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RESPONSIBLE EDITORS FOR THIS ISSUE

Inhibition of poly (ADP-Ribose) polymerase: A promising strategy targeting pancreatic cancer with BRCAness phenotype

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Abstract

The use of chemotherapeutic regimens for the treatment of pancreatic cancer is still limited because pancreatic cancer is usually diagnosed at an advanced stage as a refractory disease in which symptoms are difficult to recognize in the early stages. Furthermore, at advanced stages, there are important challenges to achieve clinical benefit and symptom resolution, even with the use of an expanded spectrum of anticancer drugs. Recently, a point of reduced susceptibility to conventional chemotherapies by breast cancer susceptibility gene (BRCA) mutations led to a new perspective for overcoming the resistance of pancreatic cancer within the framework of increased genome instability. Poly (ADP-Ribose) polymerase (PARP) -1 is an enzyme that can regulate intrinsic functions, such as response to DNA damage. Therefore, in an environment where germline mutations in BRCAs (BRCAness) inhibit homologous recombination in DNA damage, resulting in a lack of DNA damage response, a key role of PARP-1 for the adaptation of the genome instability could be further emphasized. Here, we summarized the key functional role of PARP-1 in genomic instability of pancreatic cancer with the BRCAness phenotype and listed clinical applications and outcomes of PARP-1 inhibitors to highlight the importance of targeting PARP-1 activity.

Key Words: Pancreatic cancer; BRCAness; Poly (ADP-Ribose) polymerase-1; PARylation; Poly (ADP-Ribose) polymerase-1 inhibitor

Core Tip: The incidence of germline mutations of the breast cancer susceptibility gene (BRCA), defined as BRCAness, that can be targeted for pancreatic cancer is 9%-17%.

Mutations in BRCAs are responsible for causing genetic instability and worsening the prognosis. Therefore, inhibition of poly (ADP-Ribose) polymerase-1 has emerged as a promising therapeutic target for BRCAness pancreatic cancer within the framework of an increase in genome instability.

INTRODUCTION

Therapeutic perspectives in pancreatic cancer

Pancreatic cancer is usually diagnosed at an advanced stage as a refractory disease in which symptoms are difficult to recognize in the early stages. The 5-year survival rate is extremely low (less than 9%), and about two-thirds of all patients with pancreatic cancer die within one year of diagnosis[1]. Furthermore, at advanced stages of the disease, there are major challenges to achieving clinical benefit and symptom resolution, even after expanding the range of anticancer drugs targeting pancreatic cancer, and to date, few options for treating pancreatic cancer have been proposed, such as gemcitabine alone, gemcitabine with nanoparticle albumin-bound paclitaxel (nab-paclitaxel), or gemcitabine in combination with capecitabine, fluorouracil, leucovorin, irinotecan, and oxaliplatin[2]. The main cause of pancreatic carcinogenesis is genomic instability, and it is well established that cancer development is related to defects in DNA damage response[3]. Recent genome-wide studies have made great strides in identifying distinct subpopulations of pancreatic cancer constituent cells with unstable genomic properties due to mutations in the DNA repair gene[3,4]. Based on this background, there has been a focus on the high frequency of deleterious changes which lead to a truncated/faulty response to DNA damage in cancer cells. In particular, since breast cancer susceptibility genes (BRCA) mutations have been reported to decrease susceptibility to gemcitabine and platinum-based chemotherapy, a new perspective on the molecular mechanisms overcoming resistance in pancreatic cancer is required[5,6]. Therefore, the recent approach targeting poly (ADP-Ribose) polymerase (PARP) -1 has emerged as an encouraging therapeutic strategy for inhibiting the pathogenesis of BRCAness pancreatic cancer within the framework of an increase in genome instability[7].

PARP-1 AND DNA DAMAGE RESPONSE IN PANCREATIC CANCER

PARP-1 is an enzyme that can regulate the intrinsic functions of several cytoplasmic and nuclear proteins based on inducing poly (ADP-Ribose) synthesis[8]. In various cellular physiological functions led by PARP-1, the reaction to DNA damage is known as the most important biochemical function, and with its well-established crucial role in DNA damage repair, the upregulation of PARP-1 in cancer could lead to investigations into the potential for targeting this important enzyme[9]. PARP-1 comprises a multi-domain structure that shares the catalytic domain showing structural homology with other ADP-ribosyl transferases for DNA damage repair[10]. The N-terminal region contains a DNA-binding domain with three zinc fingers and an auto-modifying domain, and the C-terminal region comprises a protein interaction domain and a catalytic subdomain accountable for the poly ADP-ribosylation reaction[10,11]. The construction of such domains enables genetic relations by catalyzing the covalent attachment of poly-ADP-Ribose polymers to DNA repair proteins and other receptor proteins, including transcription factors and chromatin modulators. Based on these structural interactions, PARP-1 can mediate ADP-Ribose synthesis and attach it to acceptor proteins[10,11]. The PARP-1 signature motif includes an NAD+-binding site and comprises an acceptor of adenosine and the donor of nicotinamide wherein ADP-Ribose from NAD+ is transferred to target proteins for ADP-Ribose synthesis[11,12]. It

is an integrative and dynamic biochemical process defined as poly ADP-ribosylation (PARylation), and the hypothesis has recently been established that the synthesis process is determined by following potential pathways[11,12]. PARP-1 catalyzes the transfer of ADP-Ribose units from NAD+ to compose the poly ADP-Ribose branches, which is negatively charged to several amino acid residues in PARP-1 or other receptor proteins[11]. Besides, poly (ADP-Ribose) synthesis is based on the attachment of ADP-Ribose to the 2'-OH end of the growing chain by sequentially adding the next ADP-Ribose residues to the end of the ADP-Ribose moiety[11]. The biochemical action of linking the long and negatively charged poly ADP-Ribose polymer to PARP-1 itself or a variety of acceptor proteins can be attributed to its primary function of repairing DNA damage during potential changes for cancer cell survival[11,13]. In DNA damage repair, PARP-1 and PARylation are universally involved in both single-strand and double-strand DNA damage repairs, such as base excision repair, homologous recombination (HR), and non-homologous end-joining (NHEJ)[14]. PARP-1 can functionally interact with X-ray repair cross-complementing protein 1, which plays a major role in signal pathways for single-strand DNA damage repair[14,15]. The BRCA1 C-terminus directly binds to the poly ADP-Ribose chain and mediates early recruitment of DNA repair proteins to DNA lesions[16]. Further, PARP-1 has been associated with HR-mediated repair and reactivation of stalled replication forks, thus promoting DNA replication for restarting stalled replication BRCA-dependent early double-strand DNA damage repair[17]. Interestingly, the role of PARP-1 in an environment where germline mutations in BRCAs inhibit the HR-mediated repair of DNA double-strand breaks, thus resulting in a deficiency in the DNA damage response, can be further emphasized[6,18].

BRCANESS IN PANCREATIC CANCER AND PARP-1

BRCAness is defined as a set of traits in which BRCA1 or BRCA2 mutation phenocopies result in a lack of double-strand DNA damage repair, and a tumor cell has an HR obstruction with a germline BRCA1 or BRCA2 deficiency[19]. The incidence of germline mutations of BRCAs that can be targeted for pancreatic cancer is estimated to be about 9%, but the incidence of these BRCA mutations (particularly BRCA2) in familial pancreatic cancer patients has increased to about 17%[20]. Mutations in BRCA are responsible for causing genetic instability and worsening prognosis. BRCAness leading to the phenotype of HR deficiency is an indispensable marker for recognizing an increase in the pancreatic cancer risk, and the sensor defect of double-strand DNA break is an error-prone repair pathway, such as NHEJ, which accumulates increased genomic instability. In this context, the HR deficiency by BRCAness may rely on a process of overcoming genetic instability that is reliant on PARP-1 activation[21]. As mentioned above, PARP-1 is an important nuclear enzyme in cellular homeostasis as it transforms various nuclear proteins by PARylation[8,11-14]. The key feature of PARP-1 is the DNA repair responding to DNA damage by targeting the histone core and linker histone proteins in the nucleus[22]. A serine group-binding ADP-ribose relies on a protein, histone PARylation factor 1 (HPF1), which has been identified as a key protein that controls DNA damage-induced PARylation and is responsible for adaptation to genomic instability[23,24]. Because PARP-1 continuously recruits DNA repair elements through PARylation in several receptor regions during genomic instability, HPF1 is used to regulate the excessive PARP-1 transformation to avoid apoptosis[14,15,24]. Taken together, PARP-1 activity and PARylation may play an important role in adapting to genomic instability in pancreatic cancer in a tumor microenvironment undergoing persistent genomic instability by BRCAness[13-15,20, 21,23,24].

