: 97 (31/30), PV: 87 (27/31)	PoV: 10/5 mL[4], PV: 6/5 mL[4]	Number of CTCs was higher in the PoV than in the PV
Choi *et al* [32], 2022	33	All	PoV, PV	Intraoperative	SMART BIOPSY™	PoV: 75.8 (25/33), PB: 92.1 (23/28)	PoV: 2.5/7.5 mL[4], PV: 1/7.5 mL[4]	Number of CTCs was higher in the PoV than in the PV

[1]Included two patients with cholangiocarcinoma and one with ampullary cancer.
[2]Expressed as mean (range).
[3]Expressed as median (range).
[4]Expressed as median.
PoV: Portal vein; PV: Peripheral vein; PB: Peripheral blood; CV: Central vein; CTCs: Circulating tumor cells; EUS: Endoscopic ultrasound; US: Ultrasound.

0.01). OS was also unfavorable in patients with higher counts of CTCs; however, the difference was not significant (mean OS, 29.5 wk *vs* 75.4 wk, $P = 0.07$). Moreover, a Cox-proportional hazards regression model demonstrated that every 10-cell increase in CTCs in the portal venous blood was associated with an increased likelihood of progression ($P = 0.03$) and death ($P = 0.01$) by 5% and 4%, respectively. White *et al*[40] reported the superiority of portal venous CTCs over peripheral blood for predicting survival. Thirty-one and 22 samples from the portal and peripheral veins, respectively, were collected during PC operation in 34 patients. Patients with ≥ 1 portal venous CTC/7.5 mL had an OS rate of 70% at 18 mo, whereas no deaths were reported in the absence of portal venous CTCs ($P < 0.01$). However, no correlation was observed between CTCs in the peripheral blood and OS. Similar results were validated by Choi *et al*[32]: CTCs in the portal vein, but not CTCs in peripheral blood, were a significant predictor of shorter PFS and OS. Zhang *et al*[31] also reported the prognostic value of portal venous CTCs, indicating that patients with a higher number of CTCs in the portal vein had poorer OS. The studies that analyzed the impact of portal venous CTCs on prognosis of patients with PC are summarized in Table 2.

Table 2 Clinical impact of portal venous circulating tumor cells on prognosis in patients with pancreatic cancer

Ref.	Patients, *N*	Cancer stage	Blood source	PoV sample	CTC isolation method	OS, median (95%CI)	PFS, median (95%CI)	Main findings
Bissolati *et al*[36], 2015	20	Resectable	PoV, PV	Intraoperative	CellSearch	CTCs (-): 23.1 (15.1-31.1) mo, CTCs (+): 26.2 (18.7-33.8) mo	CTCs (-): 19.4 (10.9-27.8) mo, CTCs (+): 18.9 (10.4-27.3) mo	PoV CTC-positive patients had a higher rate of liver metastases than CTC-negative patients.
Tien *et al* [29], 2016	41	Resectable	PoV, PV	Intraoperative	CMx platform	Not shown	Not shown	Number of CTCs in the PoV was the only significant risk factor of liver metastases within 6 mo after surgery.
Liu *et al* [23], 2018	29	Locally advanced, metastatic	PoV, PV	Transabdominal US-guided	ClearCell FX system	CTCs < 150/7.5 mL: 19.8 (16.8-25.4) wk, CTCs ≥ 150/7.5 mL: 9.2 (7.8-11.8) wk	Not shown	Higher CTC count in the PoV was associated with liver metastases and shorter OS.
Chapman *et al*[30], 2020	14	All	PoV, PV	EUS-guided	CellSearch	CTCs < 185/7.5 mL: 40.0 wk, CTCs ≥ 185/7.5 mL: 12.8 wk	CTCs < 185/7.5 mL: 75.4 wk, CTCs ≥ 185/7.5 mL: 29.5 wk	Every 10 CTC increase in the PoV was associated with a 5% and 4% increase in the likelihood of progression and death, respectively.
Padillo-Ruiz *et al* [38], 2021	35	Resectable	PoV, CV	Intraoperative	IsoFlux™	CTCs < 185/mL: 24.5 (19.6-29.4) mo, CTCs ≥ 185/mL: 10.0 (7.4-12.5) mo	Not shown	Higher number of CTCs in the PoV was associated with poorly differentiated cancer and shorter OS.
Pan *et al* [48], 2021	32	Resectable	PoV, PV	Intraoperative	CanPatrol™	Not shown	Not shown	Mesenchymal CTCs in the PoV ≥ 1/5 mL was a significant risk factor for metastasis, PFS, and OS.
White *et al* [40], 2021	34	Resectable	PoV, PV	Intraoperative	CellSearch	Not shown	Not shown	Patients with undetectable PoV CTCs showed a higher 18-mo survival rate (100%).
Zhang *et al* [31], 2021	31	All	PoV, PV	EUS-guided	Cyttel detection kit	Not shown	Not shown	The number of PoV CTCs, especially mesenchymal CTCs, was positively correlated with the advanced stage.
Choi *et al* [32], 2022	33	All	PoV, PV	Intraoperative	SMART BIOPSY™	CTCs < 3/7.5 mL: NA, CTCs ≥ 3/7.5 mL: 16.5 mo	CTCs < 3/7.5 mL: 13.4 mo, CTCs ≥ 3/7.5 mL: 7.5 mo	Higher number of PoV CTCs was associated with higher stage, lymph node metastasis, and poorer PFS and OS.
Song *et al* [37], 2020	32	Resectable	PoV, PV	Intraoperative	Microfabricated Filter	CTCs < 1/10 mL: 40.0 mo, CTCs ≥ 1/10 mL: 17.6 mo	Not shown	CTC count in the PoV was not significantly associated with OS.

CTCs: Circulating tumor cells; PoV: Portal vein; PV: Peripheral vein; OS: Overall survival; PFS: Progression free survival; US: Ultrasound; EUS: Endoscopic ultrasound; NA: Not achieved.

RECENT TRENDS IN PORTAL VENOUS CTC STUDIES

Phenotype analysis

There are various circulating biomarkers for liquid biopsy, including CTCs, circulating tumor deoxyribonucleic acid (ctDNA), ribonucleic acid, and extracellular vesicles[41]. The major advantage of CTCs over other circulating markers is the ability to detect whole tumor cells; this enables the identification of markers associated with prognosis beyond the enumeration of CTCs. The concept of epithelial-mesenchymal transition (EMT) is one of the most important elements in CTC phenotyping. This refers to the process by which tumor cells attached to the basement membrane gain mesenchymal properties, which finally leads to vessel invasion and induces metastasis[42]. Based on this concept, CTCs can be classified into three subpopulations with distinct properties: epithelial CTCs, mesenchymal CTCs (M-CTCs), and epithelial-mesenchymal transition CTCs (EMT-CTCs)[43] (Figure 2). Correlations between disease progression and M-CTCs in solid tumors have been reported previously[44,45]. Zhao *et al*[46] analyzed the phenotype of CTCs in the peripheral blood of 107 patients with PC. Advanced stage

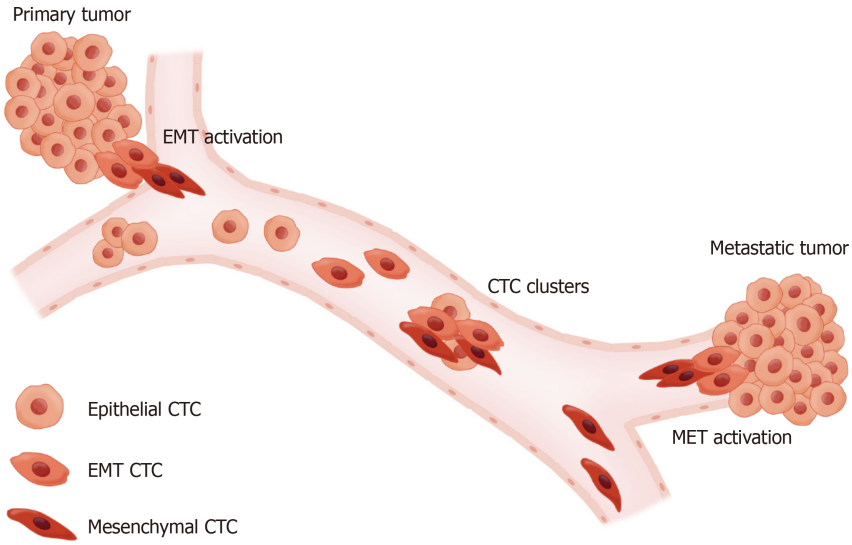

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Figure 2 Characteristic stages of circulating tumor cells during metastasis. Cells from the primary tumor undergo epithelial-mesenchymal transition, which enables them to disseminate to blood vessels. Cancer cells travel as various phenotypes of circulating tumor cells (CTCs) and extravasate the vascular system after undergoing mesenchymal-epithelial transition. This reverse process allows CTCs to escape from blood vessels into distant organs to form a metastatic tumor. CTC: Circulating tumor cell; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition.

and the presence of distant metastases were significantly associated with M-CTCs. Another study by Semaan *et al*[47] showed that prognostic variables, such as PFS and OS, were correlated with EMT-CTCs in peripheral blood but not with total CTC counts. Studies on the phenotype of CTCs have also been conducted using portal venous blood samples. According to a study by Pan *et al*[48] with 32 patients with resectable PC, M-CTCs in the portal vein were found to be a significant risk factor for metastasis-free survival and OS. Similar results were reported by Zhang *et al*[31]; in 31 patients with PC, a higher count of M-CTCs from portal venous blood was associated with advanced stage, lymph node, and distant metastases. However, this pattern was not observed in a study by Choi *et al*[32], in which no associations were observed between the phenotype of CTCs from the portal vein and prognosis. Since the abundance of CTCs in portal venous blood has been validated in previous studies, it is expected to be advantageous for phenotyping CTCs from portal venous blood. Further prospective studies with larger numbers of patients are warranted to evaluate the clinical efficacy of CTC phenotyping using portal venous samples.

Genotype analysis

Genotyping tumors for the identification of genetic mutations has become a routine practice for evaluating patients with certain solid-type cancers[49,50]. However, genotyping from primary tumor tissue has inherent limitations in that single tissue collection has a risk of selection bias from tumor heterogeneity, and acquiring tissue is not always feasible[51]. Therefore, genotypic analysis of CTCs has been conducted to reflect the genomics of primary tumors. In patients with PC, concordance of *KRAS* mutations in CTCs, which presents in over 90% of PC cases, has been reported repeatedly compared to primary pancreatic tumors[22,37,52]. However, a discordant rate of 42% for *KRAS* mutations between CTCs and primary tumors was also reported in 59 patients with PC[53]. These conflicting results may represent the natural evolution of metastatic tumors triggered by genomic instability and heterogeneity within the primary tumor[54]. Therefore, testing for other genetic mutations, such as *TP53, SMAD4,* and *P16,* which are commonly observed in PC, might be needed to confirm tumor identity[55]. Although the utility of ctDNA for tailored therapies or predicting response has been explored in many studies[56], only limited data regarding genotyping with CTCs for personalized medicine in PC are available to date. Yu *et al*[57] reported that an increase in *SMAD4* expression levels in CTCs was associated with longer PFS and favorable treatment response in 37 patients with PC treated with gemcitabine/nab-paclitaxel. Recently, the FDA approved high-throughput next-generation sequencing-based multigene biopsy that can detect genomic mutations and polymerase chain reaction-based single-gene or multigene assays[58]. Further studies are needed to validate the clinical efficacy of the newly developed genotyping techniques for tailored therapy in patients with PC.

FUTURE PERSPECTIVES

Several unmet needs, including early detection, preoperative risk stratification, and the development of effective and personalized chemotherapeutic agents, should be addressed to improve the prognosis of PC. Recently, the theory of a "three-step procedure" for pancreatic carcinogenesis has been widely adapted to the clinical field, giving rise to the opportunity for early diagnosis and intervention over a long period of time[14]. PC screening relies only on cross-sectional imaging or is accompanied by EUS-guided tissue acquisition in high-risk individuals[59]. To date, there have been no reports regarding the usefulness of CTCs for the early detection or screening of PC. CtDNA, another family of liquid biopsy, has been found to be useful for early PC detection with 64% sensitivity and 99% specificity when combined with well-selected plasma proteins[60]. A relevant study by Cohen *et al*[60]. to explore the role of CTCs in the early diagnosis of PC is registered and ongoing (ClinicalTrials.gov, NCT0207616). Further studies with larger numbers of patients are warranted to evaluate the clinical efficacy of CTCs in this field. The high number of CTCs in the peripheral blood of patients with lung and breast cancer makes CTC-guided tailored therapy more feasible to study[61-64]. By contrast, the small number of CTCs in peripheral blood is a concern for further studies on CTCs in PC. Therefore, the abundance of CTCs in portal venous blood may play a crucial role in resolving various clinical issues in the future.

Another noteworthy point is the method used to sample CTCs from the portal vein. As described in Tables 1 and 2, intraoperative sampling of portal venous blood rather than the EUS-guided approach has been more dominant. This may mean that patients who undergo portal vein sampling intraoperatively lose the opportunity to be assessed for risk of recurrence before surgery. In the future, EUS-guided portal vein CTC sampling, which preserves the patient's normal anatomy, should be performed more widely. Standardization of CTC isolation techniques and the development of new assays that can provide clinicians with comprehensive insight into CTC heterogeneity should be investigated further.

CONCLUSION

CTCs have emerged as a new biomarker for various solid cancers over the past decade. However, their role, especially in the peripheral blood, has been limited in PC. Previous midsize studies demonstrated promising results of CTCs from the portal venous blood with a higher detection rate and better prognosis prediction than those by conventional CTC research from the peripheral blood. In the future, studies with larger numbers of patients are needed to establish the role of CTCs from the portal venous blood in early detection, risk stratification of postoperative recurrence, prediction of treatment resistance, and identification of tumor-specific biomarkers for developing targeted chemotherapeutic agents.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Comprehensive analysis of prognostic value and immunotherapy prospect of brain cytoplasmic RNA1 in hepatocellular carcinoma

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Abstract

BACKGROUND

The expression of brain cytoplasmic RNA1 (*BCYRN1*) is linked to the clinico-pathology and prognosis of several types of cancers, among which hepatocellular carcinoma (HCC) is one of the most frequent types of cancer worldwide.

AIM

To explore the prognostic value and immunotherapeutic potential of *BCYRN1* in HCC by bioinformatics and meta-analysis.

METHODS

Information was obtained from the Cancer Genome Atlas database. First, the correlation between *BCYRN1* expression and prognosis and clinicopathologic characteristics of HCC patients was explored. Univariate and multivariate regression analyses were employed to examine the relationship between *BCYRN1* and HCC prognosis. Secondly, potential functions and pathways were explored by means of enrichment analysis of differentially-expressed genes. The relationships between *BCYRN1* expression and tumor microenvironment, immune cell infiltration, immune checkpoint, drug sensitivity and immunotherapy effect were also investigated. Finally, three major databases were searched and used to conduct a meta-analysis on the relationship between *BCYRN1* expression and patient prognosis.

RESULTS

BCYRN1 expression was significantly higher in HCC compared to normal tissues and was linked to a poor prognosis and clinicopathological characteristics. Enrichment analysis showed that *BCYRN1* regulates the extracellular matrix and transmission of signaling molecules, participates in the metabolism of nutrients, such as proteins, and participates in tumor-related pathways. *BCYRN1* expression was linked to the tumor microenvironment, immune cell infiltration, drug sensitivity and the efficacy of immunotherapy. Furthermore, the meta-analysis in this study showed that *BCYRN1* overexpression was related to a worse outcome in HCC patients.

CONCLUSION

Overexpression of *BCYRN1* relates to poor prognosis and may be a potential prognostic factor and immunotherapeutic target in HCC.

Key Words: Brain cytoplasmic RNA1; Immunotherapy; Prognostic; Biomarker; Hepatocellular carcinoma

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Core Tip: In this study, we combined the research methods of meta-analysis and bioinformatics analysis to comprehensively analyze and explore the prognostic value of brain cytoplasmic RNA1 (*BCYRN1*) in hepatocellular carcinoma (HCC) and the prospects of immunotherapy. Our study found that overexpression of *BCYRN1* was significantly associated with poor prognosis in HCC patients and may be an independent prognostic factor for HCC and a target for immunotherapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and has a high mortality rate[1]. Relevant statistics have shown that the number of new liver cancer cases worldwide was about 9 million in the year 2020, of which the most common type was HCC. HCC is the third-leading cause of cancer-related deaths worldwide, with a 5-year survival rate of less than 20%[2,3]. Early detection of HCC can achieve a good 5-year survival rate by surgical treatment[4], liver transplantation and radiotherapy. However, the symptoms of patients with early HCC are not apparent. Therefore, most patients are diagnosed in the advanced stage. Since only systemic chemotherapy can delay progression, the prognosis is very poor[5]. Therefore, early diagnosis and treatment is particularly important. Identifying novel sensitive tumor markers and discovering novel molecular therapeutic targets are key goals, and exploring novel immunotherapeutic drugs is another breakthrough[6,7].

With the progress of high-throughput sequencing technology, research on long non-coding RNA (lncRNA) has developed rapidly in the field of bioinformatics, especially in oncology[8]. LncRNAs are generally longer than 200 nucleotides and do not encode proteins[9]. An increasing number of studies have shown that lncRNAs can influence gene expression at translational and transcriptional levels by chromatin remodeling, affecting RNA splicing and controlling the transmission of signaling pathways [10,11]. In addition, lncRNA affects tumor and immune cell metabolism, remodeling of the immune microenvironment and promoting carcinogenesis[12]. Abnormal expression of lncRNAs can affect biological processes, including tumor cell growth, migration, invasion, angiogenesis and metastasis,

which are linked to the occurrence and prognosis of various types of cancer and has a broad research prospect[13,14].

Brain cytoplasmic RNA1 (*BCYRN1*), also known as brain cytoplasmic 200 (BC200), is mainly present in neurons, and abnormal expression of *BCYRN1* is associated with neurodegenerative diseases and malignant tumors[15]. It has previously been shown that compared with surrounding normal tissues, *BCYRN1* is overexpressed in a number of cancer types[16,17], including gastric cancer[18,19], bladder cancer[20,21], colorectal cancer[22-25] and HCC[26-28]. Furthermore, *BCYRN1* overexpression is closely related to poor patient prognosis[15]. Therefore, *BCYRN1* is a potential cancer therapeutic target and tumor prognostic marker[29]. To further clarify the prognostic value of *BCYRN1* in HCC and the prospects of immunotherapy, data related to HCC was obtained from the Cancer Genome Atlas (TCGA), and bioinformatics analysis was performed. First, expression levels of *BCYRN1* were explored in normal liver tissues and HCC and differences in survival prognosis between the low and high expression groups of *BCYRN1* were analyzed. Second, the relationship between the *BCYRN1* expression level and clinicopathologic characteristics in HCC patients was investigated. Univariate and multivariate Cox regression analysis and nomogram prognostic models for HCC were performed. In addition, co-expressed genes and differentially expressed genes (DEGs) related to *BCYRN1* were screened, and enrichment analysis was employed to further investigate the possible pathways and functions of *BCYRN1*. The correlations between *BCYRN1* expression and tumor microenvironment (TME), immune cell infiltration, immune checkpoints, tumor mutation burden (TMB), chemosensitivity and immunotherapy efficacy were investigated. Finally, to validate the prognostic value of *BCYRN1* in HCC, a relevant literature search and meta-analysis were performed.

MATERIALS AND METHODS

Data download

Gene expression data and information on clinical features from 50 normal tissue samples and 374 liver hepatocellular carcinoma (LIHC) samples were obtained from the TCGA database (https://portal.gdc.cancer.gov/). Subsequent analyses and mapping were performed on this dataset. Because the TCGA database is freely accessible, ethics committee approval was not required for this study.

Expression of BCYRN1 in pan-carcinoma and HCC

A uniformly normalized pan-cancer dataset was downloaded using the University of California Santa Cruz database (https://xenabrowser.net/), expression information of *BCYRN1* in every sample was obtained, and the expression difference plots of *BCYRN1* in pan-cancer was mapped through the online bioinformatics analysis website Sangerbox (http://sangerbox.com/, based on TCGA and GTEx databases). R software (R 4.1.3 version) was utilized to analyze the expression differences of *BCYRN1* in HCC using R packages "limma," "ggplot2" and "ggpubr." Boxplots were plotted, and difference plots of expression differences were paired.

Association between BCYRN1 expression and HCC patient survival prognosis

The online database GEPIA2 (http://gepia2.cancer-pku.cn) was used to evaluate the relationship between *BCYRN1* gene expression levels and survival prognosis of HCC patients. Second, the data downloaded from TCGA were analyzed using the "survival" and "survminer" R packages, and Kaplan-Meier (KM) curves were plotted to show the association of *BCYRN1* expression with progression-free survival (PFS) and overall survival (OS). Finally, receiver operating characteristic (ROC) curves were plotted to assess the predictive value of *BCYRN1* expression for different prognosis years using the "timeROC" R package.

Association between BCYRN1 and clinicopathologic features of HCC

The associations between *BCYRN1* and HCC clinicopathology (age, gender, tumor stage, T stage, M stage, histological grade) were analyzed using the "limma" and "ggpubr" R packages using previously downloaded clinical information from the TCGA database. Finally, a heatmap of clinical relevance was plotted using the R package "ComplexHeatmap."

Analysis of independent prognostic factors and establishment of prognostic model

First, the R package "survival" was used to accomplish univariate regression analysis and multivariate COX regression analysis of the expression of *BCYRN1* with clinicopathological information and survival information of HCC patients. Then, forest plots were drawn to determine independent prognostic indicators of HCC. Second, the R packages "survival," "rms" and "regplot" were used to summarize the clinical and prognostic information of patients. Nomograms were drawn to predict 5-year, 3-year and 1-year OS rates of HCC patients. To evaluate the accuracy of the prediction model, a calibration curve of the nomogram was drawn.

Gene co-expression and grouping DEG analysis of BCYRN1

In this study, co-expression analysis was performed using the "Pearson" method with four R packages, "limma," "ggplot2," "ggpubr" and "ggExtra." A certain index was set to screen focused co-expressed genes (correlation coefficient > 0.5, $P < 0.001$) after which visual analysis was performed. Twelve genes with the strongest correlation were selected from the co-expressed genes, and the co-expression circles were plotted using the "circlizeand" and "corrplot" R packages. Finally, *BCYRN1* expression was divided into low and high expression groups using the R package "limma," and screening conditions were set (false discovery rate < 0.05 and log2 foldchange > 1). Next, the DEGs in the groups were screened, and 50 upregulated and downregulated genes were selected, visualized using the "pheatmap" R package. A heat map of differential expression was drawn.

Enrichment analysis of BCYRN1-associated DEGs

To explore the possible enriched signaling pathways and related functions, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of *BCYRN1*-related DEGs were conducted using a series of R packages ("clusterProfile," "org.Hs.eg.db," "circlize," "RColorBrewer," "enrichplot," "dplyr," "ComplexHeatmap"). The filter condition *P* value filter was set to 0.05. In this process, a histogram, bubble diagram and GO circle diagram were drawn. Finally, the possible enriched functions and pathways of *BCYRN1*-associated DEGs were further explored using HCC using Gene Set Enrichment Analysis (GSEA).

Correlation analysis between BCYRN1 expression in HCC and TME and immune cell infiltration

Previously downloaded HCC-related expression data were entered, the scores of immune cells and stromal cells of HCC-related samples were calculated utilizing the "limma" and "estimate" R package, and the scores of the two cells were added to obtain a comprehensive score "ESTIMATEScore." The "reshape2" and "ggpubr" R packages were used to plot the resulting TME scores into violin plots for TME difference analysis between low and high *BCYRN1* expression groups. The R packages "limma" and "CIBERSORT" were used to compute the percentage composition of 22 immune cells in each sample. Next, the "reshape2," "vioplot" and "ggExtra" R packages were used to draw boxplots and correlation scatterplots of immune cell differences. Finally, lollipops were plotted according to the sorted correlation results.

Correlation analysis between BCYRN1 and immune checkpoint genes and TMB

Immune checkpoint genes associated with *BCYRN1* were selected using the "reshape2," "ggplot2," "ggpubr" and "corrplot" R packages. A correlation coefficient of $P < 0.001$ was set as the screening filter based on the downloaded gene expression data and immune checkpoint-related gene lists of HCC samples, and circular correlation heat maps and rectangular correlation heat maps were plotted, respectively. Then, the R package "ggExtra" was utilized to explore the correlation between the expression of *BCYRN1* and TMB based on genetic tumor mutation load files, and scatter plots of the correlation were drawn.