CLINICAL STUDIES ON BRCANESS PANCREATIC CANCER BY PARP INHIBITORS

BRCAness is unstable NHEJ-dependent and drives distinctive DNA repair systems creating specific genotypic and phenotypic features[19]. Therefore, it can be inferred that the sensitization of PARP-1 inhibitors has potential benefits for the treatment of BRCAness pancreatic cancer, and PARP inhibitors have recently emerged as a novel class of a targeted therapy specifically targeting BRCAness pancreatic cancer[18]. To date, five PARP inhibitors have drawn significant clinical results targeting BRCAness

Table 1 Clinical trials of Poly (ADP-Ribose) polymerase-1 inhibitor for the treatment of breast cancer susceptibility gene mutant pancreatic cancer

Drugs	Trial ID	Stage	Outcomes
Olaparib	NCT02184195	Phase II	Median OS (drug/placebo): 19.0/19.2 mo; Median PFS (drug/placebo): 16.9/9.3 mo; Toxicity: Grade ≥ 3 anemia, hyperglycemia, pain
Olaparib	NCT02677038	Phase II	5 SD, 12 PD in Israel; 2 PR, 6 SD, 3 PD in United States; PFS: 14 wk in Israel; 24.7 wk in United States; Toxicity: grade 1-2 anemia, fatigue, nausea
Niraparip	NCT03553004	Phase II	No results posted
Veliparib	NCT01585805	Phase II	4 SD, 10 PD; Median PFS: 52 d; Toxicity: Grade 3 fatigue, hematologic, nausea
Rucaparib	NCT02042378	Phase II	≥ 2 prior chemotherapy: 1 PR, 1 CR; 1 prior chemotherapy: 4 SD, 9 PD; Toxicity: Grade ≥ 3 anemia, thrombocytopenia, fatigue
Talazoparib	NCT01286987	Phase I	2 PR, 2 SD, 6 PD; Median PFS: 5.3 wk; Toxicity: Hyperbilirubinemia, fever, bacteremia

OS: Overall survival; PFS: Progression-free survival; SD: Stable disease; PD: Progression disease; PR: Partial response; CR: Complete response.

pancreatic cancer, and these agents bind to the catalytic domain of PARP and interfere with the base repair or suppress PARP synthesis[25]. Olaparib is first approved for the treatment of advanced ovarian cancer; however, presently, it is also being administered to patients having pancreatic cancer with BRCA mutations. Niraparib is a functionally selective inhibitor of PARP used for the treatment of advanced pancreatic cancer with BRCA mutations. Veliparib is being studied for its applicability to treating non-small-cell lung cancer and breast cancer with BRCA mutations, as well as advanced pancreatic cancer. Rucaparib is a small-molecule PARP inhibitor targeting germline BRCA-mutated pancreatic cancer. Talazoparib is an orally bioavailable PARP inhibitor with the potential antineoplastic activity that targets pancreatic cancer with BRCA mutations[25,26]. A pancreatic cancer olaparib ongoing (POLO) study was conducted on pancreatic cancer patients with BRCA mutations; these were the patients who did not show progression by platinum-based chemotherapy randomized to 92 patients in the phase 3 clinical trial. The results showed that median progression-free survival was increased to 31.3 mo in the olaparib group compared with 23.9 mo in the placebo group[27,28]. Another phase 2 trial has also demonstrated the efficacy of targeting metastatic pancreatic cancer with the germline BRCA mutant. A total of 32 patients was recruited, with one-two showing the partial response (PR), and eleven showing the stable disease (SD)[29,30]. Niraparib is undergoing a phase 2 clinical trial to test its safety and efficacy in patients with pancreatic cancer with HR deficiency, such as a BRCA mutation. This study is recruiting patients, and there are no interim reports[31,32]. The combination effect of cisplatin and gemcitabine with or without veliparib was reported by a phase 2 study in pancreatic cancer patients with germline BRCA mutations. A total number of 52 patients were enrolled in the trial and were randomly assigned to be treated with triple combination (gemcitabine, cisplatin, and veliparib) or double combination (gemcitabine and cisplatin). The objective response rate (ORR) in the former was higher at 74.1% compared with 65.2% in the latter[33,34]. A phase 2 trial of rucaparib in patients with pancreatic cancer with deleterious germline or somatic BRCA mutations was reported. In this study, 19 patients were treated, and the confirmed ORR was 11% (1 PR and 1 complete response). The disease control rate (PR or SD for above 12 wk) was 32% in all patients[35,36]. A dose-escalation, phase 1 study was organized to validate the antitumor activity of talazoparib. This study reported clinical benefits in 4 of the 13 patients with pancreatic cancer. The tumor response rate was 15% PR and 15% SD, and the median progression-free survival was 5.3 wk[37,38]. Table 1 presents a list of clinical trials for PARP inhibitors targeting BRCA mutant pancreatic cancer. However, while acknowledging the promising clinical outcomes of PARP-1 inhibitors, unexpected toxicity is an important concern to be considered. It can cause unacceptably high hematologic toxicity and adverse effects that are sporadically associated with acute myeloid leukemia. The combination of conventional chemotherapy, such as gemcitabine with veliparip or olaparip, was primarily associated with a marked increase in hematological toxicity above grade 3. Further, 40% of pancreatic cancer patients who received only olaparib showed gastrointestinal disorders, fatigue, and lethargy, as well as hematologic toxicity (Table 1)[25,39-42]. Therefore, potential solutions that can

optimize treatment with sophisticated applied therapies through the development of new formulations are currently unmet medical needs.

CONCLUSION

The possibility that PARP-1 inhibitors effectively improve the prognosis by targeting pancreatic cancer with the BRCAness phenotype appears to deserve scientific attention, and the accumulation of such possibilities could be a key point in understanding whether PARP inhibitors can be used as a major therapeutic strategy as a single therapeutic agent or in combination with existing DNA damage agents to overcome resistance.

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New drugs for the treatment of metastatic colorectal cancer

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Abstract

Colorectal cancer (CRC) represents one of the most frequent malignancies in terms of incidence and mortality, thus representing the third leading cause of cancer death worldwide. In the last decade, few drugs have enriched the treatment landscape of metastatic CRC and have significantly affected prognosis. Unlike other neoplasms, metastatic CRC patients who have exhausted treatment options often still maintain a good performance status. There are many challenges to increasing potential treatment options, notably a better understanding of disease biology and the mechanisms of resistance underlying cancer treatment failure. The development of new drugs for metastatic CRC certainly represents one of the most important challenges in medical oncology. This article discusses the main limitations in the development of new drugs and potential future scenarios. In particular, we addressed three questions: (1) The main limitations of targeted therapy in the treatment of metastatic CRC (mCRC); (2) New target armamentarium that could escape primary and secondary resistance and lead to more personalized mCRC therapy; and (3) Future directions.

Key Words: Colon cancer; Colon rectal cancer; New drugs; Drug resistance; Metastatic colorectal cancer

Core Tip: Although metastatic colorectal cancer (CRC) is a relevant oncological issue, few drugs have changed clinical practice in the last decade. In fact, there are many difficulties in the development of new drugs closely related to the biology of CRC; however, improved knowledge of the molecular biology of this cancer has led to a few steps forward and the hope for more targeted cancer treatments for metastatic CRC patients in the near future.

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INTRODUCTION

Targeted therapy has drastically changed the oncological landscape by modifying the natural history of numerous oncological pathologies. Colorectal cancer (CRC) patients were among the first to benefit from the introduction of targeted therapy a decade ago, following a better understanding of the molecular biology of metastatic colorectal cancer (mCRC) and the advent of anti-vascular endothelial growth factor (VEGF) and anti-epidermal growth factor receptor (EGFR) drugs, such as bevacizumab, cetuximab and panitumumab. This was followed by the introduction of a multikinase molecule, regorafenib. Despite these advances, CRC is still one of the leading causes of cancer-related deaths, being the world's fourth most deadly cancer, with almost 900000 deaths annually[1]. Furthermore, the 5-year survival for metastatic colon cancer patients to date remains below 15%[2]. For this reason, it is of fundamental importance to enrich the therapeutic scenario of mCRC, and drugs that can impact not only mCRC overall survival but also quality of life are desperately needed.

MAIN LIMITATIONS OF TARGETED THERAPY IN THE TREATMENT OF MCRC

The use of anti-EGFR target drugs, such as cetuximab and panitumumab, and anti-angiogenesis drugs, such as bevacizumab and aflibercept, are consolidated in the clinical practice of metastatic CRC as first- and second-line treatments. Regorafenib is a multikinase drug approved for third-line treatment. In recent years, numerous new agents have emerged that block various critical pathways; however, many studies involving drugs that have led to excellent results in other tumours have not yielded the expected results in the treatment of mCRC. The main causes of treatment failure in mCRC are complex downstream signalling, difficulties in completely inhibiting specific biological interactions for the compensatory activation of other signalling pathways, and innate or acquired resistance to treatment (Figure 1).

An emblematic example of the compensatory activation of other signalling pathways is explained by the history of anti-BRAF drugs in BRAF V600E-mutated mCRC compared to melanoma. Mutations in the BRAF isoform, especially V600E, of the RAF protein are present in approximately 5% to 10% of CRCs[3]. The results of BRAF inhibitors in melanoma have led to an enthusiastic development of anti-BRAF/MEK drugs in CRC; however, blockade of BRAF or BRAF/MEK did not lead to a gain in PFS (progression-free survival) or OS (overall survival) of metastatic CRC patients, although it did result in inhibition of downstream MAPK activity[4]. A possible explanation is that blocking BRAF/MEK could trigger EGFR feedback reactivation, which would bypass MAPK activation *via* RAS[5]. Based on this evidence, subsequent studies have focused on the combined use of BRAF inhibitors and EGFR inhibitors[6], ultimately leading to FDA and EMA approval of the combination encorafenib, binimetinib and cetuximab in second- or third-line mCRC. This indication is the result of the BEACON study, which demonstrated a benefit in terms of overall survival (OS: 9 *vs* 5.4 mo, HR = 0.52, *P* < 0.001) and response rate (RR: 26% *vs* 2%, *P* < 0.01) with a good safety profile[7].

Innate and acquired resistance mechanisms are very complex and affect both anti-EGFR and anti-VEGF drugs.

Concerning anti-EGFR drugs, the main known mechanisms of resistance are RAS mutations, PI3K mutations, PTEN loss, HER2 overexpression, and compensative activation of alternative pathways.