Correlation analysis of BCYRN1 expression with drug sensitivity and immunotherapy

The R package "pRRophetic" was utilized to compute the half-maximal inhibitory concentration (IC_{50}) of the drug based on downloaded gene expression data, and $P = 0.001$ was set as a filtering condition. Next, the calculation results were visualized as a difference boxplot using the R packages "ggplot2" and "ggpubr". The corresponding IC_{50} differences between the low and high *BCYRN1* expression groups were assessed from the boxplots to determine the sensitivity differences of anticancer drugs. Next, HCC-related immunotherapy-related data were downloaded from the Cancer Immunome Atlas database (https://tcia.at/). The scores of immunotherapy in the low and high expression groups of *BCYRN1* were analyzed using the "limma" and "ggpubr" R packages, and differential violin plots were drawn.

Meta-analysis search strategy

Searches were conducted as required by PRISMA guidelines[30]. With a cutoff date of October 2022, three English databases, Web of Science, PubMed, and EMBASE, were used to search for relevant studies on *BCYRN1* in HCC. The search keywords used were: ("BCYRN1" OR "BC200a" OR "LINC00004" OR "BC200" OR "Brain cytoplasmic RNA1") AND ("Hepatocellular carcinoma" OR "HCC" OR "Liver cancer"). Two authors independently performed the search, and disagreements were resolved by discussion.

Inclusion and exclusion criteria

Inclusion criteria: (1) Patients diagnosed with HCC; (2) The target gene studied was *BCYRN1*; (3) The expression level of *BCYRN1* was detected by quantitative real-time polymerase chain reaction (qRT-PCR); and (4) According to the expression level of *BCYRN1* in HCC tissues, patients were separated into a low expression group and a high expression group. Survival hazard ratios (HRs) and the 95%CI were

obtained by KM curves or multivariate regression analysis. Exclusion criteria: (1) Repeated studies; (2) The disease type was not HCC; (3) The target gene investigated was not *BCYRN1*; (3) The types of studies were reviews, meta-analyses, conference abstracts, letters and case reports; (4) Articles that did not focus on survival prognosis and focused on biological functions or mechanisms; and (5) Lack of HR or KM survival curves.

Data extraction and quality evaluation of included literature

The following information was extracted from the literature: First author's name, country or region, publication time, sample size, method of RNA detection, cutoff values for high and low expression, source of HR values, HR with 95%CI and follow-up time. If the HR of *BCYRN1* was acquired by multivariate regression analysis in the study, it was extracted directly. Otherwise, it was indirectly derived by utilizing the Engauge Digitizer tool program from KM survival curves. Articles included were graded according to the Newcastle-Ottawa scale (NOS) and were considered eligible if they scored 6 or higher.

Statistical analysis

Stata12.0 software was used to analyze the extracted data. Forest plots were plotted to combine the extracted HR and 95%CI, and heterogeneity was assessed across studies by calculating I^2 values. HR was combined using a fixed effects model if I^2 was less than 50%, thereby indicating that no obvious heterogeneity existed between studies, and a random effects model if I^2 was greater than or equal to 50%. Begg's test was used to evaluate publication bias, and sensitivity analysis was performed to investigate the stability of the results. $P < 0.05$ was considered statistically significant.

RESULTS

Differential expression analysis of BCYRN1 in pan-carcinoma and HCC

BCYRN1 expression was explored in pan-cancer, and it was discovered that *BCYRN1* expression was significantly higher in 17 tumor tissues compared to normal tissues, including breast invasive carcinoma (BRCA), lung adenocarcinoma (LUAD), esophageal cancer (ESCA), stomach and esophageal carcinoma (STES), stomach adenocarcinoma (STAD), lung squamous cell carcinoma (LUSC), LIHC, Wilms' tumour (WT), skin cutaneous melanoma (SKCM), ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), testicular germ cell tumors (TGCT), uterine carcinosarcoma (UCS), acute lymphoblastic leukemia (ALL), acute myeloid leukemia (LAML), kidney chromophobe (KICH) and cholangiocarcinoma (CHOL). In contrast, *BCYRN1* expression was significantly lower in seven tumor tissues compared to normal tissues, including glioblastoma multiforme (GBM), glioma (GBMLGG), kidney renal papillary cell carcinoma (KIRP), pan-kidney cohort (KIPAN), kidney renal clear cell carcinoma (KIRC), thyroid carcinoma (THCA), and adrenocortical carcinoma (ACC) (Figure 1A). Then, *BCYRN1* expression was explored in HCC, and the difference analysis boxplot (Figure 1B) showed that *BCYRN1* expression in HCC tissues was significantly ($P < 0.01$) higher compared to that in normal liver tissues. These findings were consistent with the result of paired difference analysis of samples ($P < 0.001$) (Figure 1C).

Correlation between prognosis and BCYRN1 expression in HCC patients

The relationship between *BCYRN1* expression and the OS and disease-free survival (DFS) of HCC patients was investigated by the GEPIA2 database. The findings showed that overexpression of *BCYRN1* was associated with a worse OS ($P = 0.0047$; Figure 2A) and DFS ($P = 0.0075$; Figure 2B) in HCC patients. Based on the TCGA database, KM survival prognosis curves were drawn, and the results showed that patients with high expression of *BCYRN1* had a worse OS ($P < 0.001$; Figure 2C) and PFS ($P = 0.025$; Figure 2D). Finally, plotted ROC curves (Figure 2E) showed that the expression of *BCYRN1* was highly predictive for the 5-year prognosis of HCC patients.

Correlation between BCYRN1 expression and clinicopathological characteristics of HCC patients

By analyzing the connection between *BCYRN1* expression and the clinicopathological characteristics of HCC patients, it was discovered that *BCYRN1* expression was not significantly correlated with age ($P = 0.26$; Figure 3A), sex ($P = 0.65$; Figure 3B) and M stage ($P = 0.17$; Figure 3F) of patients. A significant correlation was observed with pathological grade (G1 *vs* G2 and G1 *vs* G3, $P < 0.05$; Figure 3C), clinical stage (stage 1 *vs* stage 2, stage 1 *vs* stage 3 and stage 1 *vs* stage 4, $P < 0.05$; Figure 3D) and T stage (T1 *vs* T2, T1 *vs* T3 and T1 *vs* T4, $P < 0.05$; Figure 3E) of patients. In addition, a heat map (Figure 3G) was associated with clinicopathological features, and a significant relationship was observed between *BCYRN1* expression and the pathological grade ($P < 0.05$), clinical stage ($P < 0.001$) and T stage ($P < 0.0001$) of patients.

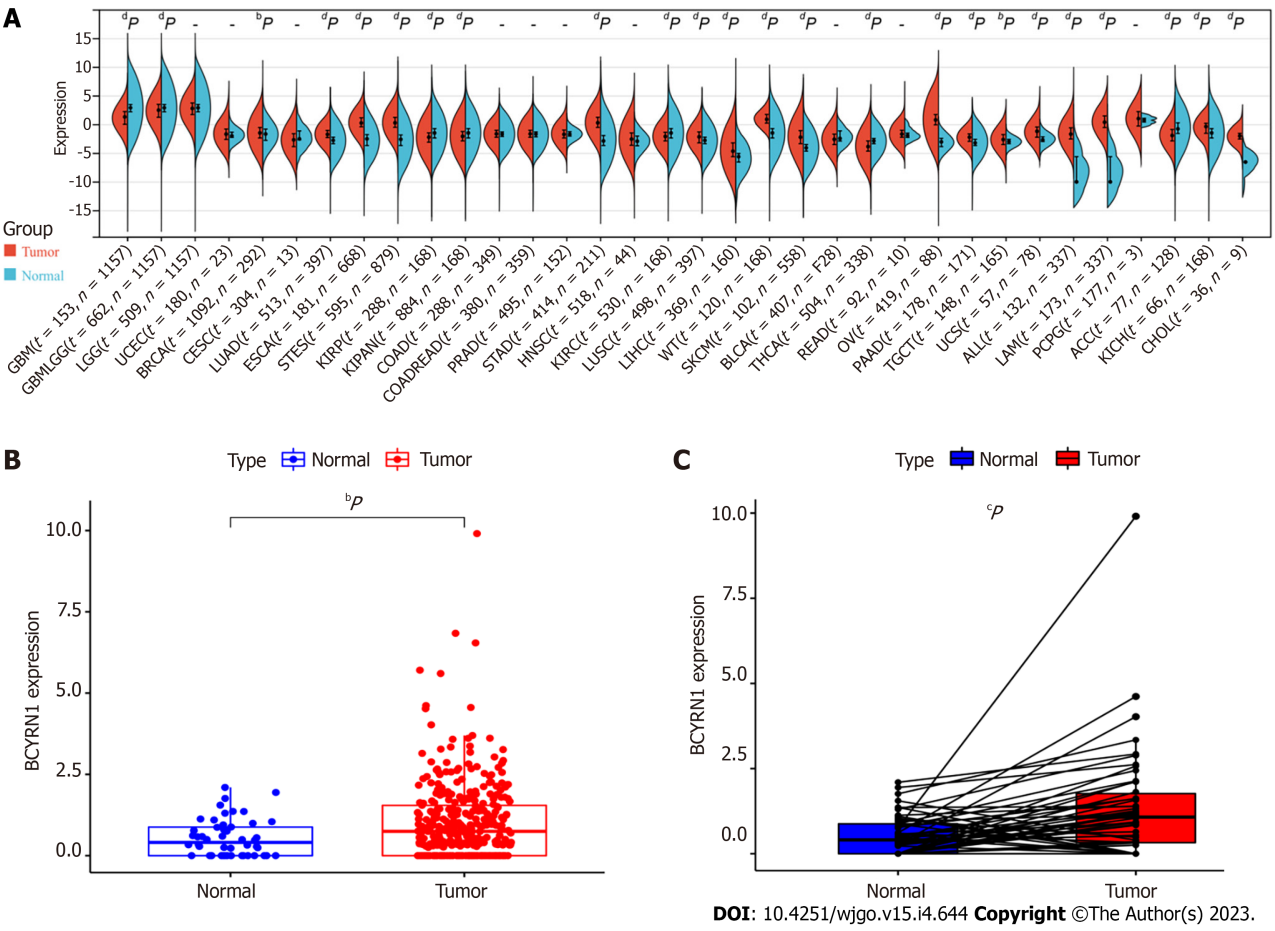

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Figure 1 Expression levels of brain cytoplasmic RNA1 in pan-cancer and hepatocellular carcinoma. A: Expression level of brain cytoplasmic RNA1 (*BCYRN1*) in pan-carcinoma; B: Differential analysis of expression of *BCYRN1* in hepatocellular carcinoma (HCC) tissues compared with normal liver tissues based on the Cancer Genome Atlas database; C: Pairwise difference analysis of *BCYRN1* expression between HCC tissues and surrounding normal liver tissues from the same patient. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$.

Analyses of independent prognostic markers for survival and establishment of nomogram prediction model

To confirm the prognostic significance of *BCYRN1* in HCC patients, univariate (Figure 4A) and multivariate regression analyses (Figure 4B) of prognostic markers in HCC patients were completed. The results of univariate and multivariate prognostic analysis were consistent, thereby suggesting that the expression of *BCYRN1* (HR = 1.16, $P = 0.038$) and the clinical stage (HR = 1.543, $P < 0.001$) of the tumor were significantly associated with OS in patients and could be used as independent prognostic markers for HCC. A nomogram prediction model (Figure 4C) for survival prediction in HCC patients was constructed using clinicopathological information and survival prognosis of patients. According to the clinicopathological information and the expression level of *BCYRN1*, the 5-year, 3-year and 1-year survival rates of patients can be predicted. Finally, calibration curves were drawn (Figure 4D) to evaluate the accuracy of the prediction model. Because the inclination of the prediction curves was close to the diagonal, the prediction model was reliable and accurate.

Analysis of co-expressed vs DEGs for BCYRN1 in HCC

An analysis of co-expressed genes of *BCYRN1* was performed. Among them, co-expressed genes that satisfied "$P < 0.001$ and correlation coefficient > 0.5" were screened to obtain a total of eight genes, and the co-expression correlation scatter plot was plotted (Supplementary Figure 1). The co-expression results were used to select the 11 genes that most closely were related to co-expression, and a co-expression circle plot was drawn (Figure 5A), in which red represented a positive correlation (*LGALS1*, *TMSB4XP4*, *TMSB10*, *CCL26*, *S100A11*, *IMPDH1*), green represented a negative correlation (*PAH*, *SLC2A2*, *F12*, *HNF4A*, *CPB2*), and the shaded area represented the magnitude of the correlation. Finally, the DEGs between groups with high and low *BCYRN1* expression were explored, and 50 DEGs were selected that were most significantly upregulated and downregulated. Finally, a heat map of DEGs was drawn (Figure 5B).

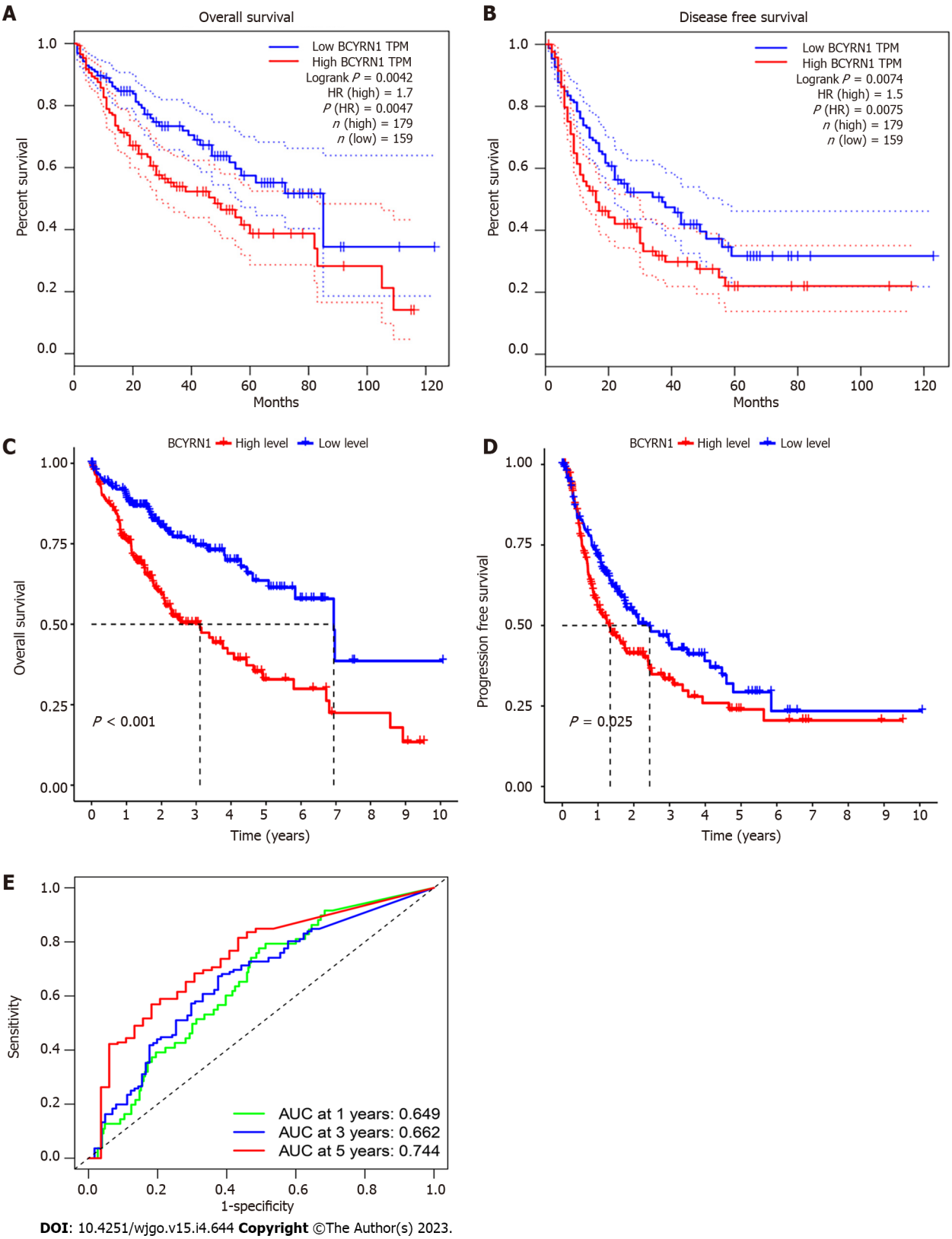

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Figure 2 Association of brain cytoplasmic RNA1 expression with prognosis in hepatocellular carcinoma patients. A and B: Association of brain cytoplasmic RNA1 (*BCYRN1*) expression with overall survival (A) and disease-free survival (B) in hepatocellular carcinoma (HCC) patients in the GEPIA2 database; C and D: *BCYRN1* expression in the Cancer Genome Atlas database in relation to overall survival (C) and progression-free (D) survival in HCC patients; E: Receiver operating characteristic curve to assess the predictive value of *BCYRN1* expression with different prognostic years. AUC: Area under the curve; HR: Hazard ratio; TPM: Transcripts per million.

GO, KEGG and GSEA enrichment analysis

A total of 1453 (GO) and 622 (KEGG) DEGs associated with *BCYRN1* were screened using HCC expression data from the TCGA database. Next, these DEGs were subjected to GO and KEGG

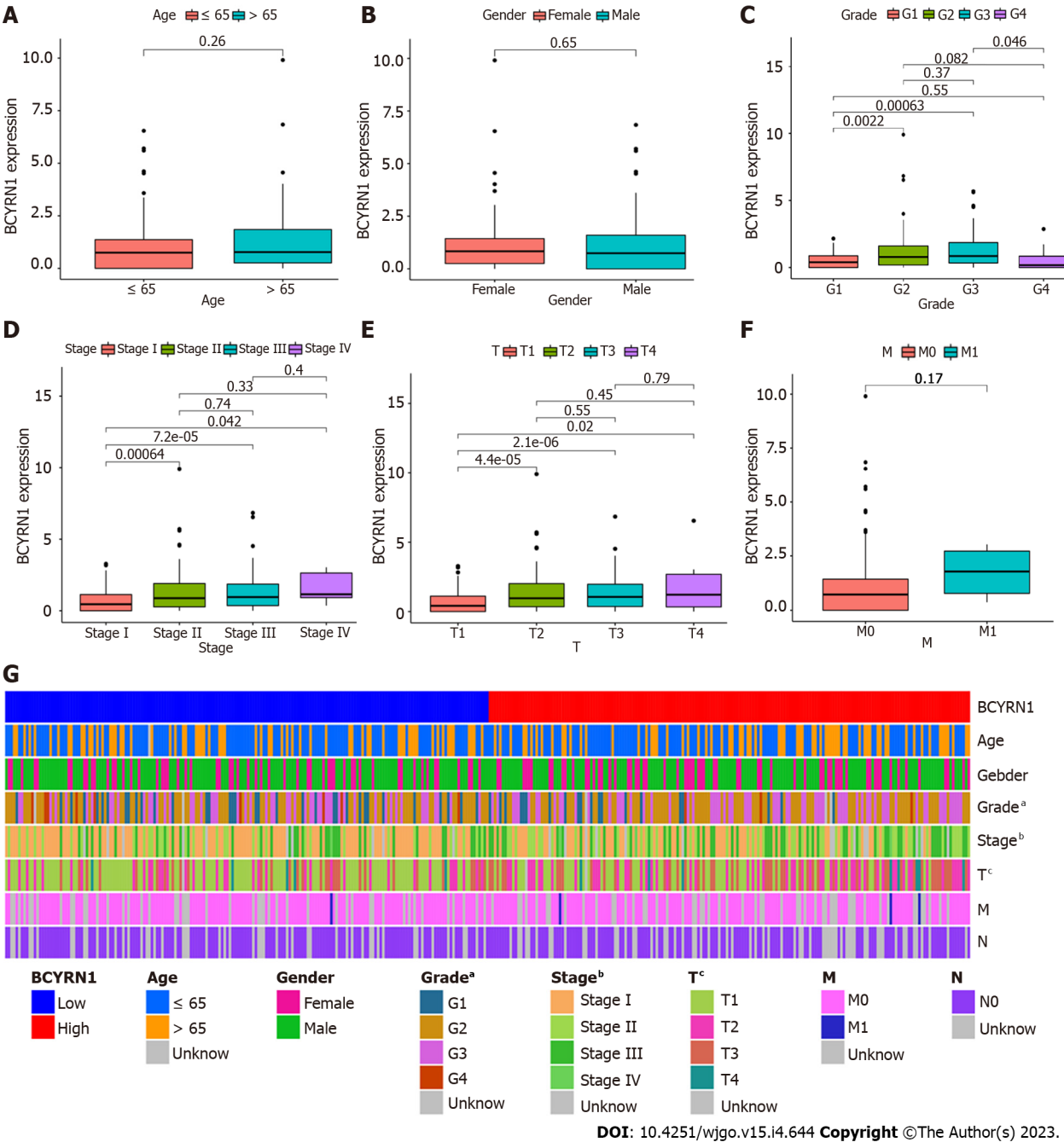

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Figure 3 Relationship between expression of brain cytoplasmic RNA1 and clinicopathological characteristics of hepatocellular carcinoma patients. A: Age; B: Sex (labeled Gender); C: Pathological grade; D: Clinical stage; E: T stage; F: M stage; G: Heat map of the correlation between the expression of brain cytoplasmic RNA1 and clinicopathological features.

enrichment analyses. GO enrichment analysis revealed that these genes were primarily involved in biological processes, including extracellular structure organization, external encapsulating structure organization and extracellular matrix organization. Cellular composition included collagen-containing extracellular matrix, the ion channel complex and the synaptic membrane. The molecular functions performed mainly involved gated channel activity, ion channel activity and signaling receptor activator activity, *etc.* (Figure 6A-C). KEGG enrichment analysis indicated that the DEGs of *BCYRN1* primarily involved pathways, including neuroactive ligand-receptor interaction, extracellular matrix-receptor interaction and protein digestion and absorption, among which protein digestion and absorption were pathways with the most annotated genes (Figure 6D and E). Finally, the result of GSEA enrichment analysis showed that pathways or functions that may be active in the low *BCYRN1* expression group were as follows: retinol metabolism, glycine, serine and threonine metabolism, peroxisome, primary bile acid biosynthesis and fatty acid metabolism (Figure 6F).

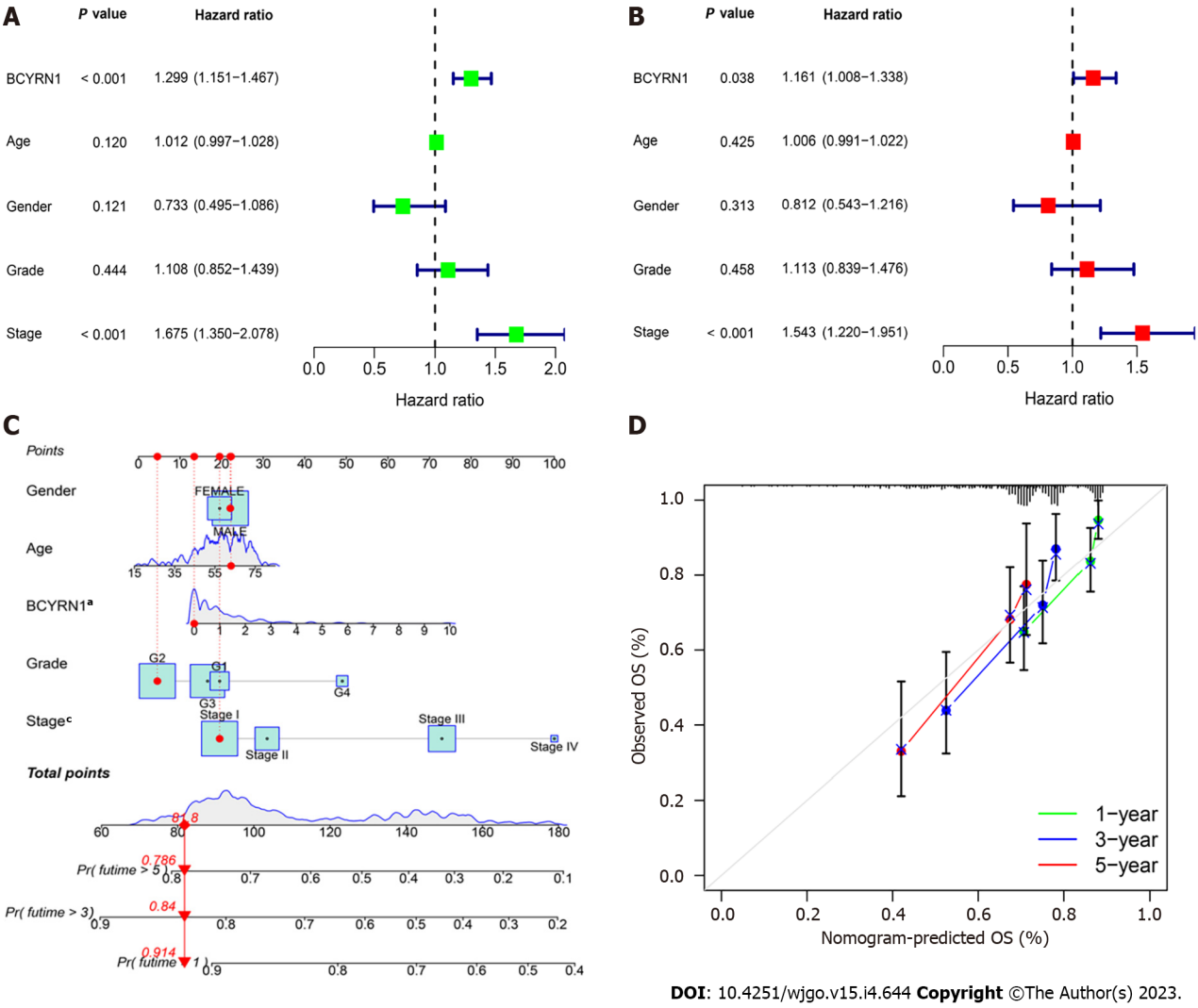

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Figure 4 Independent survival prognostic factor analysis and nomogram prediction model. A: Univariate prognostic analysis; B: Multivariate prognostic analysis; C: Nomogram prediction model; D: Calibration curve of nomogram. *BCYRN1*: Brain cytoplasmic RNA1; OS: Overall survival.