HER2 is a protein member of the Erb family (erythroblastosis oncogene B Erb)/human epidermal growth factor receptor HER). In patients with CRC, the HER2 overexpression rate is 2%–3% and it is independent of the RAS or RAF mutation. HER2 acts similarly to EGFR (HER1), sharing many downstream pathways, such as RAS/RAF/MEK and PI3K/AKT. For this reason, HER overexpression provides a logical explanation for anti-EGFR resistance[8]. Preclinical and clinical studies have shown that combined targeting of HER2 and EGFR can lead to a better result than
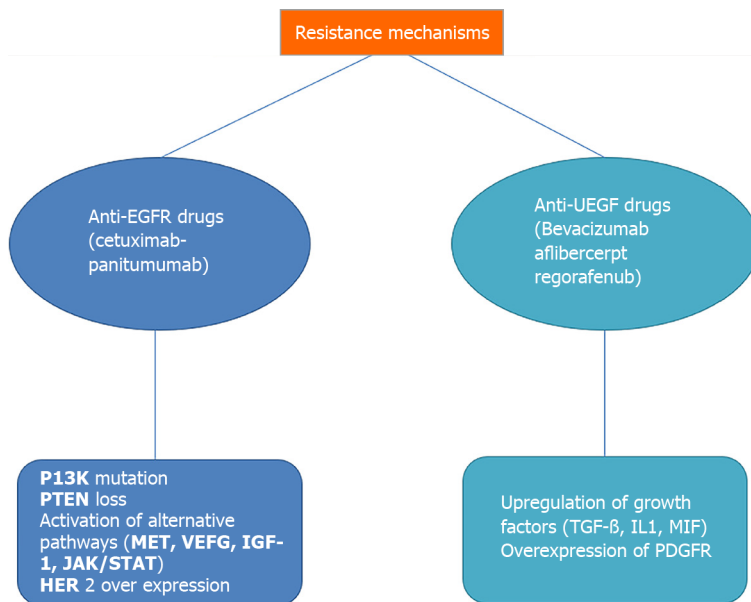
Figure 1 Main resistance mechanisms in targeted treatment for metastatic colorectal cancer. EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; TGF-β: Transforming growth factor-β; IL1: Interleukin 1.

those gained with the use of a single agent alone[9]. For Her2-overexpressing disease, unlike in breast cancer, the single inhibition of her2 does not seem to be effective in mCRC, which is likely linked to compensatory mechanisms and the activation of other pathways[10]. Further research is needed to better understand the clinical significance of *HER2* gene amplification in mCRC. An illustrative example has been reported in MyPathway, a clinical trial investigating the activity of pertuzumab + trastuzumab in patients with HER2-amplified mCRC, in which eight (8/57) patients had no response [11].

Compensatory activation of alternative pathways, such as c-MET, VEGF, insulin-like growth factor receptor 1 (IGF-1R), and JAK/STAT, could be linked to acquired resistance to anti-EGFR drugs[12].

Regarding antiangiogenic drugs, there are currently three approved drugs for mCRC: bevacizumab, a humanized monoclonal antibody that binds to vascular endothelial growth factor (VEGF-A) administered as a first- and second-line treatment; aflibercept, a recombinant fusion protein composed of fragments of VEGF receptors fused with the Fc portion of human IgG1 approved for second-line treatment; and regorafenib, an oral multikinase inhibitor approved for third-line treatment. There are several intrinsic and secondary known resistance mechanisms for antiangiogenic drugs.

In particular, these resistance mechanisms underlie the difficulty in translating preclinical successes into actual clinical advantage. For example, unlike preclinical studies, bevacizumab improves clinical outcomes only when combined with chemotherapy, with a short disease response to the withdrawal of antiangiogenic drugs, as tumour vessels rapidly grow back after stopping treatment. Compensatory mechanisms in antiangiogenic drug-resistant disease could be the upregulation of growth factors such as TGF-β, IL-1,231 MIF (macrophage migration inhibitory factor) and the overexpression of other growth factor receptors such as PDGFR[13].

Ultimately, the complexity of intrinsic and secondary resistance mechanisms in the targeted treatment of patients with metastatic CRC makes this pathology a challenging oncological dilemma.

NEW TARGET ARMAMENTARIUM THAT COULD ESCAPE PRIMARY AND SECONDARY RESISTANCE AND LEAD TO MORE PERSONALIZED MCRC THERAPY

Primary and secondary drug resistance represents the main limitation of CRC care, especially concerning targeted therapies; however, new promising drugs and drug combinations are expected to modify this complex scenario.

As mentioned before, patients with the BRAF V600E mutation have a worse prognosis, and the median overall survival (OS) is less than 1 year *vs* 2 years for patients without the non-BRAF V600E mutation[3]. In the phase III study BEACON trial, the small molecule BRAF V600E inhibitor encorafenib was combined with binimetinib, a MEK1/2 inhibitor, and cetuximab. The trial showed improved overall survival in both the triplet arm (cetuximab, binimetinib and encorafenib) and doublet arm (cetuximab and encorafenib). The median OS was 9.0 and 8.4 mo, respectively; however, the PFS was approximately 4 mo in both arms[7]. A possible explanation for this short PFS could be the reactivation of MEK and ERK signalling. An ERK 1/2 inhibitor, ulixertinib, is under investigation in a phase I trial, although data from a CRC cohort have not been reported[14].

The EGFR family also includes the HER2 receptor. Activating alterations of this receptor have been detected in approximately 2%–3% of RAS and RAF wild-type colon cancer cases[15]. Many phase II trials have explored the potential use of HER2 inhibitors in mCRC, including a combination of drugs such as trastuzumab and lapatinib in the HERACLES trial[16], trastuzumab and tucatinib, an orally administered HER2–3 inhibitor, in the MOUNTANEER trial[17], and pertuzumab and trastuzumab-emtansine (TDM1) in the HERACLES-B trial[18]. All these trials showed potential activity in terms of ORR and PFS in pretreated metastatic CRC HER2-amplificated patients with the combination of Her2 inhibitor blockade. To date, no drugs have been approved for Her2-amplified CRC.

New promising molecules are also being explored in the VEGF inhibitor setting. In particular, a multicentre phase III study, FRESCO-2, comparing placebo *vs* fruquintinib (NCT04322539), is ongoing. Fruquintinib is a highly selective small molecule inhibitor of VEGFR 1, 2, 3[19]. In the first FRESCO trial, the fruquintinib group showed a median OS of 9.3 mo *vs* 6.6 in the placebo group (*P* < 0.001) and a PFS of 3.7 mo *vs* 1.8 mo. Due to the encouraging results obtained with this trial in China, it has been extended and is now recruiting in Europe and the United States[20].

Current studies are also investigating the potential role of the combination of VEGF with conventional chemotherapy. Trifluridine/tipiracil (TAS-102) was associated with bevacizumab in a phase II study of 93 patients. The association improved both PFS and OS compared with TAS-102 alone[21]. Trifluridina/tipiracil is under investigation with many other drugs, probably due to its low toxicity profile and the absence of cross-resistance with 5-fluorouracil in pretreated patients.

Another crucial new perspective for patients with mCRC is immunotherapy. Currently, immunotherapy has been approved in the United States and will be approved in Europe for patients with microsatellite-deficient mismatch repair/micro-satellite instability-high (dMMR/MSI-high), which affects approximately 15% of all patients with mCRC[22]. The clinical trial KEYNOTE 177 comparing pembrolizumab, a PD-1 inhibitor, with standard chemotherapy showed a substantial improvement in PFS with pembrolizumab as the first line in dMMR/MSI-high mCRC[23]. This trial thus represents a practice-changing approach in the first-line therapy of patients with dMMR/MSI metastatic CRC. In the same way, the CheckMate 142 phase II trial investigated the association of nivolumab and ipilimumab in pretreated dMMR/MSI metastatic CRC. The combined treatment, such as for melanoma cancer, showed high response rates and favourable progression-free survival and OS at 12 mo with a low toxicity profile[24].

Unfortunately, immunotherapy is a missed opportunity for patients with proficient mismatch repair and microsatellite stable (pMMR/MSS) mCRC. Many ongoing studies are exploring the possibility of combining immune checkpoint inhibitors with VEGF inhibitors to enhance lymphocyte activation. In preclinical models, VEGF inhibitors showed synergistic action with immune checkpoint inhibition[25]. Based on this evidence, regorafenib was combined with nivolumab in a phase Ib trial (REGONIVO) in patients with refractory metastatic gastric and CRC, obtaining a median PFS of 5.6 and 7.9 mo, respectively[26]. Similarly, in the REGOMUNE phase II trial, the combination of regorafenib and avelumab showed a median progression-free survival of 3.6 mo and overall survival of 10.8 mo[27]. Bevacizumab has been combined with atezolizumab and the triplet chemotherapy regimen FOLFOXIRI (oxaliplatin, irinotecan and 5-fluorouracil) in the AtezoTRIBE trial for patients with unresectable or metastatic CRC. The results are not yet available[28].

Recent data showed a promising combination of avelumab and cetuximab in a rechallenge strategy for RAS and RAF wild-type mCRC patients. In a preliminary analysis, the CAVE study showed a median OS of 13.1 mo and a median PFS of 3.6 mo [29]).

The complexity of the resistance mechanisms, multiple escape pathways and disease biology make metastatic CRC a challenging disease in terms of therapeutic strategies. Fortunately, patients with mCRC maintain a good performance status even during disease progression. A very large number of new drugs or combinations are under investigation to reach an even more personalized cancer cure.

FUTURE DIRECTIONS

Currently, negative predictive markers for the response to EGFR-targeted therapies (KRAS, NRAS mutations), anti-BRAF targeted therapies (BRAF mutation) and positive predictive markers for immune checkpoint inhibitors (microsatellite instability) are standard of care in the treatment of mCRC. In all the main arms of medical oncology, the future seems to lead towards a great and ambitious goal represented by personalized medicine. A large area of research is concentrated on this trend, focusing on next-generation sequencing (NGS)[30] (Table 1).

According to the European Society for Medical Oncology (ESMO) guidelines, in colon cancers, NGS could be an alternative to PCR[31]. This method has led to the identification of mutations that could explain greater resistance to standard treatments [32,33] as well as new targets whose therapeutic effects are being studied.

Concerning the use of NGS to identify patients who are likely to respond to standard treatments, few interesting studies have been conducted. One example is the study conducted by Innocenti and colleagues that analysed the response to standard treatments with cetuximab or bevacizumab-based regimens and the results that emerged from NGS. Mutated genes that conferred worse overall survival (OS) than wild-type (WT) tumours and mutations that conferred better survival were highlighted. For example, FANCI-mutated tumours (4%) conferred worse OS than WT tumours [HR 2.0 (1.2–3.3), *P* = 0.005; OR 5.0 (1.9–14.8), *P* = 0.002][34]. These findings are very interesting, as they could provide new genes that could become predictors of response to chemotherapy regimens with cetuximab and bevacizumab combinations. If validated in other phase III trials, these mutated genes could be used to guide treatment decisions in mCRC patients.

Another interesting ongoing trial is the COLOMATE umbrella trial, which uses the genomic profiling Guardant360 NGS assay, a plasma-based assay of more than 70 genes, to detect colorectal tumour cfDNA to assign patients with advanced CRC to specific targeted treatment arms based on the molecular profiles of their tumours (NCT03765736). It is fascinating to think that this approach could become our clinical practice in the very near future.

Listed below are some new targets we believe could represent a potential innovation in the near future: KRAS, PI3K, NTRK fusions, ALK, ROS1, RET, and FGFR.

KRAS and NRAS mutations occur in a consistent percentage of mCRC cases (approximately 50% of cases) and identify tumours with a poor prognosis. Mutated KRAS tumours are also inherently resistant to anti-EGFR drugs. KRAS is considered a challenging therapeutic target (Cox AD). Nevertheless, among the different RAS mutations, the KRAS pG12C mutation, which represents approximately 1%–4% of RAS mutations in CRC[35], has been considered potentially druggable. In particular, KRAS-dependent signalling is inhibited by binding to a pocket near the nucleotide binding site and locking it in an inactive guanosine diphosphate (GDP)-bound state [36]. Two drugs are currently under investigation in colon cancer, sotorasib (AMG510) and adagrasib (MRTX849). The first phase I study, CodeBreack100, investigated the activity of sotorasib in 129 pretreated patients with the KRAS G12C mutation, including 42 patients with mCRC[37]. In the colorectal cohort, the overall response rate (ORR) was 7.1% and the disease control rate was 73.8% (DCR). The median duration of stable disease was 4 mo. Overall, these results were considered disappointing in terms of quality, duration, and adaptive signalling response to drug treatment. The authors postulated that KRAS G12C-mutant cancer cells may still become activated upstream by EGFR[38]. For this reason, ongoing studies combining KRAS G12C inhibitors and EGFR inhibitors, such as the randomized phase 3 clinical trial comparing MRTX849 in combination with cetuximab *vs* chemotherapy in patients with advanced CRC, KRYSTAL 10 (NCT04793958), are ongoing.

Another fundamental oncological driver in CRCs is the PTEN/PI3K/mTOR pathway (20% of cases). The presence of the PI3K mutation confers resistance to anti-EGFR treatments. For this reason, combinations of PI3K oral inhibitors with cetuximab are being studied. This approach, which has been successful in patients with a BRAF

Table 1 Main ongoing studies (clinicaltrial.gov) for metastatic colorectal cancer

Study	Treatment	Phase of study	Primary objectives
COLOMATE trial			
NCT03765736	Specific targeted treatment arms based on the molecular profiles	Phase II prospective trial	(1) To perform blood-based genomic profiling on patients with treatment refractory metastatic colorectal cancer (CRC) to facilitate accrual to molecularly assigned therapies; and (2) To facilitate clinically annotated genomic analyses
CALGB (Alliance)/SWOG 80405			
NCT00265850	Bevacizumab or cetuximab combined with the same chemotherapy	Phase III, randomized, open-label, multicentre study	To determine if the addition of cetuximab to FOLFIRI or FOLFOX chemotherapy prolongs survival compared to FOLFIRI or FOLFOX with bevacizumab in patients with untreated, advanced or metastatic colorectal cancer who have K-ras wild type tumours
KRYSTAL 10			
NCT04793958	MRTX849 (inhibitor of KRAS G12C) in Combination with Cetuximab *vs* Chemotherapy	Phase III, open-label, randomized	Comparing the efficacy of MRTX849 administered in combination with cetuximab *vs* chemotherapy in the second-line treatment setting in patients with CRC with KRAS G12C mutation
C-PRECISE-01			
NCT04495621	MEN1611 + Cetuximab	Phase Ib/II, open-label, multicentre study	MEN1611, a PI3K Inhibitor, and Cetuximab in Patients With PIK3CA Mutated Metastatic Colorectal Cancer Failing Irinotecan, Oxaliplatin, 5-FU and Anti-EGFR Containing Regimens
NCT04096417	Pemigatinib	phase II, multicentre, single-Arm study	To assess overall response rate (ORR) of pemigatinib in patients with metastatic or unresectable CRC harbouring activating FGFR alterations
MOUNTAINEER			
NCT03043313	Trastuzumab+tucatinib	Phase II open label study	Tucatinib combined with trastuzumab in patients with HER2+ metastatic colorectal cancer
NAVIGATE			
NCT02576431	Larotrectinib	Phase II open label study	Investigate the efficacy of larotrectinib for the treatment of advanced solid tumours harbouring a fusion of neurotrophic tyrosine receptor kinase (NTRK) of types 1–3 in children and adults
NCT03829410	Onvansertib (PCM-075)	Phase Ib/II open label study	Determine the safety and efficacy of Onvansertib in combination with FOLFIRI + Avastin, as second-line treatment in adult patients who have metastatic colorectal cancer with a Kras mutation
STARTRK-2			
NCT02568267	Entrectinib (RXDX-101)	Phase 2 basket study	Treatment of patients with Locally Advanced or metastatic solid tumours that harbour *NTRK1/2/3*, *ROS1*, or *ALK* gene rearrangements
NCT03724851	Vactosertib (TGF-β receptor I kinase inhibitor) + pembrolizumab	Phase 2, open label study	Safety, tolerability, pharmacokinetics and antitumour activity of vactosertib in combination with pembrolizumab in patients with mCRC including CMS4 or diffuse GC/GEJC

EGFR: Epidermal growth factor receptor; TGF-β: Transforming growth factor-β.

mutation[7], another driver that confers resistance to treatments with anti EGFR, could be applicable in patients with a PI3K mutation[39].

Neurotrophic receptor tyrosine kinase (NTRK) fusions are chromosomal abnormalities that result in uncontrolled TRK signalling that can lead to cancer. NTRK fusions can be identified with NGS, immunohistochemistry (IHC), polymerase chain reaction (PCR), and fluorescent in situ hybridization (FISH) techniques. NTRK fusion-positive mCRC is rare (0.9%)[40]. For this reason, a clinical indication to seek the presence of a NTRK fusion is lacking; however, with increasingly accessible NGS, this target will be necessary to evaluate. In mCRC, NTRK fusions are more frequent in elderly patients, in females, and in right-sided tumours. From the point of view of molecular biology, they are often associated with MSI-H, RAS and BRAF wild-type, as well as poor prognosis with a median overall survival (OS) of approximately 15 mo [41]. Regarding the efficacy of treatments with the oral TRK-selective inhibitors larotrectinib and entrectinib, registration studies of these two molecules, including a very low number of patients with mCRC (4 patients for larotrectinib and only 1 patient for entrectinib), are scarce given the rarity of NTRK fusion in colon cancer; however, the data are encouraging. For example, in the 4 patients included in the single-arm

study that evaluated the efficacy of larotrectinib, a partial response and disease control rate were achieved in 2 and 4 cases, respectively[42]. The data are too scarce to draw any conclusions but favourable given the poor prognosis of this category of patients.

Other very rare mutations in mCRC are rearrangements of anaplastic lymphoma kinase (ALK) and v-ros avian UR2 sarcoma virus oncogene homologue (ROS1)[43]. The future use of oral tyrosine kinase inhibitors, such as alectinib and crizotinib, would be possible in patients with mCRC who present such rearrangements. RET (rearranged during transfection) fusions are even rarer in mCRC (2% of cases)[44], although the message is the same: could a mutation, albeit rare, be successfully treated with a specific drug already used in clinical practice for other solid tumours? These are the considerations we will need to be increasingly familiar with in the near future. Unfortunately, we have no data from clinical trials on the use of selective RET inhibitor drugs in mCRC. A case report of a patient with mCRC harbouring a RET fusion treated with a selective RET inhibitor achieved a complete response to the selective RET inhibitor drug RXDX-105 and a significant PFS of 19 mo[45].

Aberrant activation of fibroblast growth factor (FGFR) signalling has been implicated in the development of various cancers, including colon cancer. Several studies have been conducted to validate the efficacy of FGFR inhibitors in mCRC and other solid tumours (NCT04096417, NCT01976741, NCT03410693, NCT03473756).

Given all these data, the use of NGS will be essential in clinical practice for the treatment of mCRC.

CONCLUSION

Limitations in the development of novel CRC drugs are due to the mechanisms of resistance to target treatments, namely, EGFR antibodies and antiangiogenic treatments, which currently represent therapeutic options for mCRC. A better understanding of mCRC molecular biology has elucidated the resistance mechanisms and consequently enabled the development of combined treatments geared towards precision medicine. The advent of new effective therapies has been very slow in CRCs; in fact, the complexity of the mechanisms involved in the carcinogenesis of CRC makes it difficult to use single biological targets for the development of new drugs. Currently, many signs give hope for new potential possibilities in the treatment of this challenging cancer.

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Extracellular vesicles: General features and usefulness in diagnosis and therapeutic management of colorectal cancer

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Abstract

In the world, among all type of cancers, colorectal cancer (CRC) is the third most commonly diagnosed in males and the second in females. In most of cases, (RP1) patients' prognosis limitation with malignant tumors can be attributed to delayed diagnosis of the disease. Identification of patients with early-stage disease leads to more effective therapeutic interventions. Therefore, new screening methods and further innovative treatment approaches are mandatory as they may lead to an increase in progression-free and overall survival rates. For the last decade, the interest in extracellular vesicles (EVs) research has exponentially increased as EVs generation appears to be a universal feature of every cell that is strongly involved in many mechanisms of cell-cell communication either in physiological or pathological situations. EVs can cargo biomolecules, such as lipids, proteins, nucleic acids and generate transmission signal through the intercellular transfer of their content. By this mechanism, tumor cells can recruit and modify the adjacent and systemic microenvironment to support further invasion and dissemination. This review intends to cover the most recent literature on the role of EVs production in colorectal normal and cancer tissues. Specific attention is paid to the use of EVs for early CRC diagnosis, follow-up, and prognosis as EVs have come into the spotlight of research as a high potential source of 'liquid biopsies'. The use of EVs as new targets or nanovectors as drug delivery systems for CRC therapy is also summarized.