Correlation analysis and differential analysis between BCYRN1 expression, TME and immune cell infiltration in HCC

Stromal cell and immune cell scores in groups with high and low *BCYRN1* expression were evaluated, and violin plots of TME scores were plotted based on the results. The data showed that immune cell score, stromal cell score and ESTIMATEScore in the group with high *BCYRN1* expression were significantly higher compared to those in the low expression group ($P < 0.05$), showing that the immune cell and stromal cell content in the group with high *BCYRN1* expression in HCC was higher (Figure 7A). Differential analysis of immune cells was performed, and the results demonstrated that the levels of plasma cells ($P < 0.05$) and CD8 T cells ($P < 0.001$) were significantly increased in the group with low *BCYRN1* expression. Moreover, the level of macrophages M0 ($P < 0.05$) was significantly increased in the group with high *BCYRN1* expression (Figure 7B). Finally, correlation analysis was performed between various immune cells and *BCYRN1* expression, and correlation Lollipop and correlation scatter plots were plotted. The results showed that *BCYRN1* expression was positively linked with the level of macrophages M0 ($P = 0.016$), macrophages M2 ($P = 0.0094$) and regulatory T cells ($P = 0.018$) and negatively correlated with the level of plasma cells ($P = 0.012$) and CD8 T cells ($P = 0.0069$) (Figure 7C and Supplementary Figure 2).

Correlation analysis of BCYRN1 expression with immune checkpoint genes and TMB

Immune checkpoint genes associated with *BCYRN1* expression were explored, and circular correlation heat maps (Figure 8A) as well as rectangular correlation heat maps (Figure 8B) were plotted. The results showed that 19 immune checkpoint-related genes (*LAIR1*, *CD70*, *TNFRSF4*, *PDCD1LG2*, *HAVCR2*, *CTLA4*, *TNFSF15*, *CD276*, *LGALS9*, *TNFRSF18*, *TNFRSF9*, *TNFRSF14*, *CD44*, *CD80*, *CD86*, *CD200R1*, *TNFRSF8*, *TNFSF9*, *VTCN1*) were significantly and positively correlated with *BCYRN1* expression, and one immune checkpoint-related gene (*ADORA2A*) was significantly and negatively correlated with

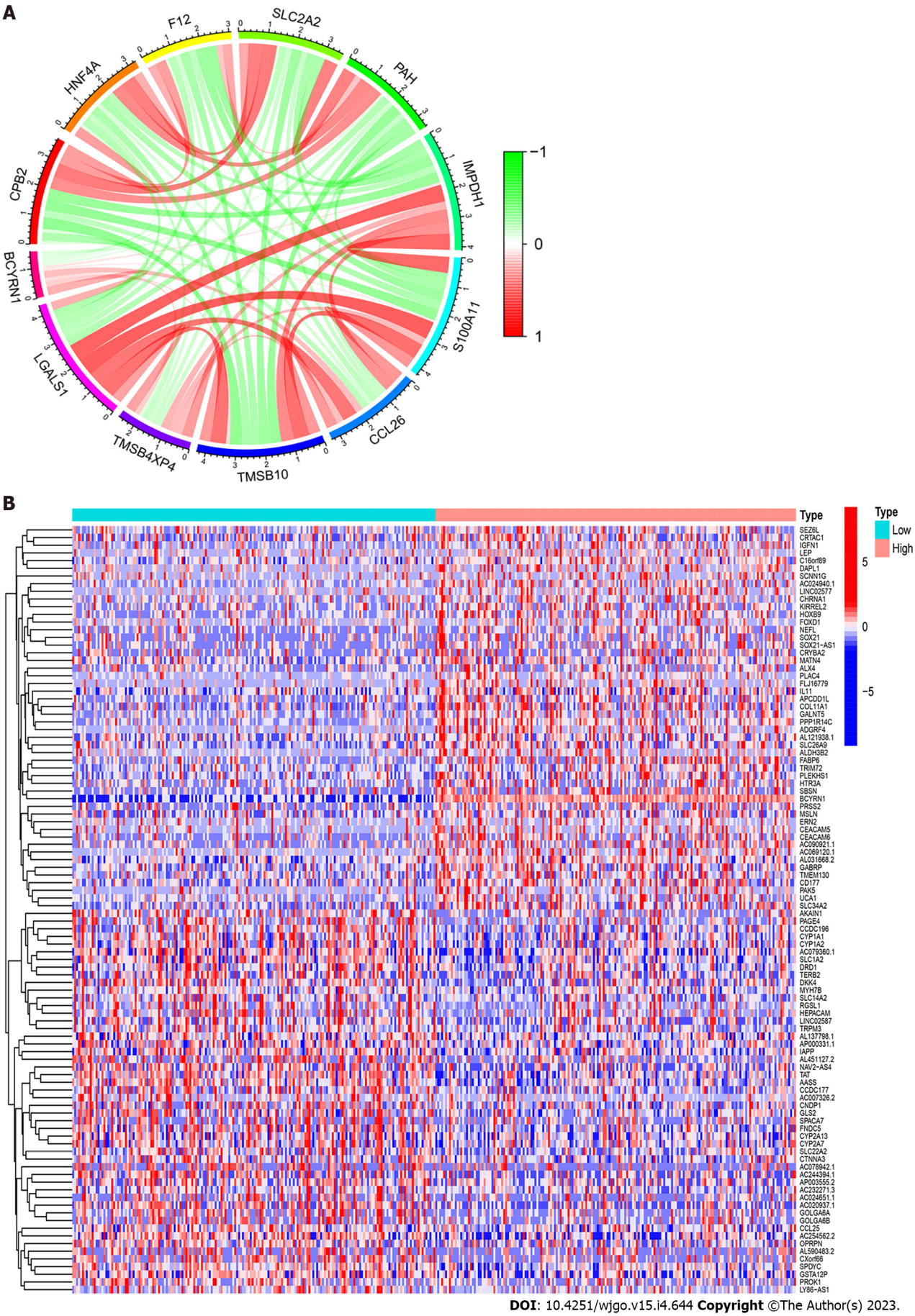

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Figure 5 Co-expressed and differential genes of brain cytoplasmic RNA1 in hepatocellular carcinoma. A: Co-expression circle plot of 11 genes

most closely related to brain cytoplasmic RNA1 (*BCYRN1*) in hepatocellular carcinoma (HCC). Red represented significant positive correlation, green represented significant negative correlation, and shade represented the magnitude of correlation; B: Heat map of differentially expressed genes of *BCYRN1* in HCC. Red represented upregulation and blue represented downregulation. Light blue represented the low expression group of *BCYRN1*, and light red represented the high expression group of BCYRN1.

BCYRN1 expression. The relationship between *BCYRN1* expression and TMB was also investigated, and correlation scatterplots were plotted (Figure 8C). Together, the data revealed that there was no significant relationship between TMB and *BCYRN1* expression (P = 0.11).

Differential analysis of BCYRN1 expression with chemosensitivity and immunotherapy efficacy

Chemotherapy is a promising therapeutic option for liver cancer. The IC_{50} of commonly used chemotherapeutic agents was evaluated in groups with high and low *BCYRN1* expression, and difference boxplots were plotted (Figure 9A). The results showed that CGP-60474, S-Trityl-L-cysteine, sunitinib, paclitaxel, VX-680 and pyrimethamine had higher sensitivity and a better therapeutic effect in the high *BCYRN1* expression group. Progression of immune checkpoint inhibitors changes the prognosis of HCC patients, and CTLA-4 and PD-1 are critical indicators to determine their therapeutic effects. Therefore, the therapeutic effects of anti-PD-1 and anti-CTLA-4 were scored in groups with high and low *BCYRN1* expression. The data revealed that the immunotherapeutic effect of anti-PD-1 was not significantly correlated (P = 0.071) with the expression of *BCYRN1*, while the immunotherapeutic effect of anti-CTLA-4 was more effective in the group with low *BCYRN1* expression (P = 0.012; Figure 9B).

Screening method for the literature and features of the included literature

Preliminary searches of three English databases yielded the following results: PubMed (n = 9), EMBASE (n = 7) and Web of Science (n = 16). Retrieval results were imported into Endnote. After removing duplicate studies, the remaining studies (n = 17) were analyzed. By reading the abstract and title, studies not conforming to the literature type and unrelated studies (n = 11) were removed. The remaining six articles were downloaded in full, and after careful examination, three publications were excluded because of a lack of essential data and poor quality. The remaining three studies were eventually included in our meta-analysis. Figure 10 depicts the above-mentioned search flowchart. All three studies were written in English, and all were from China. Specimen types were all HCC tissues, RNA expression was determined by qRT-PCR, and the source of HR values was indirectly calculated from OS curves. Follow-up time was 50 mo, 80 mo and 120 mo. NOS scores ranged from a minimum of 7 to a maximum of 8, all of which were higher quality articles. Table 1 shows essential features of the collected articles.

Relationship between BCYRN1 expression and the prognosis of HCC patients

Because there was no obvious heterogeneity (I^2 = 0.0%, P = 0.874) across studies, a fixed effects model was selected to combine the data. The forest plot results (HR = 1.66, 95%CI: 1.15-2.39; Figure 11) showed that *BCYRN1* overexpression was related to a poor prognosis in HCC patients, with individuals in the high *BCYRN1* expression group having a poorer prognosis and shorter survival. Because only three studies were included, a bias test and sensitivity analysis were not performed.

DISCUSSION

HCC is one of the most prevalent types of cancer of the digestive system. Its early symptoms are not clear, and the main characteristics of the middle and advanced stages are rapid progression, unsatisfactory treatment outcomes and low 5-year survival rates[31]. The development of HCC is an extremely complex process and includes the involvement of multiple genes and the evolution of multiple steps. Its possible underlying molecular mechanisms remain elusive. In recent years, with the discovery and exploration of lncRNAs, it has been demonstrated that lncRNAs play an important role in the development and progression of HCC[32]. Previous studies have shown that lncRNA can regulate signaling pathways associated with HCC and the expression levels of downstream target genes, thereby further affecting the activity of proteins by changing the expression levels and stability of mRNAs and miRNAs, which are closely related to a variety of malignant phenotypes of HCC[33,34]. Dysregulation of lncRNAs has been associated with precancerous lesions of HCC, such as hepatitis B virus infection, cirrhosis and fatty liver[35]. LncRNA can also evaluate and predict the efficacy of various treatment modalities for HCC patients and has a wide range of applications in HCC diagnosis and treatment[36]. *BCYRN1* is an important member of the lncRNA family and exploring its prognostic value and therapeutic prospects in HCC is a main goal.

Our study first investigated the expression level of *BCYRN1* in pan-cancer and normal tissues, and discovered that it was highly expressed in BRCA, LUAD, ESCA, LUSC, WT, STES, STAD, LIHC, SKCM, PAAD, OV, UCS, KICH, ALL, TGCT, CHOL and LAML, and lowly expressed in GBM, GBMLGG, KIRP,

Table 1 Characteristics of studies included in the meta-analysis

Ref.	Country	Sample size (high/low)	Sample	Survival analysis	Detection method	Cutoff value	Source of HR values	HR and 95%CI	Follow-up time	NOS score
Ming *et al* [28], 2019	China	55 (27/28)	Tissue	OS	qRT-PCR	Median	Survival curves	2.13 (0.77, 5.93)	50 mo	7
Lin *et al* [48], 2018	China	240	Tissue	OS	qRT-PCR	Mean	Survival curves	1.62 (1.01, 2.59)	80 mo	8
Liu *et al* [47], 2022	China	100 (50/50)	Tissue	OS	qRT-PCR	NR	Survival curves	1.56 (0.78, 3.14)	120 mo	7

HR: Hazard ratio; qRT-PCR: Quantitative real time polymerase chain reaction; OS: Overall survival; NR: Not reported; NOS: Newcastle-Ottawa scale.

KIPAN, KIRC, THCA and ACC. The expression of *BCYRN1* in HCC was independently examined, and the results revealed that *BCYRN1* was considerably overexpressed in tumors. These early findings imply that *BCYRN1* might be an oncogene in HCC. Our study continued to focus on the association between *BCYRN1* expression and the prognosis and clinicopathological features of HCC patients. Using the GEPIA database and the TCGA dataset, KM curves for survival outcomes (including OS, DFS and PFS) were generated, all of which indicated that patients with high *BCYRN1* expression had a poorer prognosis. ROC curves showed that *BCYRN1* was of great value in predicting patient prognosis.

Clinicopathologic analysis showed that *BCYRN1* expression was substantially linked with the clinical stage, pathological grade and T stage of HCC patients, and *BCYRN1* expression was higher when the clinical stage was more advanced, the pathological grade was worse and the T stage was higher. Our findings showed that the worse the pathological grade, then the stronger the proliferation and invasiveness of HCC and the higher the expression level of *BCYRN1*. However, the expression of stage G4 in Figure 3C is low, which is not in line with the expected results. The reasons behind it have been thoroughly explored. As shown by the downloaded clinical data, there were 54 patients in the G1 stage, 179 patients in the G2 stage, and 123 patients in the G3 stage, while only 13 patients were in the G4 stage. Therefore, the number of patients in the G4 stage was significantly lower than those in other stages, and the sample size was too small to be representative. Given a sufficient sample size, the results are estimated to be in line with the expectations.

Subsequently, univariate and multivariate Cox regression analysis showed that the expression of *BCYRN1* and the clinical stage of the tumor were independent prognostic factors for HCC patients. Therefore, we developed a nomogram prediction model that can predict OS in patients based on their clinicopathological characteristics and *BCYRN1* expression. According to the results of the above-mentioned study, overexpression of *BCYRN1* is related with a poor prognosis in patients and is likely to be an independent prognostic marker in HCC patients.

In addition, co-expressed genes and DEGs associated with *BCYRN1* were screened. The most strongly correlated co-expressed genes were *LGALS1*, *TMSB4XP4*, *TMSB10*, *CCL26*, *S100A11*, *IMPDH1*, *PAH*, *SLC2A2*, *F12*, *HNF4A*, and *CPB2*. Among them, genes that were positively correlated with the expression of *BCYRN1* were generally oncogenes and can lead to poor prognosis in HCC. For example, it has been found that the *LGALS1* gene is upregulated in HCC and can encode related proteins, thereby increasing tumor migration and invasion[37]. Similarly, *TMSB10* expression in HCC was significantly higher compared to that in surrounding normal liver tissues, and high TMSB10 expression was significantly associated with tumor volume and distant metastasis, which was a potential prognostic marker for predicting HCC patients[38]. It has previously been revealed that *CCL26* acts on fibroblasts, thereby affecting processes including proliferation, invasion and angiogenesis in HCC[39]. *S100A11* overexpression in HCC can enhance HCC invasiveness[40]. In contrast, genes negatively correlated with *BCYRN1* expression are generally tumor suppressor genes. Low *SLC2A2* expression was correlated with a worse DFS and OS in HCC patients, and *SLC2A2* expression was negatively associated with the degree of immune infiltration in HCC[41]. Furthermore, it has been demonstrated that *HNF4A* can inhibit the motility and metastasis of HCC cells[42]. The above-mentioned studies demonstrated the value of co-expression analysis of genes.

Moreover, DEGs associated with *BCYRN1* were screened, and KEGG, GO and Gene Set Enrichment Analysis enrichment analysis were performed. The results of comprehensive enrichment analysis demonstrated that the primary functions of *BCYRN1*-related DEGs were to regulate extracellular matrix components, regulate ion channel activity and signal molecule transmission, such as synaptic membrane. In addition, *BCYRN1*-related DEGs were involved in protein digestion and absorption as well as the metabolism of retinol, amino acids and fatty acids and involved in the regulation of tumor-related pathways (calcium signaling pathway, Wnt signaling pathway, IL-17 signaling pathway). The TME consists of three parts, including the extracellular matrix, stromal cells and cell growth factors. The extracellular matrix is crucial in the formation of tumors[43,44]. It has been demonstrated that dysregulation of the Ca^{2+} concentration increases the risk of tumor development and accelerates tumor

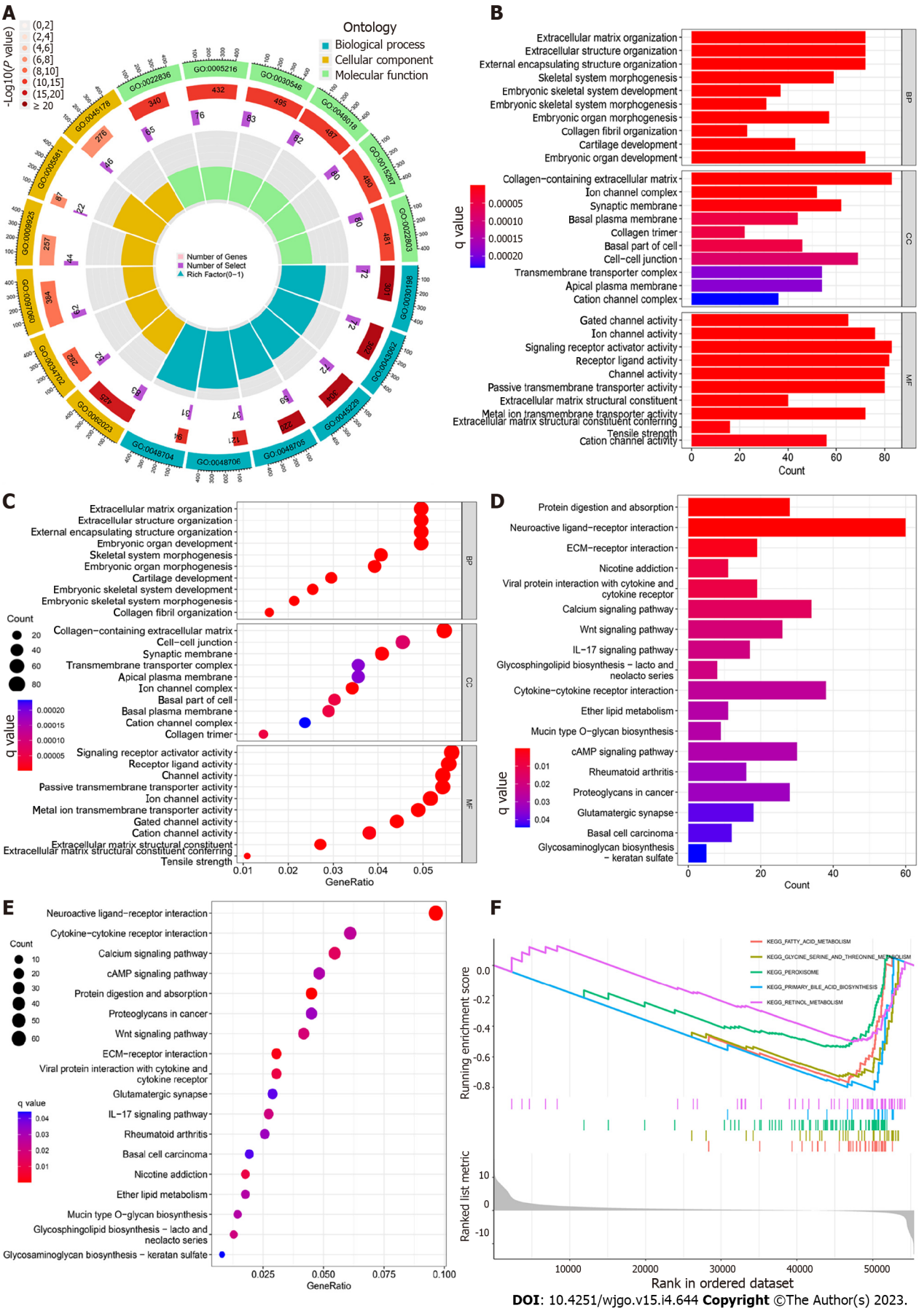

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Figure 6 Enrichment analysis of brain cytoplasmic RNA1-associated differentially expressed genes. A: Circle diagram of gene ontology (GO) enrichment analysis in biomolecular function, biological process and cellular component; B: Histogram of GO enrichment analysis; C: Bubble plots for GO enrichment

analysis; D: Histogram of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed the top 20 pathways with the most significant correlation; E: Bubble plots for KEGG enrichment analysis; F: Brain cytoplasmic RNA1-associated Gene Set Enrichment Analysis.

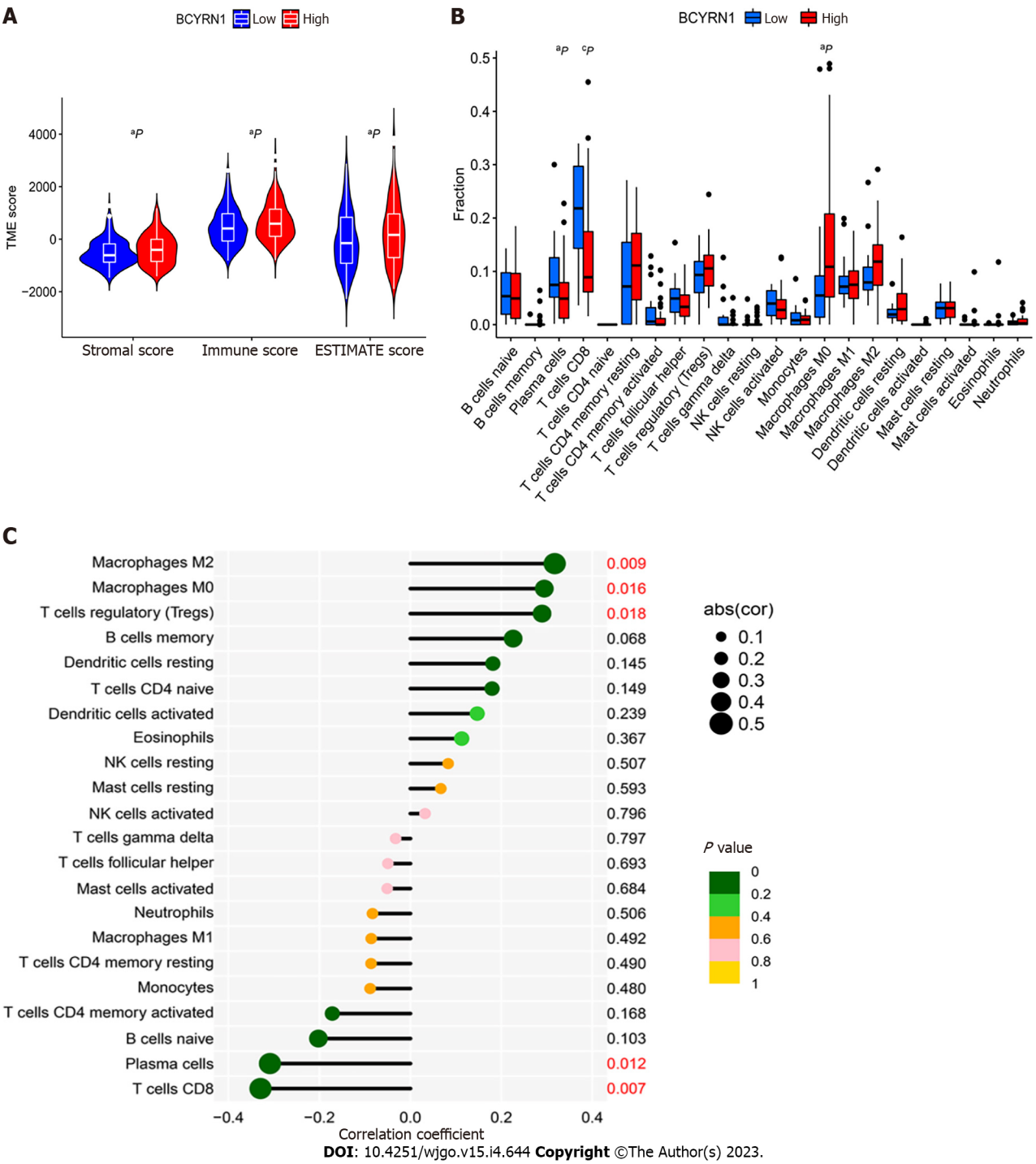

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Figure 7 Correlation analysis and differential analysis of brain cytoplasmic RNA1 expression with tumor microenvironment and immune cell infiltration in hepatocellular carcinoma. A: Violin plot of the difference in tumor microenvironment scores between the high and low brain cytoplasmic RNA1 (*BCYRN1*) expression groups; B: Box plots of differential analysis between the infiltration levels of 22 immune cells and the high and low expression groups of *BCYRN1*; C: Lollipop plot of correlation analysis between expression of *BCYRN1* and levels of 22 immune cell infiltrates. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

progression, whereas inactivation of Ca^{2+} channels accelerates the proliferation and growth of tumor cells[45]. The Wnt signaling pathway can regulate cell proliferation, differentiation, apoptosis and stem cell renewal, and dysregulation of the Wnt signaling pathway can occur at all stages of malignant tumors[46]. The above-mentioned studies and analyses on the mechanism of *BCYRN1* involved in HCC showed that *BCYRN1* is a very important regulatory gene in the formation and progression of HCC and

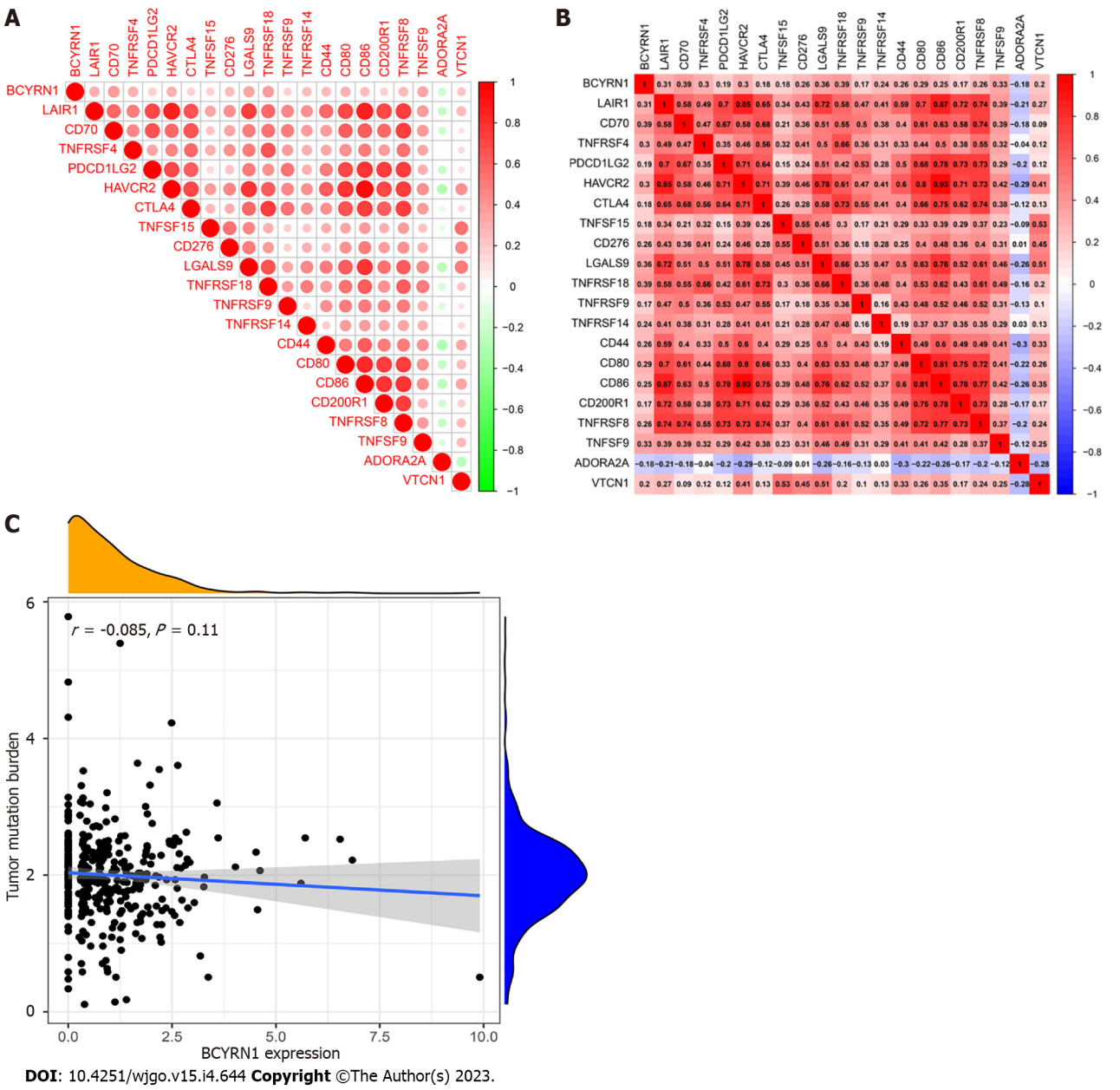

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Figure 8 Correlation analysis of brain cytoplasmic RNA1 expression with immune checkpoint genes and tumor mutation burden. A: Circular heatmap of immune checkpoint genes associated with brain cytoplasmic RNA1 (*BCYRN1*) expression; B: Rectangular heat map of immune checkpoint genes associated with *BCYRN1* expression; C: Scatter plot of the correlation between *BCYRN1* expression and tumor mutation burden.

an important possible therapeutic target for HCC.

Mechanistic studies of *BCYRN1* in HCC could validate the conclusions of the current study. It has been proven that *BCYRN1* is highly expressed in HCC and is associated with a poor prognosis in HCC patients. Together, *BCYRN1*, *POU3F2*, and miR490-3p constitute a network of competing endogenous RNAs that regulate the migration, invasion and proliferation of HCC[26]. Liu *et al*[47] discovered that *BCYRN1* can enhance the invasion and proliferation of HCC by regulating the *BCYRN1*/*BATF*/*TM4SF1* targeting axis. Moreover, Lin *et al*[48] showed that *BCYRN1* is overexpressed in HCC and promotes the growth of HCC cells and the formation of tumor tissues by regulating cell cycle-related genes and stemness markers, and prevents the degradation of cyclin E2 mRNA. Through *in vivo* and *in vitro* experiments, Tan *et al*[27] showed that *BCYRN1* could affect expression of the c-MYC protein, thereby affecting the levels of apoptotic and anti-apoptotic proteins and promoting the progression of HCC. Combined, these studies strongly confirmed that *BCYRN1* is indeed a possible therapeutic target as well as a prognostic marker for HCC.

The TME refers to the complex multicellular environment in which tumors are located during growth and development[49]. The TME is usually composed of three parts, including immune cells, the extracellular matrix and secreted factors and stromal cells, which are mixed with lymphatic vessels and blood vessels to regulate and influence the occurrence and development of tumor cells[50]. In the TME,

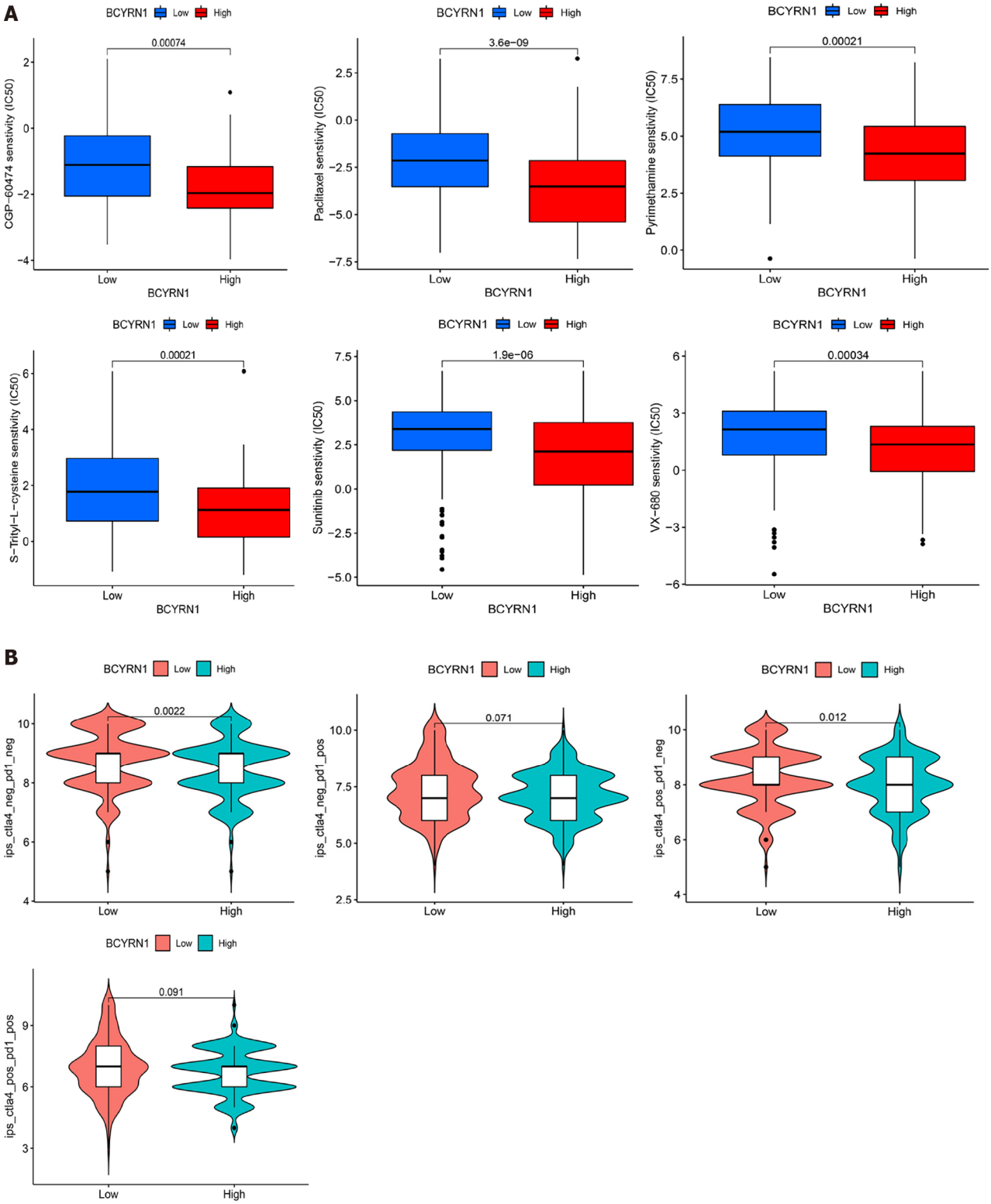

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Figure 9 Differential analysis of brain cytoplasmic RNA1 expression with chemosensitivity and immunotherapy efficacy. A: Box plots of the differences of sensitivity of the six selected chemotherapeutic agents between the high and low expression groups of brain cytoplasmic RNA1 (BCYRN1); B: Violin plot of the difference of immunotherapy effect between the high and low expression groups of BCYRN1.

various immune cells play different roles during antitumor immunotherapy, especially T cells *vs* B cells [51]. The relationship between the expression level of *BCYRN1* and the TME score was investigated. The results showed that the *BCYRN1* high expression group had a higher TME score, a higher level of immune and stromal cells and a lower purity of tumors. In addition, the relationship between *BCYRN1* expression and the level of various immune cells was investigated, and the results showed that the content of CD8 T cells and plasma cells was higher in the group with low expression, while the number

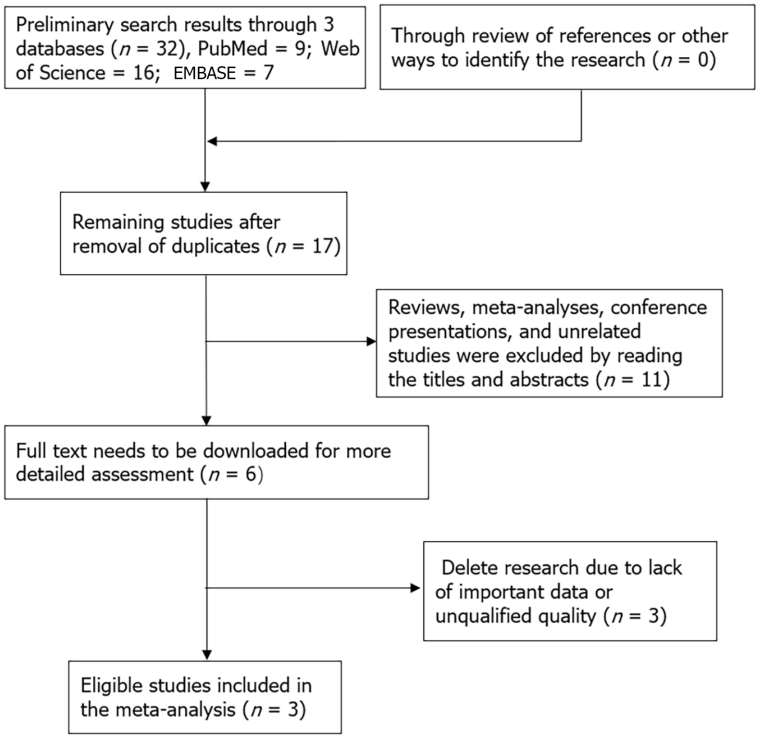

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Figure 10 Flow chart of literature retrieval and screening for meta-analysis.

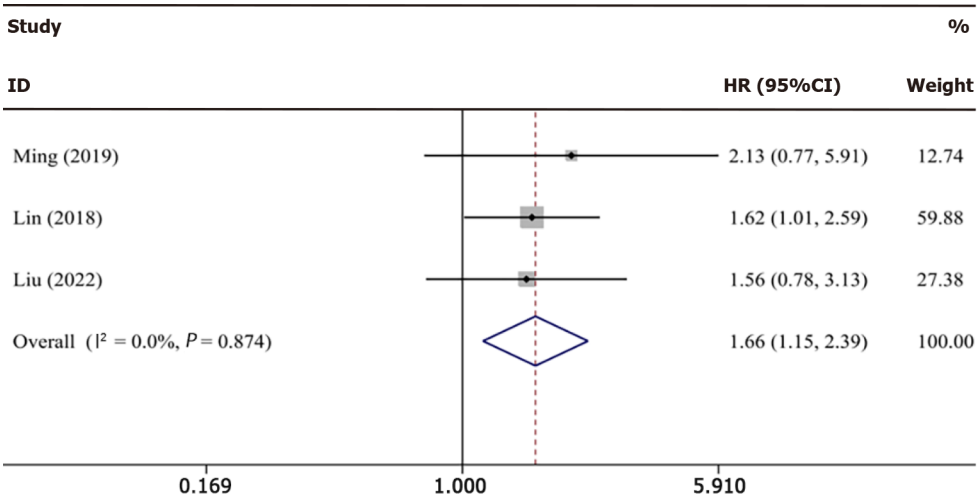

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Figure 11 Forest plot for meta-analysis of brain cytoplasmic RNA1 expression *vs* overall survival in hepatocellular carcinoma patients.

of macrophages M0 was higher in the group with high expression. Thus, *BCYRN1* expression is linked to the level of M0 and M2 macrophages, CD8 T cells and plasma cells, and *BCYRN1* can regulate the level of the above-mentioned immune cells.

Finally, the connection between *BCYRN1* expression and drug sensitivity was also investigated. It was discovered that six drugs were more sensitive in the group with high expression of *BCYRN1*, thus providing a novel idea for clinical medication. PD-1 and CTLA-4 are important immunotherapeutic targets[52]. The differences in efficacy between these two immunotherapies were analyzed in the high and low *BCYRN1* expression groups, and the results showed that the therapeutic effect of anti-CTLA-4 was more pronounced in the low *BCYRN1* expression group. Thus, our data showed that *BCYRN1* may have great potential in immunotherapy.

Our study has the following limitations. First, data were downloaded from the online database TCGA and were not validated by the laboratory and clinic. Second, although the sample size of the TCGA database is large, the type of database is relatively single. To compensate for the above-mentioned

shortcomings, the meta-analysis was performed, which combined all survival prognosis studies of *BCYRN1* in HCC and showed that high expression of *BCYRN1* in HCC was significantly associated with poor patient prognosis, which was consistent with the conclusions of the bioinformatics. The prognostic value and immunotherapeutic prospects of *BCYRN1* in HCC were explored by bioinformatics and meta-analysis, and valuable conclusions were drawn. To further explore the function and underlying mechanism of *BCYRN1* in HCC, more in-depth clinical and laboratory studies are required.

CONCLUSION

Our study revealed that the expression level of *BCYRN1* in HCC tissues was significantly higher compared to that in surrounding normal liver tissues and that high *BCYRN1* expression was related to a poor prognosis and clinicopathological progression of HCC patients. Furthermore, the expression of *BCYRN1* was significantly related to the level of immune cell infiltration, drug sensitivity and immunotherapy responses in HCC. Therefore, *BCYRN1* is likely to be a prognostic indicator and a target for the treatment in HCC patients. The prognostic value of *BCYRN1* and the promise of immunotherapy need to be further confirmed by large clinical and laboratory studies.

ARTICLE HIGHLIGHTS

Research background

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide and has a high mortality rate. Early detection of HCC can achieve a good 5-year survival rate by surgical treatment, liver transplantation and radiotherapy. Therefore, early diagnosis and treatment is particularly important, and identifying novel sensitive tumor markers and discovering novel molecular therapeutic targets are key goals. Exploring novel immunotherapeutic drugs is another breakthrough.

Research motivation

The expression of brain cytoplasmic RNA1 (*BCYRN1*) is linked to the clinicopathology and prognosis of several types of cancers, among which HCC is one of the most frequent types of cancer worldwide.

Research objectives

In this study, bioinformatics and meta-analysis were used to explore the prognostic value and immunotherapeutic potential of *BCYRN1* in HCC.

Research methods

Information was obtained from the Cancer Genome Atlas database. First, the correlation between *BCYRN1* expression and prognosis and clinicopathologic characteristics of HCC patients was explored. Univariate and multivariate regression analyses were employed to examine the relationship between *BCYRN1* and HCC prognosis. Second, potential functions and pathways were explored by means of enrichment analysis of differentially-expressed genes. The relationship between *BCYRN1* expression and tumor microenvironment, immune cell infiltration, immune checkpoint, drug sensitivity and immunotherapy effect was also investigated. Finally, three major databases were searched and used to conduct a meta-analysis on the relationship between *BCYRN1* expression and patient prognosis.

Research results

BCYRN1 expression was significantly higher in HCC compared to normal tissues and was linked to a poor prognosis and clinicopathological characteristics. Enrichment analysis showed that *BCYRN1* regulates the extracellular matrix and transmission of signaling molecules, participates in the metabolism of nutrients, such as proteins, and participates in tumor-related pathways. *BCYRN1* expression was linked to the tumor microenvironment, immune cell infiltration, drug sensitivity and the efficacy of immunotherapy. Furthermore, the meta-analysis in this study showed that *BCYRN1* overexpression was related to a worse outcome in HCC patients.

Research conclusions

Our study revealed that high *BCYRN1* expression related to a poor prognosis and clinicopathological progression of HCC patients. Furthermore, the expression of *BCYRN1* was significantly related to the level of immune cell infiltration, drug sensitivity and immunotherapy responses in HCC.

Research perspectives

Overexpression of *BCYRN1* relates to poor prognosis and may be a potential prognostic factor and immunotherapeutic target in HCC.

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FOOTNOTES

Author contributions: Han XY conceived the study; Zhao RY, Niu XD and Jin HJ performed the download and analysis of data; Yu M, Liu DM and Wang YF performed visual drawings; Han XY and Li X co-wrote the manuscript; Ma HZ and Cai H reviewed this manuscript; Han XY and Li X contributed equally to this work.

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Data sharing statement: The corresponding author may provide data obtained from this study upon reasonable request.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Nomogram established using risk factors of early gastric cancer for predicting the lymph node metastasis

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Abstract

BACKGROUND

For the prognosis of patients with early gastric cancer (EGC), lymph node metastasis (LNM) plays a crucial role. A thorough and precise evaluation of the patient for LNM is now required.

AIM

To determine the factors influencing LNM and to construct a prediction model of LNM for EGC patients.

METHODS

Clinical information and pathology data of 2217 EGC patients downloaded from the Surveillance, Epidemiology, and End Results database were collected and analyzed. Based on a 7:3 ratio, 1550 people were categorized into training sets and

667 people were assigned to testing sets, randomly. Based on the factors influencing LNM determined by the training sets, the nomogram was drawn and verified.

RESULTS

Based on multivariate analysis, age at diagnosis, histology type, grade, T-stage, and size were risk factors of LNM for EGC. Besides, nomogram was drawn to predict the risk of LNM for EGC patients. Among the categorical variables, the effect of grade (well, moderate, and poor) was the most significant prognosis factor. For training sets and testing sets, respectively, area under the receiver-operating characteristic curve of nomograms were 0.751 [95% confidence interval (CI): 0.721-0.782] and 0.786 (95%CI: 0.742-0.830). In addition, the calibration curves showed that the prediction model of LNM had good consistency.

CONCLUSION

Age at diagnosis, histology type, grade, T-stage, and tumor size were independent variables for LNM in EGC. Based on the above risk factors, prediction model may offer some guiding implications for the choice of subsequent therapeutic approaches for EGC.

Key Words: SEER; Early gastric cancer; Lymph node metastasis; Risk factors; Nomogram

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Core Tip: A model was constructed to evaluate the impact of various indicators in an integrated manner to serve as a base for predicting lymph node metastasis (LNM) in early gastric cancer (EGC) patients. Age at diagnosis, histology type, grade, T-stage, and tumor size were independent hazard elements for LNM in EGC.

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INTRODUCTION

Gastric cancer (GC), as the third most common cancer-related cause of death worldwide[1], for which risk indicators include *Helicobacter pylori* (*H. pylori*) infection, gender, eating habits, smoking and family history[2]. Screening may be done for GC using markers of atrophy in the stomach (a precursor lesion of GC), such as serum pepsinogens[3] or serum ghrelin[4]; or serum antibodies to Hp, the main risk factor for GC[5]; or examining the stomach mucosa using endoscopy[6].

Early gastric cancer (EGC) is classified as a GC limited to the mucosa or submucosa, irrespective of the presence of territorial lymph node metastasis (LNM)[7]. Compared to advanced GC, EGC has a better opportunity to be surgically removed successfully, which resulting in a better survival status. Endoscopic resection (ER), which is suitable for low LNM rate of EGC, is the first-choice therapy for EGC. Endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) are two main operations of ER[8]. Operable advanced GC could be radically resected by surgery including D2 Lymphadenectomy[9].

Although the incidence of GC has decreased in the past 3 decades in developed countries[10], the general prognosis for GC was still poor. For example, the five-year survival rate for GC is about 20 percent[11]. LNM had good predictive value for prognosis[12]. Therefore, in patients with EGC, the presence or absence of LNM is a crucial factor to be evaluated comprehensively.

Corresponding clinicopathological information of a large sample size of EGC patients was obtained from the Surveillance, Epidemiology, and End Results (SEER) database[13], including clinicopathological parameters and information of patients. Factors that may be associated with the prognosis of patients with EGC were enrolled into our research to explore their influence. There are very few researches, to our knowledge, exploring the factors influencing LNM in EGC patients. Therefore, we plotted a predictive model that allows a comprehensive assessment of the effects of various indicators and provides a platform for prediction of LNM of patients with EGC.

MATERIALS AND METHODS

Data source and patient selection

Clinicopathological information were obtained from the SEER database. The standards used for exclusion are listed below: (1) Patients who have undergone pre-operative neoadjuvant therapy; (2) patients with residual GC; (3) patients without complete clinical and pathological data; (4) retrieved unknown lymph nodes; and (5) patients without confirmed as EGC *via* biopsy.

Finally, a total of 2217 patients participated in this study and were analyzed in the next step. According to the ratio of seven to three, all patients were separately assigned to training and testing sets (1550:667).

Clinicopathological parameters

The relationship between individual clinicopathological features and LNM was evaluated to identify independent influencing variables for LNM in EGC. The clinicopathological features were examined as follow: Race, age when EGC is confirmed, gender, tumor location, histological type, degree of differentiation, TNM stage, T-stage, tumor size, LNM, survival months, status, first malignant primary indicator, sequence number, insurance recode, marital status. First malignant primary indicator, which means whether it is the first primary tumor, was divided into two subgroups: No and yes.