Key Words: Extracellular vesicles; Colorectal cancer; Diagnostic; Prognosis; Vector;

Therapy

Core Tip: New efficient screening and treatment approaches are strongly mandatory to increase colorectal cancer (CRC) patients' prognosis. Extracellular vesicles (EVs) represent a promising mean to diagnose and treat colorectal cancers. This review summarizes the most recent literature on the use of EVs in the management of CRC.

INTRODUCTION

In the world, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. In 2018, 1.8 million new cases were reported with almost 861000 related deaths according to World Health Organization[1]. In Europe and United States, approximately 748000 and 148000 new cases of large bowel cancer are diagnosed annually, two third being colon cancers, the remainder being rectal ones [2,3]. Respectively 242000 and 53000 died of CRC-related diseases. While still treated first by surgery and chemotherapy, despite a better understanding of its natural history and the development of new therapies (immune checkpoint inhibitors, *etc.*), CRC recurrence and metastasis are still the main causes of death[4]. Thus, determining relevant factors involved in disease progression is strongly mandatory to drive development of new effective strategies for therapies against CRC, *etc.*). In tumor evolution, recent studies have shown the weight of continuous interplay between surrounding cells (cancer cells with themselves, cancer cells with stromal cells[5]. Such communication strategies require specific mechanisms including direct cell to cell contacts but also autocrine, juxtacrine, paracrine and even endocrine secretion of specific factors (growth factors, matrixins, cytokines, chemokines, *etc.*)[6]. Among such secreted means figure extracellular vesicles (EVs), a generic consensus term used to describe any type of lipid bilayer-delimited particles, unable to replicate, and extracellularly released by every cell (including microorganisms)[7-9]. EVs surface receptors allow their targeting and capture by a broad range of recipient cells that will incorporate either proteic, lipidic, or genetic messages resulting in modifications of their physiological behavior. These EVs have been recently proved to be efficient communication means in human diseases[10], especially in cancer. As the field of EVs is extremely active[11,12], we aimed to review the respective roles of colonic cells EVs as well as stromal derived-EVs in colon cancer to better understand cellular and molecular mechanisms underlying its occurrence and development. We also underline EVs as powerful and early tools to diagnose colon cancer, to accurately define its aggressiveness, and to better design, in a personalized approach, treatment strategies.

EVS GENERAL PROPERTIES

Either eukaryotic or prokaryotic cells produce continually various amounts of 40-1000 nm membrane vesicles that are released into local environment. Such EVs can be evidenced in the conditioned media of every cultured cell, but also in almost all biological fluids (including blood, cerebrospinal fluid (CSF), urine, saliva, seminal plasma, and breast milk)[13,14]. EVs definition embodies different terms, sometimes used indifferently in literature, including exosomes, microvesicles, microparticles, multivesicular bodies, apoptotic particles, apoptotic bodies, oncosomes, *etc.* As not yet defined biomarkers can specifically categorize each vesicle, as a rule the 2018 minimal information for studies of extracellular vesicles consensus recommends to label bilayered vesicles smaller than 200 nm as small EVs (SEVs) and those larger than 200

nm as medium large EVs (MLEVs)[15]. Alternatively, the original process of the cell can also be mentioned: Oncosomes specifically refer to oncogene containing EVs, large oncosomes being massive EVs (over 1000 nm) produced by oncogenically transformed cells[16]. As they lack bilayered membrane, this definition should exclude the recently discovered sub-50 nm nanoparticles exomeres[17].

EVs natural history

MLEVs production: MLEVs, so called ectosomes, are heterogeneous membranous vesicles generally originating from outward plasma membrane budding (ectosomal release)[18]. In contrast with apoptotic bodies or necrotic blebs of the plasma membrane (PM) that are the consequences of complex structural transformations resulting in dying cells disassembly[19], ectosomes are shed by living cells.

SEVs synthesis & release: Unlike ectosomes, SEVs stemmed from the endosomal compartment. SEVs biogenesis starts with the inward budding of small portions of the plasma membrane containing outer membrane exposed material. These small intracellular vesicles form the early endosome. Inward budding of the limiting membrane of the early endosome then occurs, resulting in the progressive assemblage of intraluminal bilayered vesicles (ILVs) within so-called large multivesicular endosomes (MVEs) (Figure 1). During this process, cytosolic proteins as well as nucleic acids can be trapped into ILVs through the action of the endosomal sorting complex required for transport (ESCRT) machinery[20]. ESCRT is a family of proteins that associate in successive complexes (ESCRT-0, -I, -II and -III) at MVEs membrane to sort ubiquitinated cargos into late endosomes[21]. ESCRT is also essential for ILVs generation and cargo targeting driving through deubiquitinating enzymes recruitment [22,23]. Interestingly, such protein sorting can also follow a ceramide ESCRT-independent pathway suggesting a critical role for lipid raft microdomains in MVEs formation[24]. Most of MVEs are further directed for cargo degradation into lysosomes by fusing with them. Nevertheless, MVEs also contain intralumenal proteins and lipids, which are not intended for lysosome degradation. ILVs can release their content into the cytoplasm by undergoing direct back-fusion with the endosome limiting membrane[25]. Progressive acidification along the endocytic pathway seems to be required for degradation and recycling of internalized components suggesting that pH could be a major determinant of MVEs degradation *vs* secretion functions[26]. Indeed, concerning MVEs secretory function, a subset of MVEs fuse to PM and release their content into the extracellular space, in the form of SEVs, a process called exosome biogenesis[27]. MVEs that are fated for exocytosis are transported to PM along microtubules by the molecular motor kinesin[28]. MVEs docking to PM are strongly regulated by the Rab family of small GTPases proteins. Depleting Rab27a prevented MVEs to efficiently fuse with the PM while Rab27b knockdown resulted in perinuclear MVEs accumulation, both observations suggesting that Rab27 was responsible for trafficking MVEs to the cell surface[29]. Once docked, secretory MVEs couple to the SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) membrane fusion machinery[30]. SNARE complex formation and membrane fusion are tightly controlled by multiple regulatory mechanisms[31] among which figure phosphorylation profile of SNARE proteins that influence either SNARE complex localization or interaction with SNARE partners[32].

EVs capture: Once released by the secreting cell, EVs distribute to extracellular matrix (ECM) then circulate locoregionally or distantly to deliver their molecular cargo to recipient cell. EVs cargo is protected from degradation and is rapidly taken up by different organs, such as liver, spleen and lymph nodes[33]. Circulating labelled EVs half-life has been evaluated in mice to be about 2 min but it remains possible to detect EVS in the bloodstream hours after injection[34]. Although still globally unknown, differences in EV size and presence of outer surface membrane components probably could account for their recognition and capture by target cells[35]. Once recognized, strongly depending on recipient cell type[36], EVs will enter through a variety of endocytic routes, either through clathrin dependent or independent pathways (caveolin-mediated uptake, lipid raft-mediated internalization, *etc*.). Also, both phagocytosis and macropinocytosis can been involved in EVs uptake[37], the latter being very efficient for specific EVs like those harboring CD47 at their surface[38]. After internalization, while endosome seems one of the best candidate locations for EVs membrane fusion then cargo delivery, EVs intracellular fate remains a matter of debate (Figure 1).