Statistical analysis

Numerical variables were represented as mean ± SD and examined using *t*-test. Categorical variables were represented as frequency and proportion and analyzed by Pearson's χ^2 or Fisher's exact tests. In the logistic regression, variables that were significantly different in the univariate analysis were included in the multivariate analysis. Factors of influence of training sets were determined and results were displayed as odds ratio (OR) and 95% confidence intervals (CIs).

Furthermore, the LNM prediction model was plotted. In addition, 850 patients in the testing set, as the external validation sets, were included in the follow-up validation analysis. The power of identification of the prediction model is calculated using the consistency index, which corresponds to the area under the receiver-operating characteristic curve (AUC) in the logistic regression.

SPSS software (version 22.0; IBM Corp.) and R software (version 4.0.5) were used to analyze the data. Two-sided $P < 0.05$ was considered to be statistically significantly different.

RESULTS

Characteristics of patients

Two thousand two hundred and seventeen suitable patients were included in the present research (Figure 1). Of the included EGC patients, 1214 (54.8%) were male and 1003 (45.2%) were female. 1247 (56.2%) were white, 355 (16.0%) were black, and 615 (27.7%) were put in the "other" race subgroup. Moreover, T stage, 356 (16.1%) were T1/T1NOS, 801 (36.1%) were T1a, 1060 (47.8%) were T1b. Of the EGC patients, 337 (15.2%) were diagnosed with LNM totally, 1880 (84.8%) were not. The LNM rates of EGC patients were 15.6% (242/1550) in the training sets and 14.2% (95/667) in the testing sets, respectively (Table 1).

Prognostic variables of patients with EGC

Univariate logistic regression analysis showed that some factors, such as age when EGC is confirmed, histology type, grade, TNM stage, T-stage, size, primary, were influenced variables of LNM of EGC (Table 2). Those variables treated as significant prognostic factors for LNM were included in the multivariate logistic regression model. Age at diagnosis [odd ratio (OR): 0.003, $P = 0.012$], histology type (OR: 1.382, $P = 0.019$), grade (OR: 1.825, $P < 0.001$), T-stage (OR: 1.985, $P < 0.001$), and size (OR: 1.319, $P < 0.001$) were independent influenced variables for LNM (Table 3).

Construction of the prediction model for EGC patients

A nomogram prediction model was constructed (Figure 2). In the model, the points of each variable ranged from 0 to 100. Each indicator has its corresponding score row, in which each patient has a score that is derived from the corresponding first row. The total point is the sum of the points of all variable. And then, the total score for each patient corresponds to the probability of the bottom which is the probability of occurrence of LNM.

Evaluation of the nomogram

The calibration curves of the training and testing sets used to compare the forecasted situation with the actual situation, both showed satisfactory consistency (Figure 3). The AUC of internal validation was 0.751 (95%CI: 0.721-0.782) and of external validation was 0.786 (95%CI: 0.742-0.830), respectively (Figure 4).

Table 1 Characteristic of 2217 patients with early gastric cancer from Surveillance, Epidemiology, and End Results

Variables	Level	LNM (-)	LNM (+)	*P* value
		n = 1880	*n* = 337	
Age (mean ± SD)		70.87 (12.82)	68.74 (12.59)	0.005[1]
Race (%)	White	1065 (56.6)	182 (54.0)	0.554
	Black	295 (15.7)	60 (17.8)	
	Other	520 (27.7)	95 (28.2)	
Gender (%)	Male	1025 (54.5)	189 (56.1)	0.638
	Female	855 (45.5)	148 (43.9)	
Location (%)	Body of stomach	314 (16.7)	51 (15.1)	0.116
	Gastric antrum	710 (37.8)	133 (39.5)	
	Fundus of stomach	97 (5.2)	13 (3.9)	
	Greater curvature of stomach NOS	127 (6.8)	25 (7.4)	
	Lesser curvature of stomach NOS	264 (14.0)	47 (13.9)	
	Stomach, NOS	64 (3.4)	14 (4.2)	
	Pylorus	186 (9.9)	21 (6.2)	
	Overlapping lesion of stomach	118 (6.3)	33 (9.8)	
Histologytype (%)	Neuroendocrine carcinoma	80 (4.3)	1 (0.3)	0.002[1]
	Signet ring cell carcinoma	336 (17.9)	57 (16.9)	
	Adenocarcinoma	1349 (71.8)	250 (74.2)	
	Others/unknown	115 (6.1)	29 (8.6)	
Grade (%)	Well	377 (20.1)	12 (3.6)	< 0.001[1]
	Moderate	638 (33.9)	115 (34.1)	
	Poor	865 (46.0)	210 (62.3)	
Stage (%)	I	80 (4.3)	0 (0.0)	< 0.001[1]
	IA	1793 (95.4)	0 (0.0)	
	IB	7 (0.4)	225 (66.8)	
	IIA	0 (0.0)	80 (23.7)	
	IIB	0 (0.0)	32 (9.5)	
T-stage (%)	T1/T1NOS	317 (16.9)	39 (11.6)	< 0.001[1]
	T1a	745 (39.6)	56 (16.6)	
	T1b	818 (43.5)	242 (71.8)	
Tumorsize (%)	0-1 cm	449 (23.9)	15 (4.5)	< 0.001[1]
	< 2 cm	496 (26.4)	71 (21.1)	
	< 3 cm	338 (18.0)	75 (22.3)	
	< 4 cm	228 (12.1)	64 (19.0)	
	< 5 cm	132 (7.0)	42 (12.5)	
	> 5 cm and more	237 (12.6)	70 (20.8)	
Primary (%)	No	387 (20.6)	54 (16.0)	0.063
	Yes	1493 (79.4)	283 (84.0)	
Order (%)	One primary only	1323 (70.4)	259 (76.9)	0.046[1]
	1st of 2 or more primaries	146 (7.8)	21 (6.2)	
	2nd of 2 or more primaries	310 (16.5)	47 (13.9)	

	3rd of 3 or more primaries	74 (3.9)	6 (1.8)	
	4th of 4 or more primaries	20 (1.1)	3 (0.9)	
	5th of 5 or more primaries	6 (0.3)	0 (0.0)	
	6th of 6 or more primaries	1 (0.1)	0 (0.0)	
	7th of 7 or more primaries	0 (0.0)	1 (0.3)	
Maritalstatus (%)	Married (including common law)	1057 (56.2)	188 (55.8)	0.386
	Divorced	127 (6.8)	26 (7.7)	
	Separated	20 (1.1)	5 (1.5)	
	Single (never married)	243 (12.9)	49 (14.5)	
	Widowed	353 (18.8)	55 (16.3)	
	Unmarried or Domestic Partner	2 (0.1)	2 (0.6)	
	Unknown	78 (4.1)	12 (3.6)	
Insurance (%)	Insured	1129 (60.1)	201 (59.6)	0.575
	Insured/nospecifics	338 (18.0)	53 (15.7)	
	Any medicaid	344 (18.3)	69 (20.5)	
	Uninsured	39 (2.1)	10 (3.0)	
	Insurance status unknown	30 (1.6)	4 (1.2)	

[1]It means statistically significant.
LMN: Lymph node metastasis; EGC: Early gastric cancer.

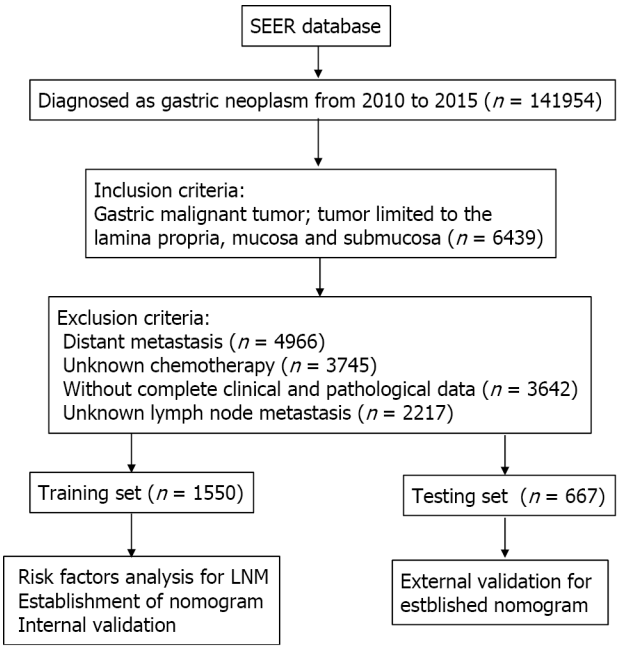

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Figure 1 The flowchart of data collection and grouping for patients with early gastric cancer. LNM: Lymph node metastasis; SEER: Surveillance, Epidemiology, and End Results.

DISCUSSION

GC has a significant impact worldwide[14]. GC, occurs in the epithelium of the gastric mucosa, tendency to undergo hematogenous or LNM even in the early stages[15]. As the understanding of GC becomes more comprehensive and deeper, the rate of occurrence and mortality is decreasing year by year[16]. The average of age at diagnosis of GC patients was lower and lower in recent year[1].

Table 2 Univariate analysis for lymph node metastasis of patients with early gastric cancer

Variables	Level	Training sets			Testing sets		
		LNM (-)	LNM (+)	*P* value	LNM (-)	LNM (+)	*P* value
		n = 1308	*n* = 242		*n* = 572	*n* = 95	
Age at diagnosis (mean ± SD)		71.07 (12.82)	68.64 (12.82)	0.007[1]	70.42 (12.80)	69.02 (12.03)	0.319
Race (%)	White	733 (56.0)	122 (50.4)	0.237	332 (58.0)	60 (63.2)	0.584
	Black	202 (15.4)	45 (18.6)		93 (16.3)	15 (15.8)	
	Other	373 (28.5)	75 (31.0)		147 (25.7)	20 (21.1)	
Gender (%)	Male	710 (54.3)	136 (56.2)	0.631	315 (55.1)	53 (55.8)	0.985
	Female	598 (45.7)	106 (43.8)		257 (44.9)	42 (44.2)	
Location (%)	Body of stomach	215 (16.4)	38 (15.7)	0.239	99 (17.3)	13 (13.7)	0.444
	Gastric antrum	479 (36.6)	90 (37.2)		231 (40.4)	43 (45.3)	
	Fundus of stomach	67 (5.1)	12 (5.0)		30 (5.2)	1 (1.1)	
	Greater curvature of stomach	86 (6.6)	19 (7.9)		41 (7.2)	6 (6.3)	
	Lesser curvature of stomach	192 (14.7)	35 (14.5)		72 (12.6)	12 (12.6)	
	Stomach, NOS	51 (3.9)	10 (4.1)		13 (2.3)	4 (4.2)	
	Pylorus	131 (10.0)	13 (5.4)		55 (9.6)	8 (8.4)	
	Overlapping lesion of stomach	87 (6.7)	25 (10.3)		31 (5.4)	8 (8.4)	
Histology type (%)	Neuroendocrine carcinoma	53 (4.1)	1 (0.4)	0.003[1]	27 (4.7)	0 (0.0)	0.088
	Signet ring cell carcinoma	238 (18.2)	43 (17.8)		98 (17.1)	14 (14.7)	
	Adenocarcinoma	940 (71.9)	173 (71.5)		409 (71.5)	77 (81.1)	
	Others/unknown	77 (5.9)	25 (10.3)		38 (6.6)	4 (4.2)	
Grade (%)	Well	252 (19.3)	7 (2.9)	< 0.001[1]	125 (21.9)	5 (5.3)	< 0.001[1]
	Moderate	448 (34.3)	85 (35.1)		190 (33.2)	30 (31.6)	
	Poor	608 (46.5)	150 (62.0)		257 (44.9)	60 (63.2)	
Stage (%)	I	53 (4.1)	0 (0.0)	< 0.001[1]	27 (4.7)	0 (0.0)	< 0.001[1]
	IA	1250 (95.6)	0 (0.0)		543 (94.9)	0 (0.0)	
	IB	5 (0.4)	166 (68.6)		2 (0.3)	59 (62.1)	
	IIA	0 (0.0)	54 (22.3)		0 (0.0)	26 (27.4)	
	IIB	0 (0.0)	22 (9.1)		0 (0.0)	10 (10.5)	
T-stage (%)	T1/T1NOS	216 (16.5)	30 (12.4)	< 0.001[1]	101 (17.7)	9 (9.5)	< 0.001[1]
	T1a	514 (39.3)	37 (15.3)		231 (40.4)	19 (20.0)	
	T1b	578 (44.2)	175 (72.3)		240 (42.0)	67 (70.5)	
Tumor size (%)	0-1 cm	313 (23.9)	12 (5.0)	< 0.001[1]	136 (23.8)	3 (3.2)	< 0.001[1]
	< 2 cm	331 (25.3)	51 (21.1)		165 (28.8)	20 (21.1)	
	< 3 cm	241 (18.4)	59 (24.4)		97 (17.0)	16 (16.8)	
	< 4 cm	158 (12.1)	45 (18.6)		70 (12.2)	19 (20.0)	
	< 5 cm	89 (6.8)	28 (11.6)		43 (7.5)	14 (14.7)	
	> 5 cm and more	176 (13.5)	47 (19.4)		61 (10.7)	23 (24.2)	
Primary (%)	No	268 (20.5)	34 (14.0)	0.025[1]	119 (20.8)	20 (21.1)	0.956
	Yes	1040 (79.5)	208 (86.0)		453 (79.2)	75 (78.9)	
Order (%)	One primary only	918 (70.2)	190 (78.5)	0.146	405 (70.8)	69 (72.6)	0.332
	1st of 2 or more primaries	104 (8.0)	16 (6.6)		42 (7.3)	5 (5.3)	

	2nd of 2 or more primaries	215 (16.4)	31 (12.8)		95 (16.6)	16 (16.8)	
	3rd of 3 or more primaries	51 (3.9)	3 (1.2)		23 (4.0)	3 (3.2)	
	4th of 4 or more primaries	14 (1.1)	2 (0.8)		6 (1.0)	1 (1.1)	
	5th of 5 or more primaries	5 (0.4)	0 (0.0)		1 (0.2)	0 (0.0)	
	6th of 6 or more primaries	1 (0.1)	0 (0.0)		0 (0.0)	0 (0.0)	
	7th of 7 or more primaries	0 (0.0)	0 (0.0)		0 (0.0)	1 (1.1)	
Marital status (%)	Married	743 (56.8)	131 (54.1)	0.444	314 (54.9)	57 (60.0)	0.431
	Divorced	80 (6.1)	15 (6.2)		47 (8.2)	11 (11.6)	
	Separated	13 (1.0)	4 (1.7)		7 (1.2)	1 (1.1)	
	Single	165 (12.6)	36 (14.9)		78 (13.6)	13 (13.7)	
	Widowed	251 (19.2)	46 (19.0)		102 (17.8)	9 (9.5)	
	Unmarried or Domestic Partner	2 (0.2)	2 (0.8)		0 (0.0)	0 (0.0)	
	Unknown	54 (4.1)	8 (3.3)		24 (4.2)	4 (4.2)	
Insurance (%)	Insured	784 (59.9)	149 (61.6)	0.515	345 (60.3)	52 (54.7)	0.771
	Insured/no specifics	234 (17.9)	34 (14.0)		104 (18.2)	19 (20.0)	
	Any medicaid	243 (18.6)	48 (19.8)		101 (17.7)	21 (22.1)	
	Uninsured	28 (2.1)	8 (3.3)		11 (1.9)	2 (2.1)	
	Insurance status unknown	19 (1.5)	3 (1.2)		11 (1.9)	1 (1.1)	

[1]It means statistically significant.
LMN: Lymph node metastasis; EGC: Early gastric cancer.

Table 3 Multivariate analysis for lymph node metastasis in training set with early gastric cancer

Variables	*P* value	OR	95%CI
Age	0.012	0.986	0.975-0.997
Histology type	0.019	1.382	1.057-1.813
Grade	0.000	1.825	1.452-2.315
T-stage	0.000	1.985	1.596-2.494
Size	0.000	1.319	1.208-1.442
Primary	0.152	1.344	0.907-2.040

95%CI: 95% confidence interval; OR: Odds ratio.

Based on Japanese Gastric Cancer Treatment Guidelines 2018[17], EGC can be treated by EMR or ESD, with acceptable results in the west[18]. EMR is primarily indicated for mucosal cancers without ulcer and with a mucosal diameter of ≤ 2 cm to be excised, which was the first endoscopic treatment for EGC. Compared to EMR, ESD is not limited by tumor size or ulceration, which is facilitate curative tumor resection[19]. The operation is judged to be a radical resection if all of the followings are met: en bloc resection, intestinal-differentiated-type, pathological-T1a, tumor size ≤ 2 cm, negative surgical cut edge (both lateral and vertical), and absence of lymphovascular invasion[20].

LNM has a clear correlation with poor prognosis in patients with EGC[21]. The presence or absence of LNM determines the choice of treatment. Precisely predicting the presence or absence of LNM in EGC patients helps to select the best treatment modality, which is of great importance in the clinical treatment process. Therefore, construction of the prediction model for EGC patients may help find those who were be prone to LNM and prolong survival time after surgery[22].

The process of nomogram development was clarified in previous study[22]. In our study, age when EGC is confirmed (OR: 0.003, $P = 0.012$), histology type (OR: 1.382, $P = 0.019$), grade (OR: 1.825, $P < 0.001$), T-stage (OR: 1.985, $P < 0.001$), and tumor size (OR: 1.319, $P < 0.001$) were independent influenced variables for LNM. Those variables were used to construct the predict model. Our clinical prediction models are more believable and more convincing because they are internally validated and externally

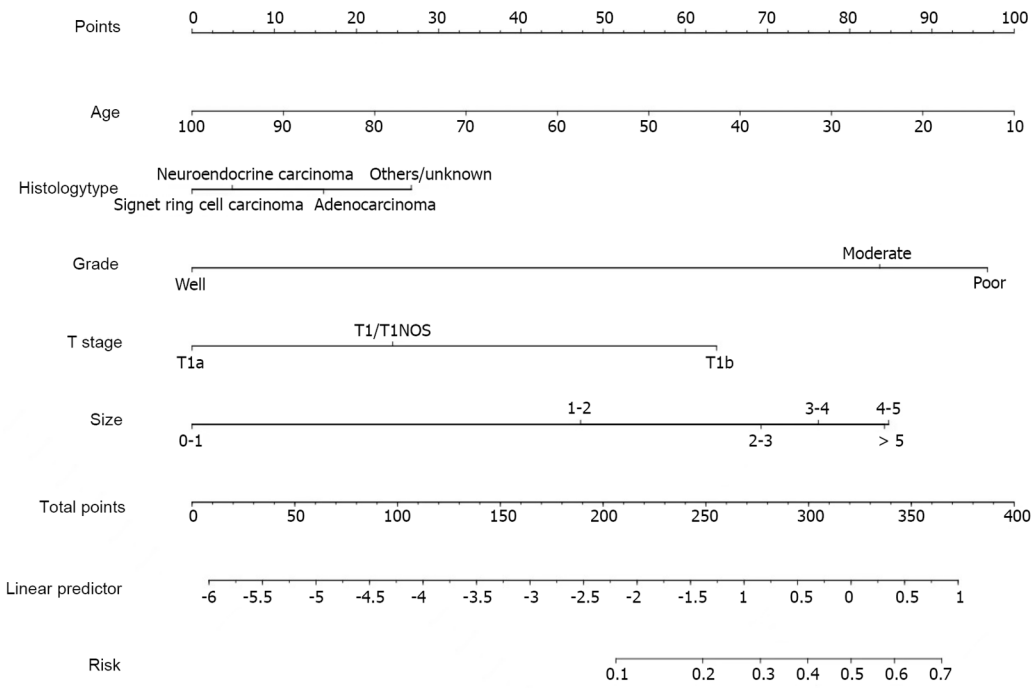

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Figure 2 Nomogram prediction model for lymph node metastasis in early gastric cancer patients.

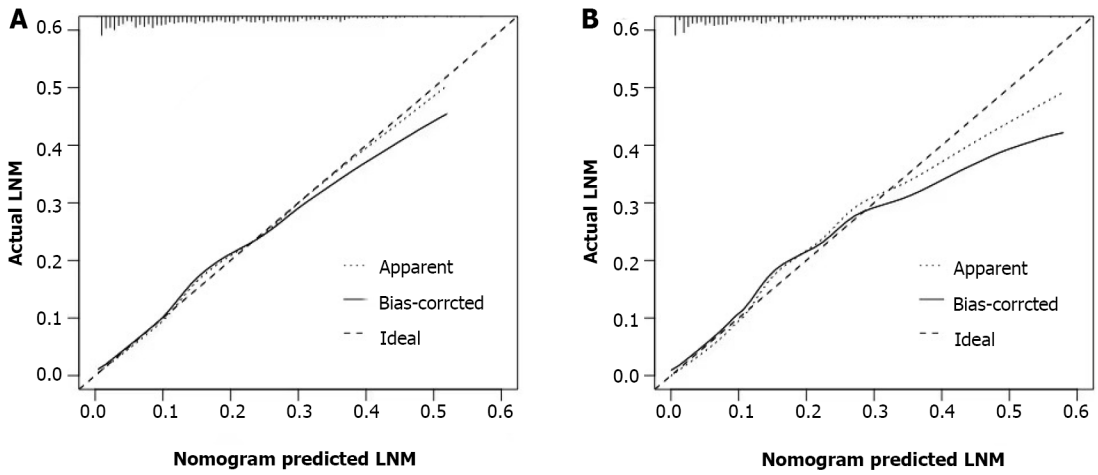

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Figure 3 Calibration curve of the nomogram prediction model for early gastric cancer patients. A: Internal validations for the nomogram prediction model for the training set of early gastric cancer (EGC) patients; B: External validations for the nomogram prediction model for the testing set of EGC patients. LNM: Lymph node metastasis.

validated.

Among the categorical data, the degree of differentiation is the most important influencing factor, which was consistent with previous findings[23]. Xiang *et al*[24] indicated that miR-145-5p was capable to induce the differentiation of GC and affect the LNM of GC.

Early-stage cancers less than 4 cm have a very low LNM rate and can be evaluated for local excision [25]. Other study showed that tumor with large diameter and deep invasion were independent risk factors for LNM[26]. Sekiguchi *et al*[27] reported that tumor with large diameter, depth, and histological type were confirmed to be the independent influencing element of LNM.

Besides, age at diagnosis, tumor size, T-stage, and histology type were also the independent influenced variables for LNM. Gurzu *et al*[28] found that in younger patients with GC, the expression of VEGF is more active, which increases the probability of tumor invasion and LMN in GC. Bao *et al*[29]

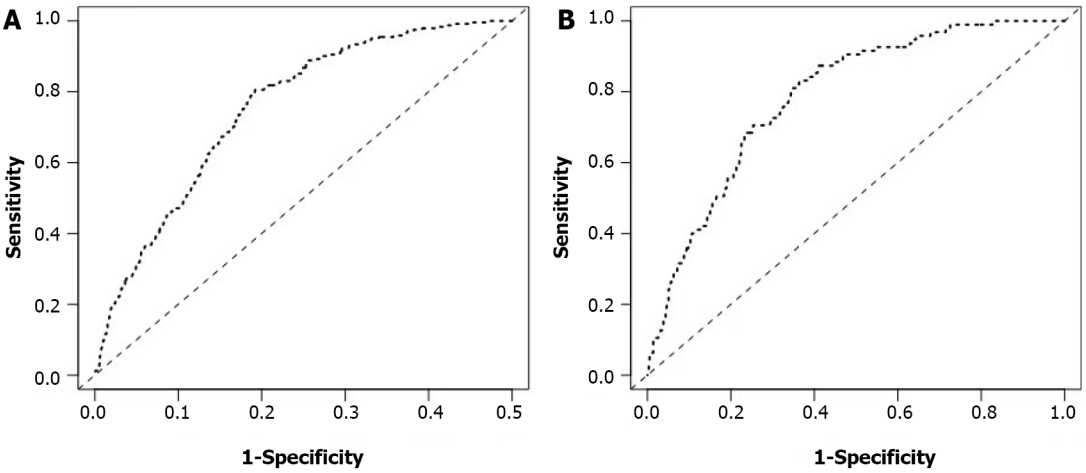

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Figure 4 Receiver operating characteristic curve of the nomogram prediction model for early gastric cancer patients. A: Internal validations for the nomogram prediction model for the training set of early gastric cancer (EGC) patients; B: External validations for the nomogram prediction model for the testing set of EGC patients.

argued that increased expression of MDM4 could correlate with LNM and lead to poorer survival status of GC especially in younger patients. Park *et al*[30] study revealed that the tissue of GC is more invasive in younger patients than in older patients. The LNM rates in young EGC patients were higher than in other patients probably related to the higher malignant potential of their tumors[31].