Altogether, due to the multiple sorting mechanisms that determine specific molecules incorporation into EVs, the distinct vesicle subpopulations carrying
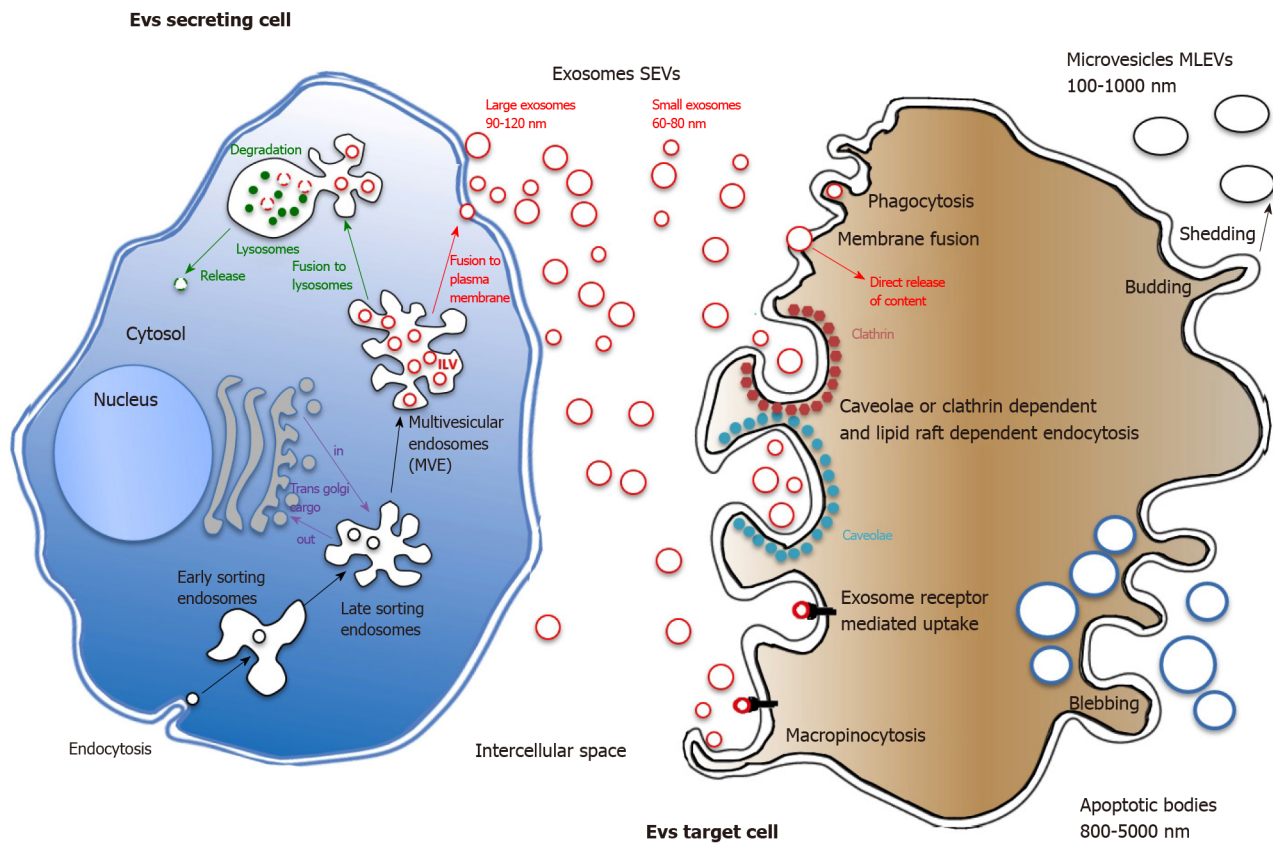
Figure 1 Extracellular vesicles biogenesis and interaction with recipient cells. Extracellular vesicles (EVs) may have multiple origins. They can originate from plasma membrane blebbing during the apoptotic process giving rise to large apoptotic bodies or by membrane budding that leads to heterogeneous membranous EVs shedding. Small EVs (SEVs, exosomes) originate from internal budding of plasma membrane giving rise to early endosomes. By complex maturating interactions with the Golgi apparatus, early become late endosomes. The membranes of late endosomes form intraluminal vesicles (ILVs), small cargos containing proteins from plasma membrane and Golgi as well as nucleic acids. ILVs are contained in multivesicular endosomes that will fuse with either plasma membrane, releasing SEVs in the extracellular space or with lysosomes for further internal degradation. The endosomal sorting complex required for transport is the key machinery of protein sorting into SEVs. Once recognized, strongly depending on recipient cell type, EVs will enter through a variety of endocytic routes, either through clathrin-dependent or independent pathways (caveolin-mediated uptake, lipid raft-mediated internalization, *etc.*). Phagocytosis, macropinocytosis and simple membrane fusion can also be involved in EVs uptake. MLEVs: Medium large extracellular vesicles; SEVs: Small extracellular vesicles; MVEs: Multivesicular endosomes; ILVs: Intraluminal vesicles; MVP: Multivesicular particles; ESCRT: Endosomal sorting complex required for transport.

different cargo that can be evidenced, and the complex pathways/factors that regulate EVs export and secretion, EVs biogenesis threshold is likely to greatly vary between cell types according to their physiological/pathological status. The high rate of SEVs secretion found in transformed cells suggests that the balance between EV degradation and secretion is disrupted in cancer towards EVs cargo release[39]. This kind of change is not specific to cancer cells but may also occur in non-transformed cells. In antigen-presenting cells, large amounts of SEVs are found to be released upon stimulation[40].

EVs cargo content
EVs are highly heterogeneous and likely reflect the phenotypic state of the cell that generates them[41]. Every EVs behave as a multi-molecular cargo whose bilayered membranes regulate its stability by protecting bioactive content from degradation[42]. Alike cells, EVS can contain inside their lipid bilayer every basic constituent of a cell including metabolites[43], functional proteins (enzymes, receptors, transporters, *etc.*) [44-46], but also nucleic acids molecules such as mRNAs[47], interfering microRNAs (miRNAs)[48], small and long non-coding RNAs (snRNAs & lncRNAs)[49], and even mitochondrial DNA[50] or more recently genomic DNA[51] (Figure 2).

EVs protein cargo: Because of their endosomal origin, and since they derived from the ILVs in MVEs, SEVs biogenesis is heavily dependent on the mechanisms that regulate MVEs maturation and trafficking. SEVs mostly contain proteins originating from the cytosol and either endosomes then PM components[7]. As budding and release of EVs require inner PM actin polymerization then actomyosin cytoskeleton contraction, cytoskeleton proteins such as actin and tubulin are generally found in EVs[52,53].
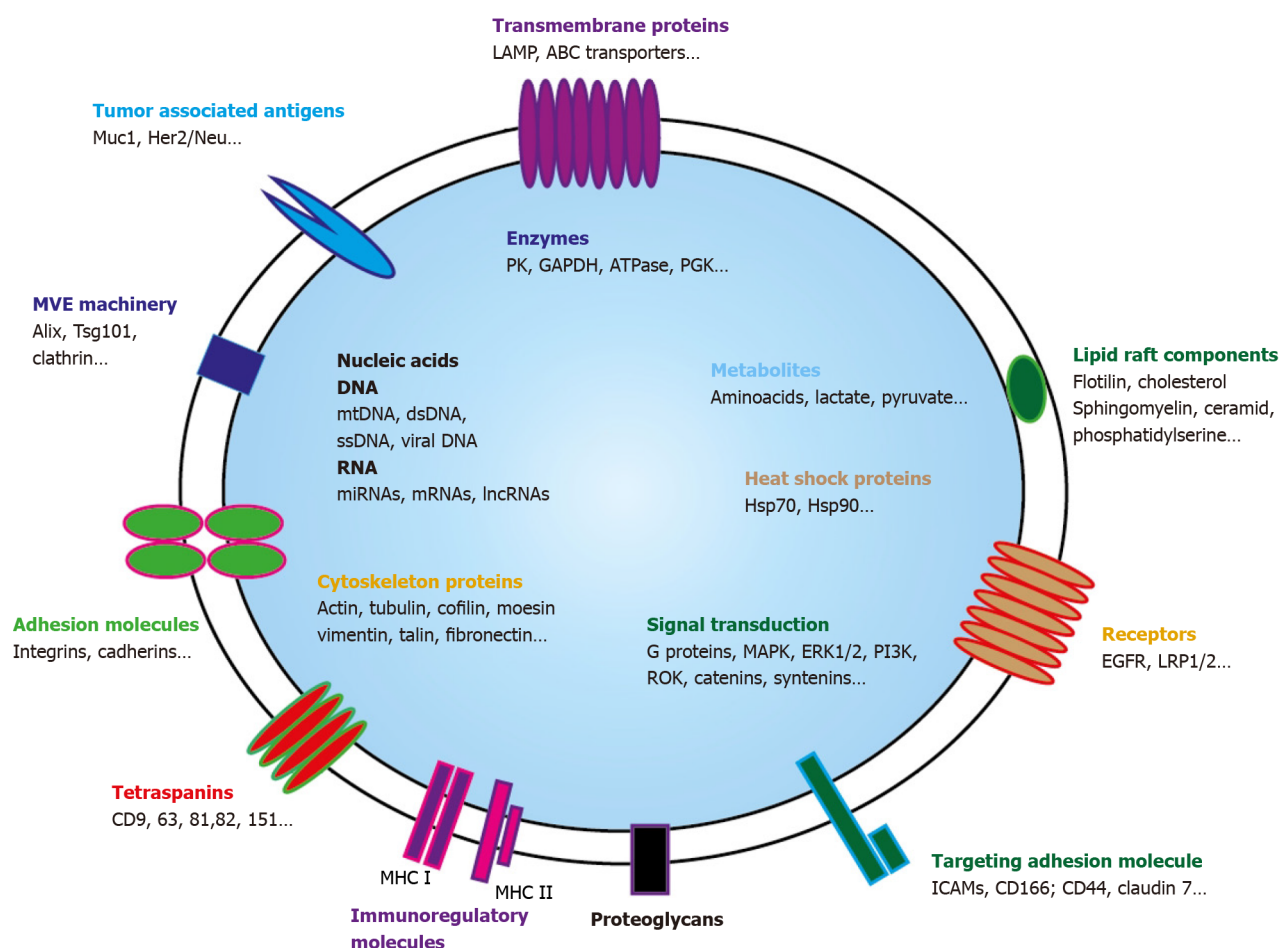
Figure 2 Exosome and its cargo content. Small extravesicles (SEVs) are nano-sized membrane vesicles released by a variety of cell types and are thought to play important roles in intercellular communications. SEVs contain many kinds of proteins, either cytosolic or plasma membrane ones. Transporters, receptors, signaling proteins… but also enzymes can be evidenced. Metabolites are also present as well as nucleic acids. Genomic and mitochondrial DNAs, and multiple RNAs (mRNAs, miRNA, lncRNA, circRNA…) can be detected. Through horizontal transfer of these bioactive molecules, SEVS are emerging as local and systemic cell-to-cell mediators of oncogenic information. MHC: Major histocompatibility complex; MVE: Multivesicular endosomes.

Among highly representative proteins that can also be found in SEVs figure important regulators of EVs trafficking: (1) Members of the Rab family that play well-established roles in vesicle transfer between intracellular compartments such as MVEs driving to PM for SEVs secretion[54,55]; (2) SNARE membrane fusion machinery, through SNARE complexes recruitment, that is specifically required for MVEs docking then fusion with PM[30,35,56]; (3) ESCRT proteins and important ESCRT side molecules implicated in ESCRT assembly or nucleation like ALIX[57]; and (4) Tetraspan transmembrane proteins (tetraspanins), highly enriched in SEVs, that are also involved in ESCRT-independent EVs release[58,59]. Tetraspanins display high affinity for cholesterol and sphingolipids such as ceramides which may create PM microdomains as it occurs in membrane reconstitution experiments[60]. Their interaction with PM proteins, either by direct association or by entrapment in tetraspanin-enriched PM microdomains, facilitates their sorting into EVs[58,61-63].

Interestingly, EVs can also transport mitochondrial proteins that may be active. Two mitochondrial inner membrane proteins MT-CO2 (encoded by the mitochondrial genome) and COX6c (encoded by the nuclear genome) were highly prevalent in the plasma of melanoma patients, as well as in ovarian and breast cancer patients defining a new EVs subtype[64]. As not only mitochondrial membrane proteins but also mitochondrial enzymes are present in EVs, mt-EVs could affect the metabolic output of the recipient cells by either preventing inflammation[65] or promoting tumor growth[66-68].

SEVs specific endosomal-driven content allows their distinction from ectosomes that can directly bud and shed from PM at lipid-raft-like domains[69]. These vesicles, now generically referred to as MLEVs, are extremely heterogeneous in size, ranging from 200 nm to as large as 10 μm. They are generally enriched in cell surface or integral

transmembrane proteins, reflecting their PM origin[70,71]. For example, during reticulocyte maturation, autophagosomal exocytic event is coupled with plasma membrane blebbing that release glycophorin A, an integral plasma membrane protein, into budding vesicles[72].

Last, SEVs content is also distinct from apoptotic microparticles or apoptotic bodies (apoBD). ApoBDs are larger than SEVs and MLEVs as they have a diameter of 800–5000 nm[73]. ApoBDs encapsulate residual ingredients of dying cells. They are enriched with autoantigens and pro-inflammatory factors[74,75] and bear key markers of cell disassembly such as ROCK1 and PANX1 and apoptotic markers such as CD31 or Annexin V.

EVs metabolite cargo: Aside proteomic studies that try to unravel the complex protein repertoire in EVs, metabolomic studies reveal that EVs contain different classes of low-molecular-weight compounds. Organic acids, nucleotides, sugars and their derivatives, carnitines, vitamins and related metabolites, and amines are frequently evidenced in EVs[43]. Of course, most of these metabolites were generally derived from cytosolic cellular pathways, as large portions of cytosol are engulfed in ILVs then EVs[76]. Nevertheless, metabolites presence could also result from either specific metabolite sorting or ILVs/EVs in situ synthesis through residing metabolic enzymes as high metabolite concentrations over the cellular levels were reported in EVs[77]. Complete but more often partial metabolic routes can be evidenced in EVs explaining why EVs metabolite identification does not generally cover the whole parental cell metabolome but represents a miniature subset of it.

Lipids are also frequently found in EVs. EVs lipidome analysis allows characterization of different classes of lipids, including glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, and fatty acids confirming similarity between EVs lipid content and their parental cells membranes composition[78]. As it is important to preserve functional flexible lipid bilayer as well as right ion composition and pH-homeostasis[60], numerous ATP-driven transporters and ion-pumps are also found in EVs. To be fully functional, these elements need energy supply that may be given either by glycolytic enzymes[79] or even mitochondrial ATP synthase that is frequently found in EVs[64]. To optimize energy thresholds, such enzymes and substrates seems to be organized in metabolons that have been found to be fully functional in EVs[80].

Every cell may send out a range of messages to distinct still unknown targets, and both messages and targets may vary depending on the metabolic state of the producing cell. In EVs metabolic composition is of importance as it may represent a specific environment ("climate") the parental cell is going to transfer to the recipient one. By providing substrates for biosynthesis, EVs-transported aminoacids (glutamine, leucine…) have been shown to strongly affect the tricarboxylic acid (TCA) cycle of the recipient cancer cells thus improving nutrient status of fast growing and proliferating cells[81]. By providing both enzymes and substrates, adipocytes EVs stimulate melanoma fatty acid oxidation (FAO) that increase mitochondrial activity redistributes mitochondria to membrane protrusions of migrating cells, which is necessary to increase cell migration[82]. Interestingly, using various cell culture protocols, several reports have shown that EVs production in quantity and composition is largely influenced by external factors[83], the most striking variation being in the EVs metabolomes[84]. As slight metabolic variations could drive cancer cell reprogramming[85], the role of EVs seems central in that process.

EVs RNA cargo: Valadi and Skog both demonstrated that EVs transported mRNAs that can be translated into protein, providing the first evidence of virus-independent genetic material horizontal transfer between cells[86]. Since these pioneering studies, the presence of RNAs, within EVs have been reliably shown with either microarrays or real-time quantitative polymerase chain reaction techniques in numerous reports[47]. This presence can easily be explained as cytosolic proteins engulfment, resulting from a microautophagy process[87], involve proteins located close to the MVE outer membrane during its inward budding and can comprise RNAs molecules[86]. Those RNA species include not only mRNAs but also rRNA, tRNA, snRNA, snoRNA, piRNA, Y-RNA, scRNA, SRP-RNA, 7SK-RNA and lncRNAs. All these RNAs can be transferred to the recipient cells[88,89]. In addition, two major components of the RNA-Induced Silencing Complex, namely DICER and Argonaute, aimed at producing miRNAs have been shown to associate with MVE and to be sorted into exosomes[48, 90]. This suggests that miRNAs are likely to be packaged into EVs along with proteins required for their processing or function)[91]. As largely protected from RNAses when packaged in EVs, miRNAs driven-gene regulation will be able to generate a

multifaceted signaling response in the target cell. As EVs mRNAs are also functional and can be translated in the target cell[86], both mechanisms provide a direct modulation of recipient cell protein production. This new signaling pathway play specific roles in intercellular communication during various physiological[14,92] or pathological processes. Indeed, numerous reports have described the ability of EVs RNAs to impact the functional properties of cells that incorporate them[93], especially in the cancer field where such mechanism may drive apoptosis resistance[94], drug resistance[67,95,96], and metastatic behavior[89].

EVs DNA cargo: Extracellular DNA is present in the circulation and may represent an attractive marker issue for liquid biopsies. In plasma, DNA is found both in free form and enclosed in EVs[97,98]. Rather than being packaged within EVs membrane-bound space, DNA seems mostly attached to the outer surface of EVs[99]. Quantities as well as properties of packaged DNA may largely vary in different subsets of EVs even originating from the same source. It is likely that the heterogeneity of DNAs in EVs is related to the size of EVs. In contrast to SEVs that are more frequently devoid of DNA, large size intact DNA (> 2 Mbp), generally associated to histones, is commonly found in LEVs[100,101]. EVs DNA fragments may represent and even cover all chromosomes of parental cells[51,97]. As DNA sometimes harbor mutations, it may reflect the mutational status of parental DNA[102-104] and thus serve as a relevant oncologic biological marker.

Beside single stranded and/or double stranded genomic DNA, mtDNA can also be found in EVs extracted from cell culture medium[105,106] but also in plasma EVs[107] where presence of complete mitochondrial genome has been evidenced. Transfer of this complete mtDNA molecule seems to drive recipient cells fate[108].

EVS ROLE IN LARGE BOWEL TISSUES AND COLORECTAL CANCER

Considering the many cell types that interact at the mucosal interface, the intestinal lumen could be a rich source for EVs in large bowel tissues as well as an interesting source of disease-specific EVs in pathological conditions.

EVs production in normal large bowel tissues

Normal colonic cells as a primary source of EVs: As most of our tissues, colonic tissue may be an important source of EVs. Intestinal epithelial cells (IEC) are located at the strategic interface between external environment and the body most extensive lymphoid compartment. Aside their essential role in nutrients absorption, IEC have been shown to play a key role in immune response by promoting and regulating luminal antigens presentation to mucosal immune cells[109] through EVs release at both apical and basolateral sides as IEC display all the elements needed for either antigen processing or EVs production[110]. These EVs contain molecules that are implicated in adhesion and antigen presentation, such as major histocompatibility complex (MHC) class I molecules, MHC class II molecules, CD63…[111]. As these EVs may also contain CD133, whose presence in lipid rafts play a pivotal role in the maintenance of stem cell features[112], it has been suggested that CD133-containing EVs release may contribute to cell differentiation by reducing and/or modifying stem cell characteristic membrane microdomains composition within IEC apical plasma membrane[113].

Maintenance of the intestinal stem cell can be driven by niche-derived EVs: The intestinal epithelium is continuously renewed by a small proliferating intestinal stem cell (ISC) population residing at the bottom of the intestinal crypts in a specific microenvironment, the stem cell niche[114]. Niche surrounding cells including intestinal subepithelial myofibroblasts, endothelial cells and macrophages, generate Wnt, Notch, hedgehog and epidermal growth factor (EGF) signals that maintain ISC as a stem cell[115,116]. Mutations within these key signaling pathways can deregulate ISCs from the control of regulatory signals, allowing them to develop precursor lesions [117]. Once induced, intestinal regeneration through ISC symmetric division is strongly dependent on specific signals such as the recently evidenced IL-22[118]. In that intestinal homeostasis general regulatory process, EVs can also largely participate as intestinal fibroblast-derived EVs are involved in forming the ISC niche by transmitting Wnt and EGF activity[119] as well as intestinal macrophage-derived EV-packaged Wnt are essential for regenerative response of intestine against radiation [120]. EVs can also drive ISC differentiation as Rab8a vesicles regulate Wnt ligand

delivery then Paneth cell maturation at ISC niche[121]. Such EVs-driven mechanism has also been shown to impose quiescence on residual hematopoietic stem cells in the leukemic niche[122].

Microbiota as an important source of EVs: Intestinal tract is a specific place where communication between many different species (bacteria, fungi, parasites…) occurs continually. Not only human IEC but also commensal bacteria are known to release signaling vesicles[123]. Interestingly, many studies have shown that intestinal microbiota can be shaped either by food plant-derived EVs[124] or host-derived EVs [125] suggesting multidirectional influences on each other of all intestinal tract living species. Such interspecies communication has also been evidenced between resident helminths and host IEC[126,127]. Every bacteria, parasite, fungi… generate a huge reservoir of antigen that can induce host immune response. Thus, once initiated, this response can be tailored through complex cross reacting EVs modulation leading to either immune tolerance or inflammatory reaction.

Deregulation of EV release in colorectal diseases

Numerous studies have demonstrated that circulating EVs increased in patients with intestinal pathologies while EVs fractions are different in cancers, compared to patients with inflammatory intestinal diseases such as Crohn's or inflammatory bowel diseases (CD or IBD)[128].