This is the fact that tumor infiltrating into the submucosa of the stomach is related to the increased significantly incidence of LNM[32,33], which our study came to the similar findings. Radical surgical resection and lymph node dissection are suitable for deeply infiltrated GC[34].

However, our research has some limitations. First of all, only patients with EGC who underwent surgery were included in this study for retrospective analysis Secondly, "others/unknown" expanded applicability of the predicted model which could be influenced the precision of the model. Thirdly, the molecular pathologic characteristics, family history, and *H. pylori* infection are not enrolled in analysis.

CONCLUSION

Age at diagnosis, histology type, grade, T-stage, and tumor size were independent risk variables for LNM in EGC. Based on these, the predictive model was built for predicting possibilities of LNM in EGC patients. Both internal and external validation proved the credibility and persuasiveness which demonstrated by the receiver operating characteristic and the calibration curve.

ARTICLE HIGHLIGHTS

Research background

Lymph node metastasis (LNM) has a major influence on the postoperative survival status of patients with early gastric cancer.

Research motivation

Our aim was to improve early gastric cancer (EGC) patients' prognosis.

Research objectives

To improve EGC patients' prognosis.

Research methods

Clinical information and pathology data of 2217 EGC patients were collected and analyzed. Based on a 7:3 ratio, 1550 people were grouped to training sets and 667 people were assigned to testing sets, randomly. The predictive model was built based on the training set for predicting possibilities of LNM in EGC patients. Both internal and external validation proved the credibility and persuasiveness which demonstrated by the receiver operating characteristic (ROC) and the calibration curve.

Research results

Age at diagnosis, histology type, grade, T-stage, and size were risk factors of LNM for EGC. Besides, nomogram was drawn to predict the risk of LNM for EGC patients. Among the categorical variables, the effect of grade (well, moderate, and poor) was the most significant prognosis factor. For training sets and testing sets, respectively, area under the receiver-operating characteristic curve of nomograms were 0.751 [95% confidence interval (CI): 0.721-0.782] and 0.786 (95%CI: 0.742-0.830). In addition, the calibration curves showed that the prediction model of LNM had good consistency.

Research conclusion

Based on these independent risk variables, the predictive model was built for predicting possibilities of LNM in EGC patients. Both internal and external validation proved the credibility and persuasiveness which demonstrated by the ROC and the calibration curve.

Research perspectives

We analyzed the independent influenced variables for LNM in EGC patients. Based on the independent risk factors, the prediction model was plotted. After internal validation and external validation, the ROC and the calibration curve were built, which validated the credible and persuasive of the nomogram.

FOOTNOTES

Author contributions: Jiang XC, Yao XB, Xia HB, and Su YZ provided the databases, conducted the statistical analysis, and drafted the manuscript; Jiang XC, Yao XB, Xia HB, and Su YZ made the contribution to the main work equally and share the first authorship; Luo PQ, Sun JR analyzed the data and revised the manuscript; Song ED helped them; Xu AM, Wei ZJ, Zhang LX, and Lan YH designed the main study and critically revised the manuscript; Xu AM, Wei ZJ, Zhang LX, and Lan YH are all the correspondence author; all authors read and approved the final manuscript.

Institutional review board statement: Institutional review board statement was not acquired since data were obtained from the SEER database that covering approximately 28% of the cases in the United States.

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

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

Data sharing statement: The data can be obtained from the correspondence. The collection of patient information did not require informed consent nor institutional review because such information was publicly available.

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

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Retrospective Cohort Study

Role of adjuvant chemotherapy on recurrence and survival in patients with resected ampulla of Vater carcinoma

Se Jun Park, Kabsoo Shin, In-Ho Kim, Tae Ho Hong, Younghoon Kim, Myung-ah Lee

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Abstract

BACKGROUND

Owing to rarity of disease and lack of prospective studies, data supporting the role of adjuvant chemotherapy in ampulla of Vater (AoV) carcinoma is limited.

AIM

To evaluate whether adjuvant chemotherapy cases for AoV carcinoma had better disease-free survival (DFS) rates than cases of observation following curative surgery.

METHODS

We retrospectively analyzed the association between adjuvant chemotherapy and DFS and overall survival (OS) in patients with stage IB-III AoV carcinoma who underwent curative surgical resection. Fluorouracil-based adjuvant chemotherapy was administered after surgery at the discretion of the physician. Adjusted multivariate regression models were used to evaluate the association between adjuvant chemotherapy and survival outcomes.

RESULTS

Of the total 104 patients who underwent curative surgery, 52 received adjuvant chemotherapy. Multivariate analysis revealed that higher histologic grade [hazard ratio (HR) = 2.24, P = 0.046], advanced tumor stage (HR = 1.85, P = 0.030), and vascular invasion (HR = 2.14, P = 0.010) were associated with shorter DFS. Adjuvant chemotherapy improved DFS compared to the observation group (HR =

0.50, P = 0.015) and tended to be associated with a longer OS, although the difference was not statistically significant (HR = 0.58, P = 0.098).

CONCLUSION

Among patients with resected AoV carcinoma, the adjuvant chemotherapy group was not associated with a significant survival benefit compared to the observation group. However, on multivariate analysis adjusting for prognostic factors, adjuvant chemotherapy following surgery was an independent prognostic factor for DFS in patients with resected AoV carcinoma. Further studies are needed to investigate the effectiveness of adjuvant chemotherapy according to histologic phenotype.

Key Words: Ampulla of Vater carcinoma; Adjuvant chemotherapy; Prognosis; Recurrence

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Core Tip: To date, there is no standard adjuvant treatment for patients with ampulla of Vater (AoV) carcinoma after surgical resection. In this study, we evaluated the effectiveness and safety of adjuvant fluorouracil-based chemotherapy. Of 104 patients investigated, 52 were received 5-fluorouracil-based adjuvant chemotherapy and 52 were observed without any adjuvant treatment. Adjuvant chemotherapy could improve disease-free survival in patients with AoV cancer following surgery, but overall survival was not associated with adjuvant chemotherapy. Treatment related adverse events were manageable. Further studies are warranted to identify patients with resected AoV cancer who might benefit from adjuvant chemotherapy.

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INTRODUCTION

The ampulla of Vater (AoV) carcinoma arises in the mucosa of the common channel composed of the confluence of the pancreatic and common bile ducts (CBD). Thus, it is difficult to differentiate from malignancies originating in the pancreatic head, distal CBD, or duodenum. AoV carcinoma is a rare form of periampullary cancer, accounting for approximately 7% of cases[1]. Surgical resection remains the primary curative approach for patients with localized AoV carcinoma; however, recurrence is frequent, and the 5-year survival rate following resection is 45%[2].

Various clinicopathological features are known to be associated with recurrence and poor prognosis, including advanced tumor stage, regional lymph node metastasis, poorly differentiated tumors, perineural invasion, and pancreatobiliary histology[3,4]. Given the rarity of this disease, there is absence of large-scale controlled trials investigating adjuvant treatment for patients with AoV cancer. Till date, no standard adjuvant treatment has been accepted globally for resected AoV cancers with a high risk of recurrence. Several previous retrospective studies have shown a significant benefit of adjuvant treatment in selected patients with stage IIB or higher disease[5]. Similarly, patients with regional lymph node involvement may have a survival benefit with adjuvant chemoradiation compared to those with surgery alone[6]. Although there is no clear guideline for adjuvant treatment, concurrent chemoradiation is often performed for patients with resected AoV cancer who have stage IB or higher disease [1]. However, in a previous study that included 113 patients with resected AoV cancer, adjuvant concurrent fluorouracil chemoradiation did not improve survival outcomes or decrease recurrence rates [7]. In terms of adjuvant chemotherapy, European Study Group for Pancreatic Cancer (ESPAC-3), the largest phase III randomized trial to investigate the role of adjuvant chemotherapy in resected periampullary adenocarcinoma, included 428 patients with periampullary carcinoma (297 patients with ampullary cancer) who were randomly assigned to each treatment group: observation, fluorouracil/leucovorin (FL), or gemcitabine. Among patients with AoV cancer, those who received adjuvant gemcitabine treatment had better survival outcomes than those in the observation group[8]. However, data supporting the role of adjuvant chemotherapy from the ESPAC-3 trial are limited because the trial enrolled patients with biliary tract or other periampullary cancers, in addition to AoV cancer. The inclusion of a significant number of patients with stage I or IVA cancer was another limitation.

In this study, we investigated the association between clinicopathological features and recurrence or mortality in patients with resected AoV cancers. Furthermore, we examined the role of adjuvant chemotherapy in patients with resected true AoV cancers.

MATERIALS AND METHODS

Patients

We reviewed the medical records of patients with histologically confirmed adenocarcinoma of the AoV who underwent curative pancreaticoduodenectomy with regional lymph node dissection at the Catholic University of Korea, Seoul St. Mary's Hospital between June 01, 2008 and December 31, 2020. Patients aged ≥ 19 years were eligible for this study based on the following criteria: (1) Histologically confirmed adenocarcinoma of the AoV; (2) pathological stage IB-III according to the American Joint Committee of Cancer Staging (AJCC), 8th edition[9]; and (3) recurrence and survival confirmation at the time of data collection. Patients with the following conditions were excluded: (1) Pathological tumor, node, metastasis (TNM) stage IA and IV; (2) no regional lymph node examination; (3) macroscopically remaining tumors (R2 resection); (4) received previous preoperative chemotherapy; (5) did not fully recover from surgery; and (6) newly diagnosed with secondary malignancies after surgery for AoV cancer.

Treatment

Pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy with standard lymph node dissection was performed at the surgeon's discretion. The decision to administer adjuvant chemotherapy following surgery and the regimen of adjuvant chemotherapy to be followed were at the physician's discretion; patients received adjuvant FL or fluorouracil and cisplatin (FP) within 12 wk of surgery. In the FL group, patients received a bolus of 20 mg/m^2 leucovorin, followed by a 2-h infusion of 425 mg/m^2 fluorouracil for five consecutive days every 28 d for six cycles (24 wk). In the FP group, each cycle consisted of cisplatin at a dose of 70 mg/m^2 (day 1 of every cycle) delivered as a 1-h intravenous infusion, followed by fluorouracil at a dose of 1000 mg/m^2 (per day) administered by intravenous infusion over 8-h for three consecutive days every 28 d for six cycles (24 wk). The chemotherapy dose and schedule modifications were at the physician's discretion. The relative dose intensity (RDI) of chemotherapy was defined as the ratio of the delivered dose to the planned dose and presented as a percentage. Adverse events were assessed according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 4.03.

Surveillance

Patients were evaluated every three months after surgery for two years, every six months for the following three years, and annually thereafter. Imaging evaluations were performed using computed tomography with contrast, and carbohydrate antigen 19-9 (CA 19-9) levels were also checked at every visit. If any evidence suggested the possibility of recurrence, additional imaging or biopsy was performed to confirm recurrence.

Statistical analysis

Descriptive statistics are reported as proportions and medians with ranges. Categorical variables were compared using the Chi-squared or Fisher's exact test, and continuous variables were compared using Student's t-test. Disease-free survival (DFS) was defined as the interval between curative surgery and recurrence or death from any cause. Overall survival (OS) was estimated from the date of surgery to the time of the last follow-up or cancer-related deaths. Survival outcomes were estimated using the Kaplan-Meier method and compared using a two-tailed log-rank test. Multivariable regression based on the Cox proportional hazard model was used to estimate the effect of clinicopathological factors as prognosticators of DFS and OS. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated for each factor. All tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance. The number of patients receiving adjuvant treatment and the relative dose intensity of the total doses received are reported. The number of patients experiencing treatment-related adverse events was reported as a percentage of the total number of patients in each treatment group. All statistical analyses were performed using SPSS for Windows (version 24.0; IBM SPSS Inc., Armonk, NY, United States) and GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, United States).

RESULTS

Characteristics of the patients

Between June 1, 2008 to December 31, 2020, 104 patients were eligible for this study. The clinical characteristics and pathological details of the patients are shown in Table 1. The median age was 64 years

Table 1 Clinicopathological characteristics of patients with surgically treated ampulla of Vater carcinoma according to the adjuvant treatment, *n* (%)

Variables	Total (*n* = 104)	AC (*n* = 52)	Surgery alone (*n* = 52)	*P* value
Age, median (range)	64 (49-83)	69 (62-83)	64 (49-83)	0.924
Gender				
Male	58 (55.8)	33 (56.9)	25 (43.1)	0.114
Female	46 (44.2)	19 (41.3)	27 (58.7)	
Tumor size (cm), mean ± SD	2.5 ± 1.1	2.6 ± 1.3	2.4 ± 1.0	0.314
Resection status				
R0	102 (98.0)	50 (49.0)	52 (51.0)	0.495
R1	2 (2.0)	2 (100)	0	
Histologic grading				
Grade 1/2	94 (90.3)	45 (47.9)	49 (52.1)	0.319
Grade 3	10 (9.7)	7 (70.0)	3 (30.0)	
Tumor stage				
T1-2	56 (53.8)	23 (41.1)	33 (58.9)	0.049
T3-4	48 (46.2)	29 (60.4)	19 (39.6)	
Node stage				
N0	45 (43.3)	14 (31.1)	31 (68.9)	0.001
N1-2	59 (56.7)	38 (64.4)	21 (35.6)	
TNM stage[1]				
Stage IB	30 (28.8)	8 (26.7)	22 (73.3)	0.002
Stage II	15 (14.4)	6 (40.0)	9 (60.0)	
Stage III	59 (56.8)	38 (64.4)	21 (35.6)	
Lymphatic invasion				
No	42 (40.4)	13 (31.0)	29 (69.0)	0.001
Yes	62 (59.6)	39 (62.9)	23 (37.1)	
Vascular invasion				
No	81 (77.9)	34 (42.0)	47 (58.0)	0.002
Yes	23 (22.1)	18 (78.3)	5 (21.7)	
Perineural invasion				
No	74 (71.2)	32 (43.2)	42 (56.8)	0.030
Yes	30 (28.8)	20 (66.7)	10 (33.3)	
Preoperative CA19-9 level				
Within normal (< 40 U/mL)	45 (43.3)	22 (48.9)	23 (51.1)	0.516
Above normal (≥ 40 U/mL)	43 (41.3)	24 (55.8)	19 (44.2)	
Missig data	16 (15.4)			

[1]According to American Joint Committee on Cancer 8th edition. AC: Adjuvant chemotherapy; TNM: Tumor, node, metastasis; CA 19-9: Carbohydrate antigen 19-9.

(range, 49-83) and 58 patients (55.8%) were male. Most patients had negative surgical margins and only two patients (2.0%) had R1 resection status. A total of 30 patients (28.8%) had stage IB, 15 (14.4%) had stage II, and 59 (56.8%) had stage III disease. Preoperative serum CA 19-9 Levels were elevated in 43 patients (41.3%) at the time of diagnosis. Of the 104 patients, half (50.0%) received adjuvant chemotherapy and the other half (50.0%) underwent surgery alone. Patient demographics, such as age,

sex, tumor size, histologic grade, and elevated preoperative CA 19-9, were similar between the two groups. With respect to pathological details, patients in the adjuvant chemotherapy group had a more advanced tumor (T3-4, 60.4% *vs* 39.6%, P = 0.049), node (N1-2, 64.4% *vs* 35.6%, P = 0.001), and TNM (stage III, 64.4% *vs* 35.6%, P = 0.002) stage than those in the surgery-alone group. Lymphatic (62.9% *vs* 37.1%, P = 0.001), vascular (78.3% *vs* 21.7%, P = 0.002), and perineural (66.7% *vs* 33.3%, P = 0.030) invasion were also significantly more common in the adjuvant chemotherapy group than in the surgery-alone group.

Survival outcomes

The median follow-up time was 30.2 mo (95%CI: 24.16-42.20). Recurrence and cancer-related deaths occurred in 64 (61.5%) and 49 (47.1%) patients, respectively. The median DFS of all patients was 16.4 mo (95%CI: 10.8-22.0) and the DFS rates at 6 mo, 1 year, and 2 years were 84.5% (95%CI: 75.9-90.2), 62.2% (95%CI: 51.7-71.0), and 40.5% (95%CI: 30.3-50.4), respectively. Among patients with confirmed recurrence, most recurrence events (56 patients, 87.5%) occurred within 2 years after surgery. The median OS of all patients was 55.0 mo (95%CI: 38.9-71.0) and estimated OS rates were 94.8% (95%CI: 87.9-97.8) at 1 year, 60.7% (95%CI: 49.4-70.2) at 3 years, and 44.1% (95%CI: 32.1-55.4) at 5 years.

Survival analysis by staging revealed recurrence in 13 (43.3%) of 30 patients with stage IB disease, 12 (80.0%) of 15 patients with stage II disease, and 39 (66.1%) of 59 patients with stage III disease. Cancer-related death events in stages IB, II, and III disease were observed in 9 (30.0%) of 30 patients, 9 (60.0%) of 15 patients, and 30 (50.8%) of 59 patients, respectively. In patients with stage IB disease, the median DFS was 34.8 mo (95%CI: 3.2-66.4) and the DFS rates at 1 year and 2 years were 81.7% (95%CI: 61.3-92.0) and 59.9% (95%CI: 37.9-76.3), respectively (Figure 1A). The median DFS was 10.8 (95%CI: 7.2-14.4) and 15.4 (95%CI: 10.5-20.3) months in patients with stage II and III disease, respectively (Figure 1A). DFS rates at 1 year and 2 years in patients with stage II disease were 40.0% (95%CI: 16.5-62.8) and 26.7% (95%CI: 8.3-49.7), and 59.4% (45.4-70.9) and 35.5% (95%CI: 22.8-48.4) in patients with stage III disease. The median DFS was numerically better in patients with a lower stage of disease than in those with a higher stage, although the difference was not statistically significant (Stage IB *vs* II, P = 0.062; stage IB *vs* III, P = 0.094; stage II *vs* III, P = 0.405). The median OS of patients with stage IB, II, and III disease was 106.7 (95%CI: 36.9-176.5), 37.8 (95%CI: 27.1-48.5), and 45.6 (95%CI: 23.5-67.8) mo, respectively (Figure 1B). Estimated survival rates at 3 and 5 years were 81.0% (95%CI: 56.9-92.4) and 56.3% (95%CI: 30.1-76.0) for stage IB, 50.8% (95%CI: 23.1-73.1) and 42.3% (95%CI: 16.5-66.2) for stage II, and 54.2% (95%CI: 39.3-66.9) and 39.0% (95%CI: 23.9-53.9) for stage III disease, respectively.

Adjuvant chemotherapy and survival outcomes

The median DFS was 17.2 mo (95%CI: 11.0-23.3) in the adjuvant chemotherapy group, as compared with 13.0 mo (95%CI: 2.38-23.7) in the surgery-alone group (P = 0.536, Figure 2A). DFS rates at 6 mo, 1 year, and 2 years were 90.4% (95%CI: 78.4-95.9), 67.7% (95%CI: 52.7-78.9), and 40.8% (95%CI: 26.6-54.5), respectively in the adjuvant chemotherapy group, as compared with 78.5% (95%CI: 64.5-87.9), 56.6% (95%CI: 41.4-69.3), and 39.6% (95%CI: 25.2-53.6), respectively in the surgery-alone group. On multivariable analysis, patients who received adjuvant chemotherapy had longer DFS than those treated with surgery alone (HR = 0.50; 95%CI: 0.29-0.88; P = 0.015, Table 2). In addition, poorly differentiated histology (HR = 2.24; 95%CI: 1.02-4.95; P = 0.046), advanced tumor stage (HR = 1.85; 95%CI: 1.06-3.22; P = 0.030), and vascular invasion (HR = 2.14; 95%CI: 1.20-3.79; P = 0.010) were associated with shorter DFS after multivariate analysis (Table 2). The median OS was 43.3 mo (95%CI: 15.2-71.4) in the adjuvant chemotherapy group, as compared with 55.0 mo (95%CI: 39.8-70.1) in the surgery-alone group (P = 0.894, Figure 2B). The OS rate at 3 years was 56.1% (95%CI: 39.9-69.5) in the adjuvant chemotherapy group and 65.3% (95%CI: 49.0-77.5) in the surgery-alone group. Multivariate analysis showed that older age at diagnosis (HR = 2.16, 95%CI: 1.18-3.98, P = 0.013) and higher tumor stage (HR = 2.50, 95%CI: 1.31-4.80, P = 0.006) were significantly associated with poor survival outcomes; however, adjuvant chemotherapy was not significantly associated with OS (HR = 0.58, 95%CI: 0.30-1.11, P = 0.098, Table 3). In stage II or III patients, although the difference was not significant, the median DFS was numerically better in the adjuvant chemotherapy group than in the surgery-alone group (median DFS 16.4 mo *vs* 10.9 mo; P = 0.058, Figure 2C). The median OS did not differ between two groups (median OS 63.5 mo *vs* 40.0 mo, P = 0.372, Figure 2D).

Adjuvant chemotherapy

The median treatment duration was 24.1 wk (range, 7.9-29.0) in the FL group and 24.1 wk (range, 7.7-29.6) in the FP group (Table 4). The median number of cycles was six (range, 2-6) in both the FL and FP groups. The RDI was 0.83 (range, 0.27-1.00) in the FL group and 0.85 (range, 0.33-1.00) in the FP group. A total of 20 patients (66.7%) in the FL group and 16 patients (72.7%) in the FP group received all planned cycles of chemotherapy. Permanent treatment discontinuation due to intolerance occurred in five patients (16.7%) in the FL group and four patients (18.3%) in the FP group.

Adverse events

Grade 3 or 4 adverse events were reported in 2 of 30 patients (6.7%) in the FL group and in 7 of 22

Table 2 Univariate and multivariate analyses of the clinicopathologic findings and adjuvant chemotherapy for disease-free survival in patients with ampulla of Vater carcinoma

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age ≥ 70 yr (*vs* < 70 yr)	1.57 (0.91-2.70)	0.102		
Histologic grade 3 (*vs* grade 1-2)	2.66 (0.98-7.18)	0.054	2.24 (1.02-4.95)	0.046
Tumor stage 3 or 4 (*vs* stage 1 or 2)	1.99 (1.21-3.28)	0.007	1.85 (1.06-3.22)	0.030
Nodal metastasis (*vs* none)	1.24 (0.76-2.03)	0.390	1.10 (0.63-1.95)	0.736
Lymphatic invasion (*vs* none)	1.76 (1.07-2.90)	0.025	1.49 (0.80-2.80)	0.212
Vascular invasion (*vs* none)	2.98 (1.53-5.80)	0.001	2.14 (1.20-3.79)	0.010
Perineural invasion (*vs* none)	1.47 (0.85-2.55)	0.164	0.97 (0.55-1.71)	0.901
Received AC (*vs* none)	0.85 (0.52-1.40)	0.536	0.50 (0.29-0.88)	0.015

HR: Hazard ratio; AC: Adjuvant chemotherapy.

Table 3 Univariate and multivariate analyses of the clinicopathologic findings and adjuvant chemotherapy for overall survival in patients with ampulla of Vater carcinoma

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Age ≥ 70 yr (*vs* < 70 yr)	2.66 (1.40-5.07)	0.003	2.16 (1.18-3.98)	0.013
Histologic grade 3 (*vs* grade 1-2)	1.91 (0.67-5.49)	0.228	1.93 (0.77-4.81)	0.159
Tumor stage 3 or 4 (*vs* stage 1 or 2)	2.32 (1.31-4.11)	0.004	2.50 (1.31-4.80)	0.006
Nodal metastasis (*vs* none)	1.36 (0.77-2.39)	0.284	1.09 (0.58-2.04)	0.799
Lymphatic invasion (*vs* none)	1.50 (0.84-2.65)	0.168	1.23 (0.62-2.45)	0.553
Vascular invasion (*vs* none)	2.89 (1.35-6.20)	0.006	1.93 (0.96-3.88)	0.064
Perineural invasion (*vs* none)	2.17 (1.13-4.17)	0.020	1.27 (0.66-2.45)	0.477
Received AC (*vs* none)	1.04 (0.59-1.82)	0.018	0.58 (0.30-1.11)	0.098

HR: Hazard ratio; AC: Adjuvant chemotherapy.

patients (31.8%) in the FP group (P = 0.033, Table 5). No serious adverse events resulted in death in either group. Grade 3 or 4 hematological toxicities were observed only in the FP group (n = 5, 22.7%). The most frequent adverse events were stomatitis (n = 11, 36.7%), fatigue (n = 9, 30.0%), and anemia (n = 8, 26.7%) in the FL group whereas fatigue (n = 19, 86.4%), nausea (n = 19, 86.4%), and neutropenia (n = 14, 63.6%) were the most frequent adverse events in the FP group. All grades of adverse events, including nausea, fatigue, neutropenia, and thrombocytopenia, were observed significantly more frequently in the FP group than in the FL group.

DISCUSSION

In this trial involving patients with stage IB-III resected adenocarcinoma of the AoV, adjuvant chemotherapy was significantly associated with longer DFS than surgery alone in multivariate analysis; however, there was no significant association with OS. Discontinuation of treatment due to intolerance to chemotherapy occurred at a similar rate in both groups. As there is no clear evidence of clinical benefit with the use of fluorouracil-based chemotherapy in combination with cisplatin, the FL regimen, which is associated with lower toxicity, may be a more feasible option for elderly patients or those with a relatively poor performance status.

Given the rarity of this disease, the availability of large-scale randomized controlled trials investigating adjuvant treatments is limited, and as a result, there is currently no consensus regarding the effectiveness of adjuvant treatments following surgery. Although the ESPAC-3 trials demonstrated a

Table 4 Relative dose intensity and dose modification of the adjuvant chemotherapy

	FL (*n* = 30)	FP (*n* = 22)
Duration of treatment, weeks, median (range)	24.1 (7.9-29.0)	24.1 (7.7-29.6)
Cycles of drug administration, median (range)	6 (2-6)	6 (2-6)
Relative dose intensity, mean (range)	0.83 (0.27-1.00)	0.85 (0.33-1.00)
Received all cycles of chemotherapy, *n* (%)	20 (66.7)	16 (72.7)
Dose reduction or interruption, *n* (%)	10 (33.3)	10 (45.5)
Discontinued adjuvant chemotherapy, *n* (%)	10 (33.3)	6 (27.3)
Relapse	5 (16.7)	2 (9.0)
Intolerance	5 (16.7)	4 (18.3)

FL: Fluorouracil/leucovorin; FP: Fluorouracil/cisplatin.

Table 5 Adverse events during treatment

Adverse event	FL (*n* = 30)		FP (*n* = 22)		*P* value[1]
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	
Stomatitis	11 (36.7)	0	3 (13.6)	0	0.112
Nausea	6 (20.0)	0	19 (86.4)	0	< 0.001
Vomiting	1 (3.3)	0	4 (18.2)	0	0.149
Diarrhea	2 (6.6)	1 (3.3)	0	0	0.253
Fatigue	8 (26.7)	1 (3.3)	19 (86.4)	0	< 0.001
Neutropenia	3 (10.0)	0	10 (45.5)	4 (18.2)	< 0.001
Febrile neutropenia	0	0	0	2 (9.0)	0.174
Anemia	8 (26.7)	0	10 (45.5)	1 (4.5)	0.084
Thrombocytopenia	2 (6.6)	0	8 (36.4)	0	0.012
Increased AST/ALT level	4 (13.3)	0	0	0	0.128

[1]Comparison of the proportion of all grades of adverse events. FL: Fluorouracil/leucovorin; FP: Fluorouracil/cisplatin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

survival benefit with adjuvant chemotherapy in a multivariable analysis, the heterogeneous clinicopathological features of the study population and the modest effect of the adjuvant chemotherapy warrant further investigation[8]. Previous retrospective studies have suggested that patients who received adjuvant chemotherapy tended to have better DFS and OS compared to those who did not receive adjuvant treatment; however, these differences were not statistically significant[4,10]. The studies were limited by the inclusion of a relatively large number of stage IA patients and the use of various adjuvant chemotherapy regimens, which may have influenced the outcomes. Patients with stage IA disease were excluded from this study as they are unlikely to experience relapse and do not typically receive adjuvant treatment. During the same period as the study, 33 patients were diagnosed with stage IA AoV cancer, and only two (6.0%) of these patients experienced recurrence. These findings may aid in the selection of patients who would benefit from adjuvant chemotherapy following surgery.

Contrary to expectations, there was no significant difference in DFS and OS between stage IB-II and stage III disease in this study. Furthermore, lymph node metastasis, a known risk factor for recurrence and poor survival, was not associated with recurrence or cancer-related death in multivariate analysis. These unexpected results may be due to the higher proportion of patients with stage III disease receiving adjuvant chemotherapy than those with stage IB or II disease. Moreover, according to the AJCC 8th staging system, involvement of one or more lymph nodes in the pathological findings, regardless of tumor stage, is indicative of at least stage III disease. For optimal node staging, it is important to harvest sufficient lymph nodes during surgery; however, in this study, a relatively low number of lymph nodes was harvested in patients with stage IB or II. In patients with pancreatic cancer, it has been recommended that at least 11-17 Lymph nodes be examined to provide accurate nodal

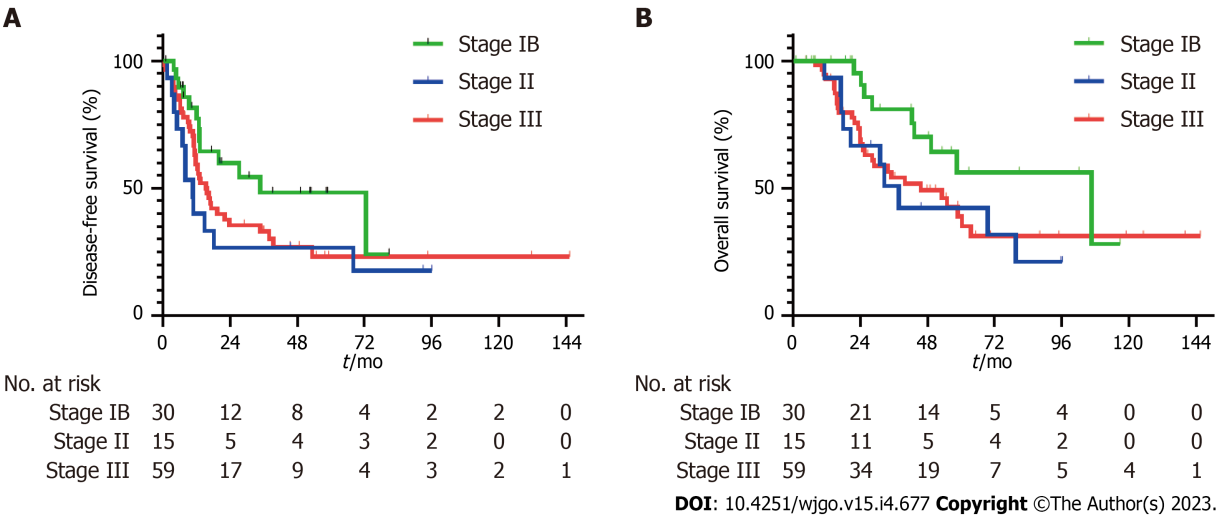

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Figure 1 Kaplan-Meier estimates of disease-free survival (A) and overall survival (B) in patients with ampulla of Vater cancer according to the tumor, node, metastasis stage.

staging[11,12]. However, less than 11 Lymph nodes were harvested in 28 (62.2%) of 45 patients with stage IB or II from this study; this result suggests that the stage of a significant portion of patients with stage IB or II might have been underestimated. A significant proportion of stage IB or II patients were understaged and did not receive adjuvant chemotherapy. These might be potential confounders that influence the effectiveness of adjuvant chemotherapy.

In the absence of consensus guidance for adjuvant treatment for physicians in real-world settings, there has been a tendency to determine the treatment regimen for patients with AoV cancer based on whether the tumor had an intestinal or pancreatobiliary histological subtype[13,14]. Notably, AoV cancer with pancreato-biliary phenotype has a worse outcome than those with intestinal phenotype[3, 15]. According to the ESPAC-3 trial, there were no statistically significant differences in response to adjuvant treatment between the group of patients with pancreato-biliary phenotype and the group of intestinal phenotype[8]. However, more than half of the patients (162 of 297 patients, 54.5%) were classified as having an intermediate phenotype and were excluded from the analysis; therefore, further studies are needed to evaluate the treatment strategy according to the histologic subtypes. For patients with resected pancreatic ductal adenocarcinoma, combination chemotherapy with fluorouracil, leucovorin, irinotecan, and oxaliplatin (modified FOLFIRINOX), and gemcitabine with capecitabine have been used as standard adjuvant treatments following surgery[16,17]. For patients with resected biliary tract cancer, adjuvant capecitabine following surgery can improve OS according to the per-protocol analysis[18]. These published prospective studies suggest that fluorouracil-based chemotherapy might have a role in resected AoV carcinoma with a pancreatobiliary phenotype. Fluorouracil-based adjuvant treatment might also provide benefits for AoV carcinoma with an intestinal phenotype because FL, FL with oxaliplatin (FOLFOX), or capecitabine with oxaliplatin (CAPOX) have already been used as standard adjuvant treatments in patients with colon cancer, which is similar to the intestinal type of AoV cancer[19,20]. In addition, chemotherapy regimens, such as gemcitabine plus cisplatin or CAPOX, which have shown survival benefit in patients with advanced AoV cancer, could be considered as one of the adjuvant chemotherapy options[21,22].

Our study has some limitations. First, this study was conducted at a single institution and had a retrospective design. Second, differences in clinicopathological factors between the two groups hampered comparison of the adjuvant chemotherapy efficacy. Adjuvant chemotherapy was associated with better DFS, but not OS, which is one of the limitations. However, the DFS rate seems to be more suitable than the OS rate for evaluating the efficacy of adjuvant chemotherapy. OS could be affected by various factors, such as whether palliative chemotherapy was performed after recurrence, the pattern of recurrence (local or systemic), and the availability of local treatment.

CONCLUSION

In summary, among patients with resected AoV carcinoma, fluorouracil-based adjuvant chemotherapy was not associated with a better survival outcome in the primary analysis, compared with surgery alone. However, multivariate analysis demonstrated that better DFS was statistically associated with adjuvant chemotherapy. Further investigations are warranted to identify patients with resected AoV cancer who might benefit from adjuvant chemotherapy.

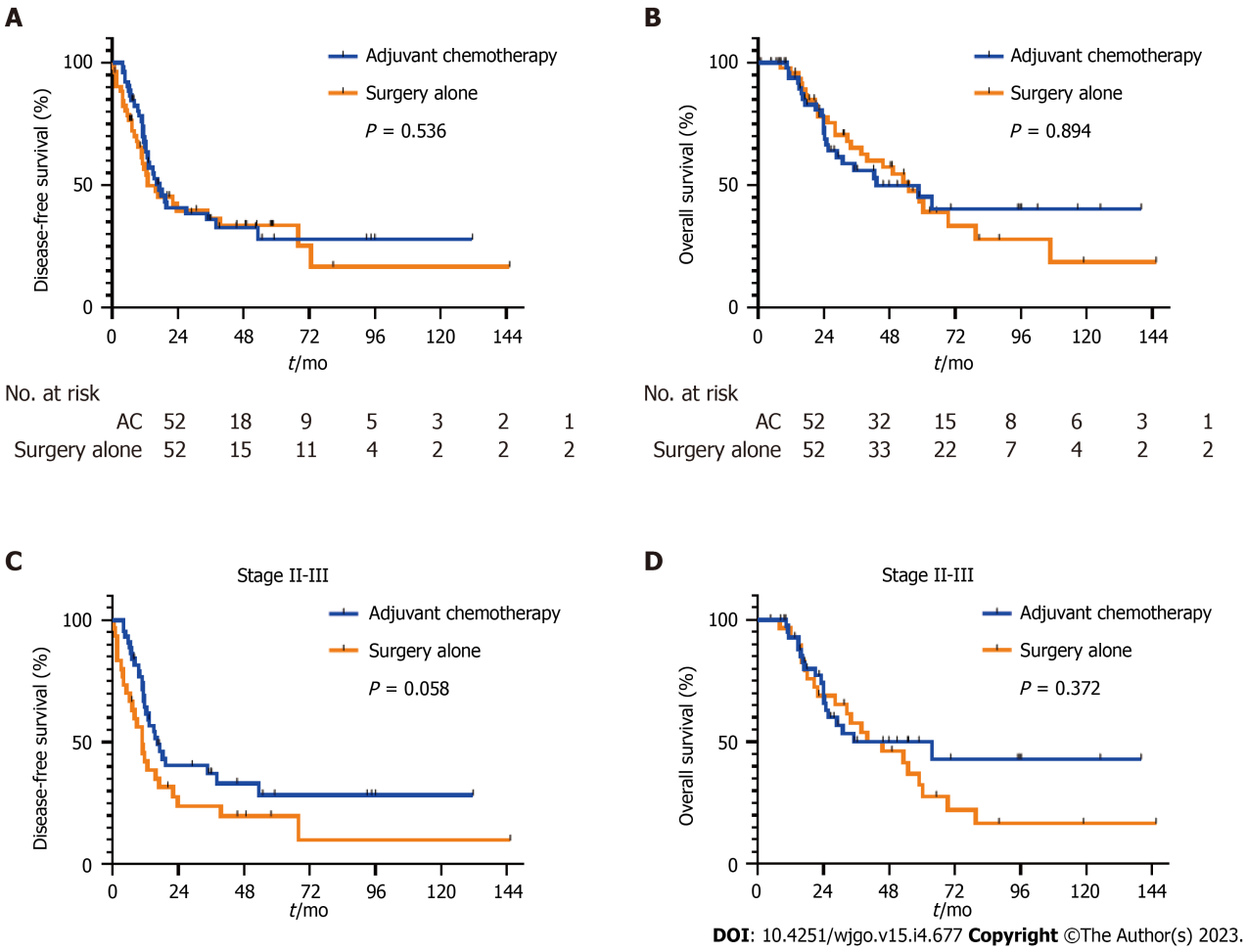

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Figure 2 Kaplan-Meier curves for disease-free survival and overall survival by adjuvant chemotherapy. A: The median disease-free survival was 17.2 mo in the adjuvant chemotherapy group, as compared with 13.0 mo in the surgery-alone group; B: The median overall survival was 43.3 mo in the adjuvant chemotherapy group, as compared with 55.0 mo in the surgery-alone group; C: The median disease-free survival; and D: Overall survival in patients with stage II-III disease according to adjuvant chemotherapy.

ARTICLE HIGHLIGHTS

Research background

Surgical resection is the primary curative approach for patients with localized Ampulla of Vater (AoV) carcinoma, but recurrence is frequent. There is no standard adjuvant treatment globally accepted for resected AoV carcinoma.

Research motivation

A significant number of surgically resected AoV carcinoma patients experience recurrence, and there is a great unmet need because standard adjuvant treatment has not been established.

Research objectives

The purpose of this study was to determine the correlation between fluorouracil-based adjuvant chemotherapy and prognosis in surgically resected AoV carcinoma patients.

Research methods

The association between adjuvant chemotherapy and survival outcomes in patients with stage IB-III AoV carcinoma who underwent surgical resection was analyzed. The administration of fluorouracil-based adjuvant chemotherapy after surgery was determined by the physician's discretion. Adjusted multivariate regression models were utilized to evaluate the correlation between adjuvant chemotherapy and disease-free survival and overall survival.

Research results

After curative surgery for AoV carcinoma, 52 patients received adjuvant chemotherapy. Multivariate

analysis showed that advanced tumor stage, higher histologic grade, and vascular invasion were linked with shorter disease-free survival (DFS). Adjuvant chemotherapy improved DFS and was linked with a longer overall survival, although this was not statistically significant.

Research conclusions

Overall, our study found no significant survival benefit of fluorouracil-based adjuvant chemotherapy in patients with resected AoV carcinoma. However, multivariate analysis revealed a positive association between adjuvant chemotherapy and improved DFS. Further research is needed to identify subgroups of resected AoV cancer patients who may benefit from adjuvant chemotherapy.

Research perspectives

This study evaluated a relatively homogenous population with a consistent chemotherapy regimen, which is considered a strength in contrast to most retrospective studies that included heterogeneous populations and used inconsistent adjuvant treatment regimens. Patients with tumors that invade beyond the sphincter of Oddi could be considered for adjuvant treatment following surgery as a considerable proportion of stage IB patients experience relapses. These findings will aid in identifying appropriate candidates for adjuvant treatment in patients with AoV carcinoma.

FOOTNOTES

Author contributions: All authors helped to perform the research; Park SJ was involved in manuscript writing, drafting conception and design, acquisition of data, performing procedures, and data analysis; Shin KS, Kim IH, Hong TH, and Kim Y contributed to writing the manuscript; Lee MA contributed to writing the manuscript, drafting conception and design, performing procedures, and data analysis; all authors have read and approved the final manuscript.

Institutional review board statement: The study conformed to Korean regulations and the Declaration of Helsinki. Ethical approval was provided by the Institutional Review Board (IRB) of The Catholic University of Korea, Seoul St. Mary's Hospital (approval ID: KC21RISI0518).

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. We received Institutional Review Board approval to conduct our study without consent process.

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Data sharing statement: The datasets used in this study are available from the corresponding author upon request.

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

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ORIGINAL ARTICLE

Retrospective Study

Correlation between immune-related adverse events and long-term outcomes in pembrolizumab-treated patients with unresectable hepatocellular carcinoma: A retrospective study

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Abstract

BACKGROUND

Although immune checkpoint inhibitor (ICI) therapy has improved the prognosis of unresectable hepatocellular carcinoma (HCC), it has also resulted in unique immune-related adverse events (irAEs). The relationship between irAE and treatment outcomes in ICI-treated unresectable HCC patients remains unknown.

AIM

To elucidate the correlation between immune-related toxic effects and prognosis in patients with unresectable HCC treated with pembrolizumab.

METHODS

From March 2019 to February 2021, a total of 190 unresectable HCC (Barcelona Clinic Liver Cancer C) patients receiving pembrolizumab treatment were retrospectively reviewed. Overall survival (OS) was the primary endpoint, while objective response rate (ORR), disease control rate (DCR), and time to progression (TTP) were secondary evaluation indexes. We assessed demographics, irAEs, and outcomes by retrospective review.

RESULTS

One hundred and forty-three males and 47 females were included in the study. The ORR and DCR were 12.1% (23/190) and 52.1% (99/190), respectively. The

median OS was 376 d [95% confidence interval (CI): 340-411 d] and the median TTP was 98 d (95%CI: 75-124 d). The overall incidence of treatment-related adverse events was 72.6% (138/190) and 10.0% of them were severe irAEs (grade ≥ 3). Child-Pugh B class, portal vein tumor thrombus, extrahepatic metastasis, and hypothyroidism were the independent risk factors for survival. Patients with hypothyroidism showed a longer OS [517 d (95%CI: 423-562) *vs* 431 d (95%CI: 412-485), P = 0.011] and TTP [125 d (95%CI: 89-154) *vs* 87 d (95%CI: 61-98), P = 0.004] than those without irAEs.

CONCLUSION

Pembrolizumab-treated patients with unresectable HCC who experienced hypothyroidism have promising ORR and durable response. Hypothyroidism, an irAE, may be used as a clinical evaluation parameter of response to ICIs in unresectable HCC.

Key Words: Hepatocellular carcinoma; Immune checkpoint inhibitors; Pembrolizumab; Immune-related adverse events; Overall survival; Retrospective study

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Core Tip: This is a retrospective study to elucidate the correlation between immune-related toxic effects and prognosis in patients with unresectable hepatocellular carcinoma (HCC) treated with pembrolizumab. One hundred and forty-three male patients and forty-seven female patients were included in the study. The overall incidence of treatment-related adverse events was 72.6% (138/190) and 10.0% of them were severe immune-related adverse events (irAEs) (grade ≥ 3). Patients with hypothyroidism were observed to have a longer overall survival and time to progression than those without irAEs. Unresectable HCC patients who experienced hypothyroidism had a better therapeutic effect.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death globally[1]. Because early-stage HCC is insidious and the tumors have a tendency to invade intrahepatic vessels and create distant metastases, 80% of patients are already in the advanced stage at diagnosis and lose the opportunity for hepatectomy. In addition, 70% of patients who receive radical liver resection will experience tumor recurrence[2]. Therefore, a large number of patients with unresectable HCC need comprehensive treatment. Locoregional therapy including transarterial chemoembolization or selective internal radiation therapy has been proven to prolong the median survival of patients with unresectable HCC[3]. Since sorafenib has been shown to improve the prognosis of unresectable HCC[4], tyrosine kinase inhibitors (TKIs) have become the first-line treatment for advanced HCC. However, the objective response rate (ORR) to TKIs is unsatisfactory, ranging from 6.5%-18.8%, and the prognosis improvement is not significant, with an average prolonged survival time of 2.8-5.8 mo[5,6]. Recent studies have shown that immune checkpoint inhibitors (ICIs) represented by pembrolizumab have become the second-line treatment for unresectable HCC due to their safety and long duration of response. KEYNOTE-224, a non-randomized, multi-center, open-label, phase 2 trial, has proven that pembrolizumab was effective and tolerable in patients with unresectable HCC who had previously been treated with sorafenib[7].

However, the response rate to both TKIs and ICIs was less than 30%. Moreover, adverse effects of the drugs may interfere with the judgment of curative effect and decrease compliance to the best treatment available. Therefore, determining patients who are mostly likely to benefit from TKIs or ICIs is very important. Researchers have been trying to find biomarkers that predict patient survival or response to drugs[8]. Previous studies have reported that unresectable HCC patients who developed hand-foot skin reaction possessed a prolonged time to progression (TTP) and better disease-control rate (DCR)[9]. Studies have suggested that vascular endothelial growth factor inhibition may rely on a stronger recruitment of inflammatory cells to take a more pronounced antitumor effect that is accompanied by stronger treatment-related adverse events[10]. Whether similar adverse events can be found to evaluate

the response of tumor to ICIs remains to be investigated. Immune-related adverse events (irAEs) have been reported to include polymyalgia, colitis, skin lesions (rash, pruritus, and vitiligo), hypophysitis, hepatitis, thyroiditis, uveitis, Guillain-Barré syndrome, and immune-mediated cytopenia. It was reported that the overall incidence of all-grade irAEs was 60%-80% and the incidence of high-grade irAEs was 20%-30%[11,12]. Several studies have suggested that autoimmune-like toxic effects are thought to represent bystander effects from activated T-cells, accompanied with antitumor effects, and are consistent with the mechanism of action of ICIs[13,14]. Herein, we retrospectively studied whether immune-related toxic effects correlated with prognosis in patients with unresectable HCC treated with pembrolizumab.

MATERIALS AND METHODS

Terminology

Overall survival (OS) was defined as the time from the start of ICIs until death or until last follow-up. ORR was defined as the proportion of patients achieving complete response (CR) and partial response (PR). DCR was defined as the proportion of patients achieving CR, PR, and stable disease (SD). TTP was defined as the time from the start of ICIs to the radiological confirmation of tumor progression. Tumor burden score (TBS) was calculated as $\sqrt{a^2 + b^2}$ [a = maximum tumor diameter (cm), measuring the area of arterial enhancement and excluding the area of internal necrosis[15]; b = tumor number, the lesion is at least 1 cm in size][16].

Patients

Between March 2019 and February 2021, a total of 190 patients who received ICI treatment were included in this retrospective study at Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology. All patients included in the study met the following criteria: (1) Child-Pugh A/B; (2) Advanced HCC [Barcelona Clinic Liver Cancer (BCLC) stage C]; and (3) Not receiving previous antitumor therapy. HCC was diagnosed by the European Association for the Study of Liver criteria and American Association for the Study of Liver Disease guidelines[17,18]. Demographics, including age, sex, *etc.* laboratory examination, including levels of alanine aminotransferase, aspartate aminotransferase, alpha fetoprotein (AFP), *etc.* imaging examination, and survival status were reviewed retrospectively. The study was approved by the Ethical Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology. All procedures performed in this study abided by the Declaration of Helsinki.

Usage of ICIs

All of the patients received treatment with pembrolizumab (KEYTRUDA, Merck Sharp & Dohme Co., Inc.), 200 mg/time, every 3 wk, *via* intravenous infusion[7] according to the guidelines or expert consensus, with dose modification according to toxic effects, as needed.

Follow-up and irAE assessment

An experienced radiologist blinded to patient information assessed tumor response according to modified response evaluation criteria in solid tumor criteria every 6 wk by contrast-enhanced computed tomography or magnetic resonance imaging examination. Common Terminology Criteria for Adverse Events (CTCAE) were used by two experienced hepatologists to independently assess irAEs. Corresponding examinations or imaging examinations were performed to diagnose irAEs and assess the grades according to CTCAE. Follow-up was terminated on April 24, 2022. The survival status was confirmed by governmental death registration or telephone. The flow diagram of the study is displayed in Figure 1.

Statistics analysis

Continuous variables are presented as the median and interquartile range (IQR) and categorical data as numbers and percentages. Survival analysis was carried out using the Kaplan-Meier method and log-rank test. Univariate and multivariate Cox proportional regression analyses were used to evaluate risk factors for OS or TTP. A two-sided P value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 19.0 for Windows (SPSS, Chicago, Illinois, United States). GraphPad Prism 7 software was used for all graphical drawings.

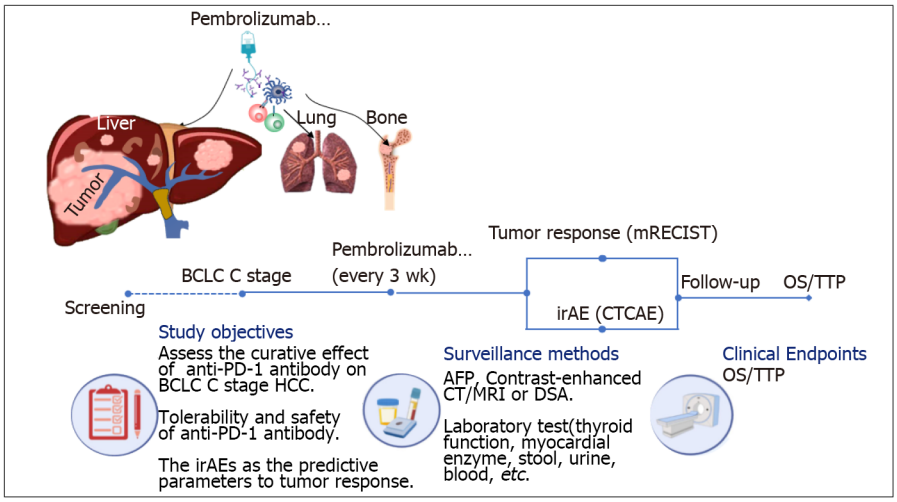

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Figure 1 Flow diagram of the study. mTECIST: Modified response evaluation criteria in solid tumors criteria; CTCAE: Common Terminology Criteria for Adverse Events; HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein; DSA: Digital subtraction angiography; OS: Overall survival; TTP: Time to progression; BCLC: Barcelona Clinic Liver Cancer; PD-1: Programmed death 1; CT: Computed tomography; MRI: Magnetic resonance imaging; irAE: Immune-related adverse event.

RESULTS

Demographics

The baseline characteristics of 190 unresectable HCC (BCLC stage C) patients receiving pembrolizumab as an initial treatment are summarized in Table 1. One hundred and fifty-four (81.1%) of the patients were male, and the primary cause of disease was chronic hepatitis B virus infection (75.8%). One hundred and forty-six (76.8%) of the patients had Child-Pugh class A and 44 (23.2%) had Child-Pugh class B. All of the patients were at BCLC stage C.

Tumor response

Follow-up was terminated on April 24, 2022. The mean follow-up time was 814 d (median, 748 d; range, 438-1146 d). No patients achieved CR. Twelve percent (23/190) of the patients achieved PR and 40% (76/190) had SD. The DCR was 52.1% (99/190), and 47.9% (91/190) of the patients had progressive disease (PD). All patients who experienced disease progression permanently discontinued ICI treatment; none of the patients discontinued ICIs because of adverse effects. At the end of the follow-up, 130 patients (68.4%) died. The median OS was 376 d [95% confidence interval (CI): 340-411 d] and the median TTP was 98 d (95%CI: 75-124 d) (Figure 2).

Incidence of irAEs

The overall incidence of irAEs was 72.6% (138/190) and 10.0% of them were severe irAEs (grade ≥ 3). Table 2 summarizes the observed representative irAEs. Elevated transaminase (> 3 times upper limit of normal) was the most common adverse reaction to ICIs seen in 69 (36.3%) patients, followed by diarrhea in 40 (21.1%), cutaneous toxic effects in 37 (19.5%), hypothyroidism in 25 (13.2%), and proteinuria in 24 (12.6%). Relatively rare adverse events were myocarditis in 3 (1.6%) patients and hypoadrenocorticism in 5 (2.6%). The elevated transaminase was the earliest irAE, and the median (IQR) time to onset was 4.8 (3.5-7.2) wk, followed by cutaneous toxic effects [5.9 (3.8-7.9) wk], proteinuria [6.8 (4.3-8.1) wk], diarrhea [7.1 (5.2-8.9) wk], hypoadrenocorticism [7.9 (6.7-9.5) wk], hypothyroidism [8.7 (6.9-10.9) wk], and myocarditis [10.2 (8.6-11.9) wk]. Figure 3A shows that the percentage of tumor burden score (TBS) changed from baseline by treatment of 91 patients who had PD. Figure 3B shows that the percentage of TBS changed from baseline by treatment of 99 patients who achieved CR, PR, or SD. We found that those patients who developed myocarditis or hypothyroidism tended to achieve PR and have a more significant decrease in tumor burden. In addition, patients without irAEs were more likely to enter a PD status. Figure 4 shows a spider plot which depicts the percentage change in TBS of 25 hypothyroidism patients from baseline by treatment over time. We observed substantial reductions in tumor burden and several responders exhibited deep responses.

irAEs and prognosis

In order to determine independent risk factors affecting OS, the clinical parameters including demographics, laboratory results, and adverse events were included in the univariate and multivariate Cox proportional regression analyses. We found that Child-Pugh class B [hazard ratio (HR) = 1.321;

Table 1 Clinical characteristics of 190 patients

Clinical characteristic	No. of patients
Age (yr), median (IQR)	51 (42-58)
Sex	
Male, *n* (%)	154 (81.1)
Etiology	
Hepatitis B	144 (75.8)
Hepatitis C	4 (2.1)
Hepatitis B & C	2 (1.1)
Non-hepatitis B, non-hepatitis C	40 (21.0)
ALT (U/L), median (IQR)	75 (51-254)
AST (U/L), median (IQR)	57 (47-302)
TBiL (μmol/L), median (IQR)	20.1 (11.2-31.2)
AFP (ng/mL), median (IQR)	205 (19.2-4692)
Child-Pugh class	
A	146 (76.8)
B	44 (23.2)
Portal vein tumor thrombosis	131 (68.9)
Extrahepatic metastasis	
Lung	64 (33.7)
Bone	31 (16.3)
Peritoneum	25 (13.1)
Multiple locations	15 (7.9)
BCLC stage	
C	190 (100)
Tumor response	
Complete response	0 (0)
Partial response	23 (12.1)
Stable disease	76 (40.0)
Progressive disease	91 (47.9)
Follow-up time, median (range)	748 (438-1146) d
TTP, median (95%CI)	98 (75-124) d
OS, median (95%CI)	376 (340-411) d

IQR: Interquartile range; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBiL: Total bilirubin; AFP: Alpha fetoprotein; TTP: Time to progression; OS: Overall survival; BCLC: Barcelona Clinic Liver Cancer; CI: Confidence interval.

95%CI: 1.112-1.711], portal vein tumor thrombus (PVTT) (HR = 3.125; 95%CI: 3.021-3.568), extrahepatic metastasis (HR = 2.871; 95%CI: 2.579-3.052), and hypothyroidism (HR = 0.641; 95%CI: 0.489-0.901) were the independent risk factors for survival. Similarly, AFP > 400 ng/mL (HR = 1.872; 95%CI: 1.357-2.135), PVTT (HR = 2.472; 95%CI: 2.243-2.891), extrahepatic metastasis (HR = 1.489; 95%CI: 1.246-1.574), and irAE of hypothyroidism (HR = 0.613; 95%CI: 0.362-0.886) were the independent risk factors for TTP (Table 3). The OS and TTP of patients who developed hypothyroidism (n = 25) were compared with those patients without irAE using the Kaplan-Meier method and a log-rank test. We found that the median OS was 517 d (95%CI: 423-562 d) in patients with hypothyroidism, which was longer than that of patients without irAE [431 d (95%CI: 412-485 d), P = 0.011] (Figure 5A). Similarly, the median TTP was 125 d (95%CI: 89-154) in patients with hypothyroidism, which was longer than that of patients without irAE [87 d (95%CI: 61-98 d), P = 0.004] (Figure 5B).

Table 2 Incidence of treatment-related adverse events

Adverse event	Any grade	Grade 3	Grade 4
Cutaneous toxic effects	25 (13.2)	3 (1.6)	2 (1.1)
Mucositis	8 (4.2)	2 (1.1)	1 (< 1)
Rash	7 (3.7)	1 (< 1)	1 (< 1)
Pruritus	10 (5.3)	2 (1.1)	0 (0)
Diarrhea	27 (14.2)	4 (2.1)	1 (< 1)
Elevated transaminases	35 (18.4)	10 (5.3)	2 (1.1)
Hypothyroidism	25 (13.2)	4 (2.1)	2 (1.1)
Myocarditis	3 (1.6)	1 (< 1)	2 (1.1)
Hypoadrenocorticism	5 (2.6)	1 (< 1)	0 (0)
Proteinuria	18 (9.5)	5 (2.6)	0 (0)
Overall incidence	138 (72.6)	12 (6.3)	7 (3.7)

Table 3 Univariate and multivariate analyses of prognostic factors for predicting overall survival and time to progression

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
OS				
Child-Pugh, class B	1.446 (1.044-1.547)	0.039	1.321 (1.112-1.711)	0.021
AFP > 400 ng/mL	1.651 (1.351-1.782)	0.021	1.446 (0.875-1.836)	0.239
PIVKA > 40 mAU/mL	1.324 (1.023-1.472)	0.043	1.568 (0.411-1.687)	0.085
PVTT, yes	2.145 (1.897-3.587)	0.003	3.125 (3.015-3.568)	0.008
Extrahepatic metastasis, yes	1.784 (1.254-2.571)	0.013	2.871 (2.581-3.052)	0.028
Cutaneous toxic effects, yes	0.741 (0.654-0.968)	0.025	0.845 (0.425-1.751)	0.129
Hypothyroidism, yes	0.623 (0.487-0.912)	0.034	0.641 (0.489-0.901)	0.017
TTP				
AFP > 400 ng/mL	1.757 (1.271-1.972)	0.023	1.872 (1.357-2.135)	0.017
PVTT, yes	2.595 (1.377-3.889)	0.003	2.472 (2.243-2.891)	0.008
Extrahepatic metastasis, yes	1.536 (1.296-1.765)	0.031	1.489 (1.246-1.574)	0.012
Cutaneous toxic effects, yes	0.874 (0.621-0.925)	0.036	0.785 (0.358-1.258)	0.157
Hypothyroidism, yes	0.741 (0.514-0.870)	0.024	0.613 (0.362-0.886)	0.018

AFP: Alpha fetalprotein; PIVKA: Protein induced by vitamin K absence or antagonist-II; OS: Overall survival; PVTT: Portal vein tumor thrombus; HR: Hazard rate; TTP: Time to progression; CI: Confidence interval.

DISCUSSION

Previous study has reported that among the irAEs manifesting as endocrine dysfunctions, hypothyroidism (6.07%) and hyperthyroidism (2.82%) were most common[19]. Thyroid events occur in approximately 10% of patients treated with anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) monotherapy[20,21]. The median time to onset of thyroid dysfunction, most of which is hypothyroidism, is 6 wk after ICI initiation[22]. In our study, we found that patients who developed hypothyroidism had a longer OS and TTP than those without irAE. Multivariate analysis showed that hypothyroidism was an independent prognostic factor. In addition, we found that patients with hypothyroidism had a significant reduction in TBS from baseline by treatment, which was an intuitive manifestation of the effectiveness of immunotherapy. Many previous studies have also shown that patients who experienced irAEs had a superior progression free survival and OS compared to those who did not experience irAEs[23-25]. A study of 270 non-small cell lung cancer (NSCLC) patients

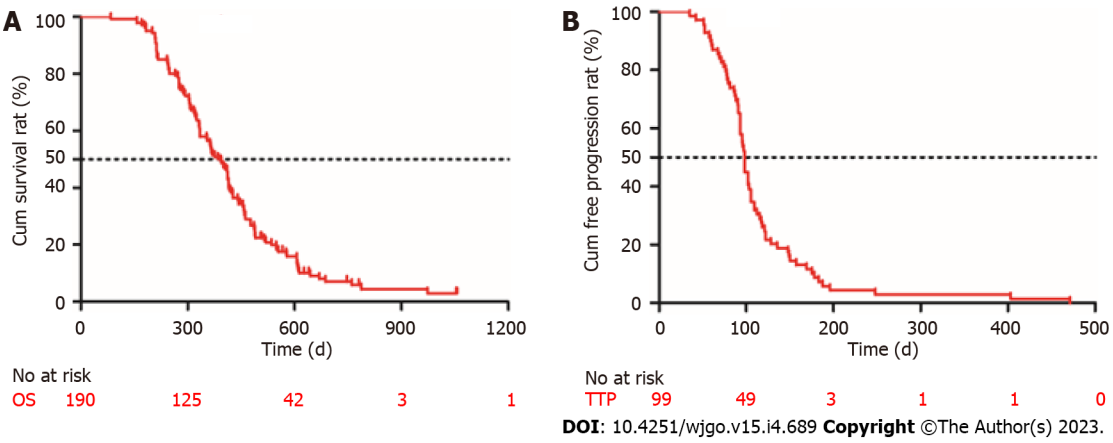

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Figure 2 Kaplan-Meier curves describing the overall survival of all patients (*n* = 190) and the time to progression of patients who achieved disease control (*n* = 99). A: Survival curve; B: Time to progression curve. OS: Overall survival; TTP: Time to progression.

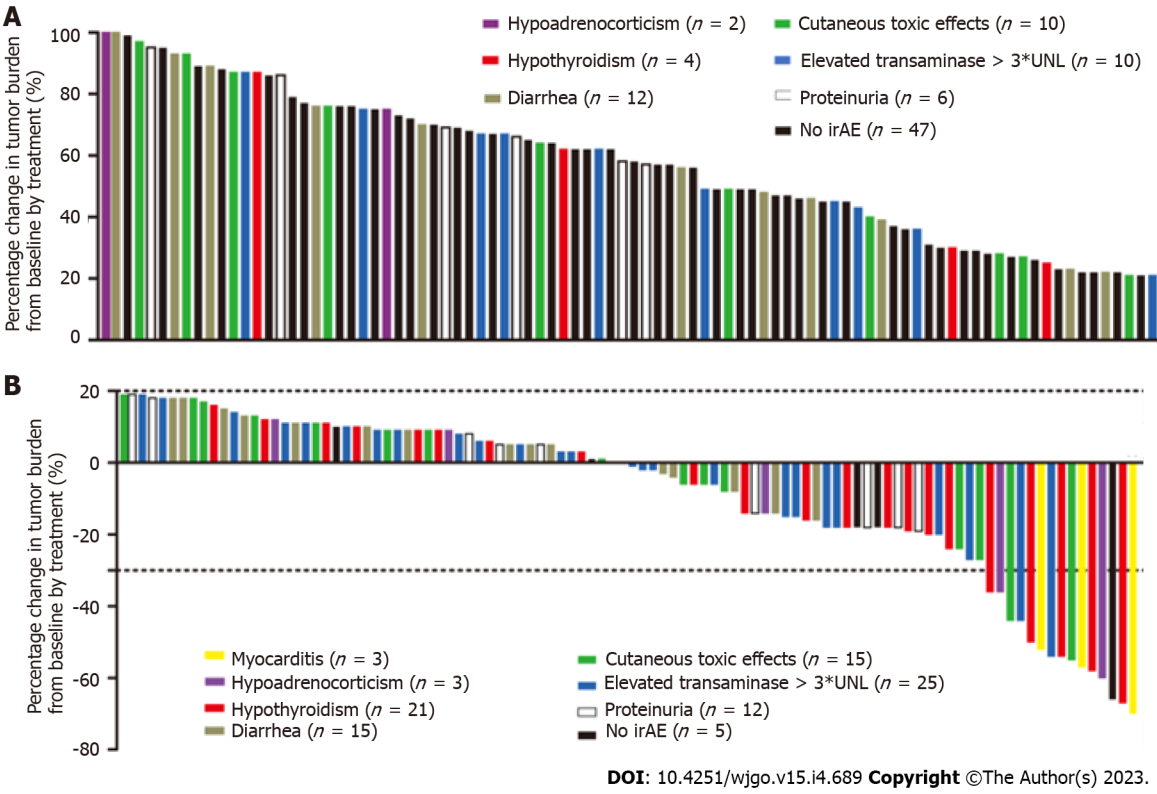

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Figure 3 Waterfall plots of percentage change in tumor burden from baseline (*n* = 190). The "y" axis represents the percentage change in tumor burden from baseline by treatment. The immune-related adverse events are distinguished by different colors. Negative/positive values represent maximum tumor reduction or minimum tumor increase, respectively. A: 91 patients had progressive disease; B: 99 patients achieved complete response, partial response, or stable disease. irAE: Immune-related adverse event.

treated with at least one dose of anti-PD-L1 or anti-PD-1 antibodies showed that patients who experienced thyroiditis had statistically significant improvements in OS compared to patients who did not (P = 0.01)[26]. A meta-analysis of 12 randomized controlled trials identified 3815 metastatic head & neck and lung cancer patients treated with ICIs and showed a significant correlation between endocrine irAEs and OS was observed (P = 0.019)[27]. In addition, a retrospective study reviewed 318 advanced melanoma patients and found that patients who experienced irAEs had a superior OS compared to those who did not[13].

Although the precise mechanisms by which irAEs occur have not been fully uncovered, they are thought to represent bystander effects from activated T-cells and are consistent with the mechanism of action of ICIs[14,28]. It is now generally accepted that the pathogenesis of ICI-induced dysthyroidism involves both immune and non-immune mechanisms[29]. It is traditionally believed that thyroperoxidase (TPO) and thyroglobulin (Tg) antibodies may play an important role in mediating thyroiditis.

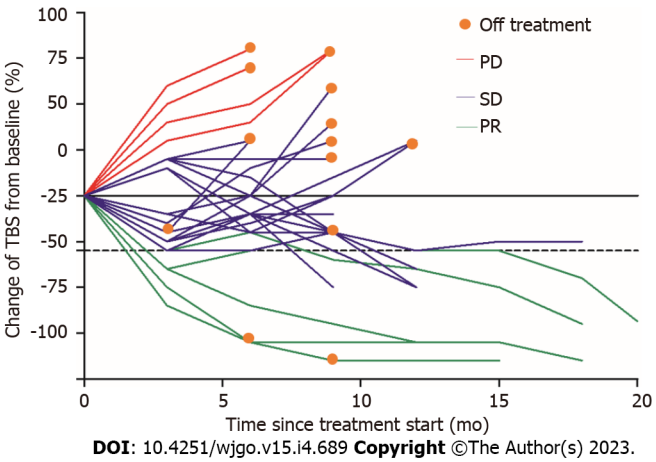

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Figure 4 Spider plot displaying tumor response in 25 patients with hypothyroidism. PD: Progressive disease; SD: Stable disease; PR: Partial response; TBS: Tumor burden score.

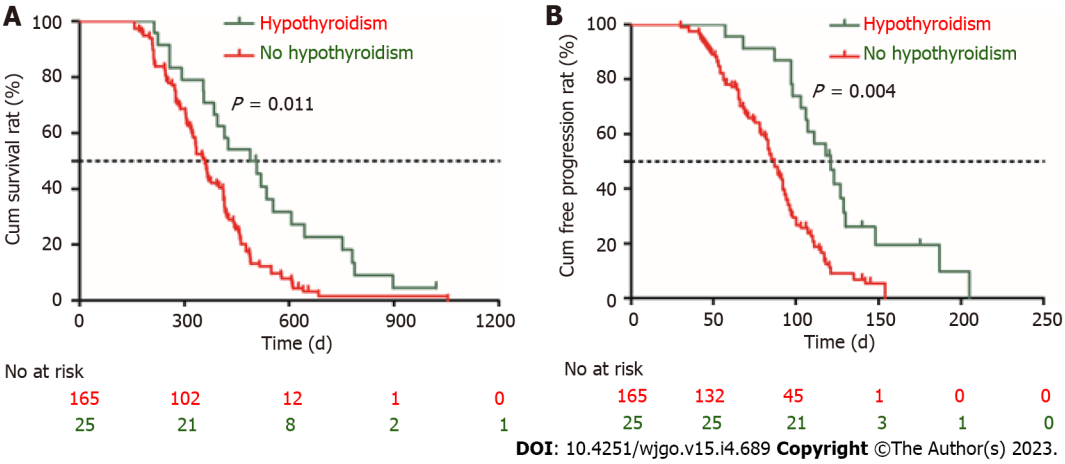

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Figure 5 Prognosis comparison between hypothyroidism group and non-hypothyroidism group. A: Survival curve; B: Time to progression curve.

Maekura *et al*[30] considered that the presence of anti-thyroid antibodies such as TPO and Tg antibodies is a positive predictive factor for developing hypothyroidism in a Japanese cohort of 64 patients with advanced NSCLC treated with nivolumab. Recent study found that the mechanism of thyroid destruction by PD-1 antibodies may be mediated by T cells, natural killer (NK) cells, and monocytes[31-33]. Delivanis *et al*[31] showed that patients treated with anti-PD-1 therapy had an increasing circulating number of $CD56^{+}CD16^{+}$ NK cells and high HLA-DR surface expression in $CD14^{+}$ $CD16^{+}$ monocytes that mediate the inflammation. In addition, Das *et al*[34] considered that B lymphocytes also played a role in mediating dysthyroidism. They showed that patients with advanced melanoma treated by a combined checkpoint blockade who developed high-grade irAEs, compared to those who did not, had a decreased total peripheral B lymphocyte count with increased plasmablasts and a subset of B lymphocytes[34,35].

There are some limitations to our study. First, the study is retrospective and conducted only in one center; therefore, multiple centers should be evaluated in further studies. Second, the sample size was relatively small so that the sample of irAEs was small, resulting in large confidence intervals and imprecise results. Third, the effect of different ICI agents on adverse reactions and prognosis in patients was not strictly excluded in this study. Fourth, our study did not include those patients with autoimmune disease, meaning that the correlation between irAEs and prognosis in patients with autoimmune disease needs further exploration. Finally, some adverse events which tended to be ignored, such as fever, weakness, and anorexia, were not recorded, resulting in a lower incidence of irAEs than in previous studies.

CONCLUSION

In conclusion, dysthyroidism is the most common irAE related to a good prognosis and involves T and

B-lymphocytes, multiple cytokines, and diverse factors. Further clinical and laboratory studies should be conducted to clarify the mechanism of ICI-related dysthyroidism. Additionally, the clinical diagnosis and management of thyroid irAEs should be enhanced to avoid life-threatening complications. In addition, the long-term effects of ICIs on dysthyroidism should be further researched to better understand thyroid irAEs and autoimmune thyroid diseases.

ARTICLE HIGHLIGHTS

Research background

Unresectable hepatocellular carcinoma (HCC).

Research motivation

Immune-related adverse events (irAEs) have a high incidence in immune checkpoint inhibitor (ICI) treatment of unresectable HCC. The relationship between irAEs and treatment outcomes in ICI-treated unresectable HCC patients remains unknown.

Research objectives

A retrospective study was conducted to elucidate the correlation between immune-related toxic effects and prognosis in patients with unresectable HCC treated with pembrolizumab.

Research methods

A total of 190 unresectable HCC (Barcelona Clinic Liver Cancer stage C) patients receiving pembrolizumab treatment were retrospectively reviewed. All irAEs were reviewed and the relationship between irAEs and prognosis was analyzed.

Research results

In our study, we found that the overall incidence of irAEs was 72.6% (138/190) and 10.0% of them were severe irAEs (grade ≥ 3); elevated transaminase (> 3 times upper limit of normal) was the most common adverse reaction to ICIs. Patients who developed myocarditis or hypothyroidism tended to achieve partial response and have a more significant decrease in tumor burden. In addition, patients without irAEs were more likely to have progressive disease. It suggested that irAEs are indeed closely related to antitumor effects. In addition, hypothyroidism was the independent risk factors for time to progression and overall survival.

Research conclusions

irAEs, especially hypothyroidism, could be used as an indicator to evaluate the effect of immunotherapy.

Research perspectives

The study could help doctors in identifying patients who are responding to immunotherapy. In general, the response rate to both tyrosine kinase inhibitors and ICIs is less than 30%. Serious adverse events may put patients at risk of death. Therefore, timely identification of the right patients can not only reduce the side effects of immunotherapy but also improve the effectiveness of treatment.

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FOOTNOTES

Author contributions: Zhou JM and Xiong HF analyzed the data and wrote the manuscript; Zhu LP, Zhang ZW, and Chen XP designed the research; Wu B modified the manuscript; and all authors read and approved the final manuscript.

Institutional review board statement: The study was approved by the Ethical Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology. All procedures performed in this study abided by the Declaration of Helsinki.

Informed consent statement: All study participants or their legal guardian provided informed written consent about

personal and medical data collection prior to study enrolment.

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

Data sharing statement: The datasets (data) for this study can be found in the (figure) https://figshare.com/s/ee63fa5271448d51104e. The other datasets were available on request from the corresponding author at wuhanyywb@163.com.

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