EVs deregulation in intestinal inflammatory diseases: Chronic inflammation pathologies of gastrointestinal (GI) such as IBD, CD, *Helicobacter pylori*-associated inflammation and chronic pancreatitis have been identified as strong risk factors for cancer development[129]. Interaction of different genetic, microbiome, and environmental factors with the immune system drives IBD complex characters. The balance between immune suppression and stimulation against environmental factors is largely disturbed in IBD patients, resulting in inflammation and compromised integrity of the intestinal barrier. Elevated levels of EVs and/or EV content have been identified in IBD patients. EVs can modulate the immune response[130]. Among immune cells, macrophages are essential for the maintenance of intestinal homeostasis[131]. Serum EVs isolated from the dextran sulphate sodium-induced acute colitis mouse model could activate macrophages[132]. as well as EVs derived from the colonic luminal fluid of IBD patients that contained high mRNA and protein levels of several inflammatory cytokines could promote macrophage migration[133]. Dysfunction of regulatory T cells (Tregs) has been shown to be associated with a failure of intestinal tolerance, and contributes to the pathogenesis of IBD[134]. EVs derived from Tregs were shown to induce other T cells to develop into the Treg phenotype[135].

EVs release in colorectal cancer: Acidity and hypoxia are key features in cancer that could affect exosome release. Tumor pH may range from 6.0 to 6.8, and the level of acidity is directly associated to the tumor level of malignancy as it selects among cancer cells those that will resist[136]. One consequence of acidity-driven cancer cell selection pressure is an increased EVs release by human cancer cells[137,138].

Hypoxia is also a common characteristic of solid tumors and is associated with cancer progression and poor outcomes. It is generally associated with hypoxic environment that has also been shown to be an important cause of EVs release[139]. Hypoxic CRC cells can transfer Wnt4 mRNA to normal CRC cells by exosome, which can activate β-catenin signal and potentiate the invasive ability of normal CRC cells [140]. In hypoxic microenvironment, CRC cells-secrete miR-410-3p in EVs that promotes progression and metastatic potential of normoxic CRC cells *via* PTEN/ PI3K/Akt pathway[141].

EVs and cancer stem cells

Epithelial cancers may be driven by a relatively rare sub-population of self-renewing, multipotent cells, named cancer stem cells or cancer-initiating cells (CSCs). Increasing data show that CSCs play a crucial role not only in primary colorectal tumor formation but also in metastasis[142]. In addition, CSCs play a critical role in CRC relapse[143]. They display unique properties of self-renewal, infinite division and multi-directional differentiation potential[144]. Asymmetrical growth and slow-cycling cellular turnover renders them resistant to therapies that target rapidly replicating cells[145]. Not all CSCs in primary lesions are metastatic, allowing distinction between stationary cancer stem cells (SCSCs) and migrating cancer stem cells (MCSCs)[146]. SCSCs exist in colonic epithelial tissues and are active even in benign precursor lesions, contributing to tumor mass proliferation in situ[147]. On the contrary, MCSCs, which have

undergone EMT, possess motility characteristics and are able to spread in other tissue to form metastatic tumor mass[148,149].

Untreated colorectal tumors contain a population of quiescent/slow cycling cells resembling CSCs and overexpressing EMT markers such as Zeb2[150]. As for ISC, maintenance of these scarce CSCs generally resides in very specialized niches[151], allowing them to stay dormant for various to long periods of time[152,153]. These niches represent a positive specific microenvironment which is able to maintain stemness and pluripotency[154]. The release of EVs by mesenchymal stromal niche surrounding cells drive hematopoietic stem cell clonogenic potential maintenance and survival, by preventing apoptosis through EV gene expression regulation[155].

This continuous crosstalk between CSC and their surrounding microenvironment is critical as a tiny variation in its modulation could induce important deregulation and subsequent tumor progression[156]. For example, miR-196b-5p, which is highly enriched in CRC patients serum EVs[157] has been shown to promote either CRC cells stemness or chemoresistance to 5-fluorouracil (5-FU) *via* targeting negative regulators of the STAT3 signaling pathway. Understanding the importance of EVs transfer in that context is a key feature for future CRC therapy[158].

Bidirectional contribution of colorectal tumor and microenvironmental cells EVs to CRC changes

Tumor microenvironment (TME) is a complex and dynamic network including both cancer and stromal cells. Stress conditions such as hypoxia, starvation, and acidosis increase tumor cells EVs release leading to TME changes and expansion. Such specific behavior is the consequence of a complex combinatory of bioactive molecules present in EVs[159]. Not only different form of RNAs but also proteins or lipids could account for these important changes. The release of CD133+ EVs by poorly differentiated CRC cells was found to increase Src and ERK phosphorylation in surrounding cells, with subsequent MAPK intracellular signaling activation and promotion of tumor growth [113]. In response to CRC cells, TME modifications induce EVs-driven stromal cells response that subsequently results in tumor progression by further modifying CRC cells[160]. This continuous dual EVs-driven interplay between stromal and CRC cells is central in tumor behavior as it may drive either tumor cells proliferation or migration[161] (Figure 3).

Among TME, fibroblasts such as cancer associated fibroblasts (CAFs), endothelial cells and infiltrating immune cells are likely to be the major cell types that interacts with tumor cells through EVs signaling[162,163]. Both nature and composition of TME-derived EVs is of importance as cellular origin of the EVs cargo will determine specific changes within the recipient cell[164]. Analyzing their effect on CRC tumor cells, TME-originating EVs have been evidenced to play a central role in cell proliferation[165], acquisition of invasive properties and increased migration[166,167], resistance to chemotherapy[168], angiogenesis development[169], and escape from the immune system.

On the other side, several tumorigenic signals are derived from CRC cells and conveyed to stromal cells through EVs. From the very beginning of CRC progression, CRC cells secrete EVs that can deeply modify TME cells[170]. CAFs are prompted by CRC cells EVs to harbor a highly pro-proliferative and pro-angiogenic phenotype [171]. These important stromal changes are driven by CRC cells EVs composition that is itself largely modulated by different factors such as differentiation or hypoxia[113].

Promotion of cancer cell expansion

Accumulated genetic and epigenetic changes often activate the expression of oncogenes while silencing tumor suppressors during carcinogenesis. In CRC, several protooncogene mutations affecting *KRas*, *BRaf*, *PTEN*, *PIK3CA* or *TP53* are now well known to promote CRC cells proliferation through cell cycle key players deregulation [172]. Interestingly, mutant *KRas* expression in donor cell alter EVs cargo composition [173,174]. Such KRas mutation can be transferred through EVs cargo to non-transformed neighboring recipient cells leading to enhanced growth of these newly *KRas*-expressing cells[175]. However, aside these genetic transfers, most of the profound changes that drive cancer cell proliferation remains of epigenetic origin. Many different mechanisms can be used to alter gene expression, among which figure transfer of EVs cargo content that can increase cell proliferation by their oncosuppressive properties[176]. By suppressing fibroblast *TP53* expression, CRC cells EVs miRNAs promote tumor progression[177]. This holds also true for DeltaNp73 enriched EVs that promote oncogenic potential of recipient cells[178]. Such CRC cells EVs transfer can play a role in a synergistic manner with classical factors acting on CRC cell
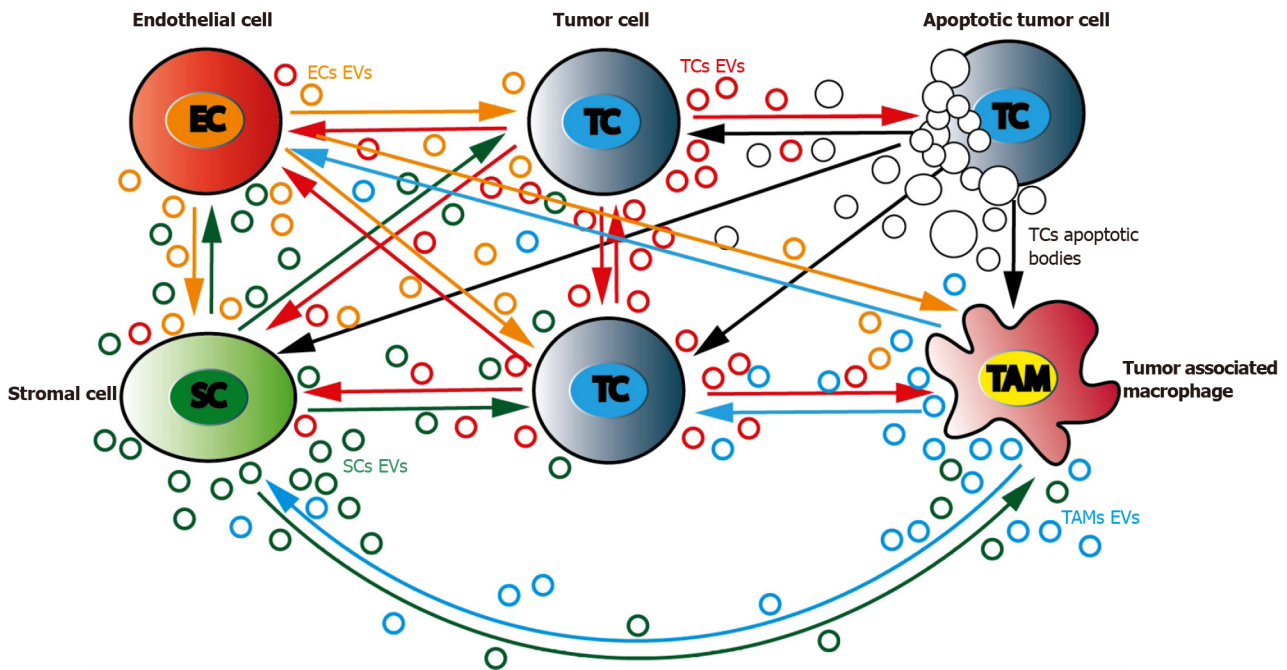
Figure 3 Bidirectional communications between tumor cells and their surrounding environment. Tumor microenvironment is a complex and dynamic network that include tumor (TC), stromal (SC), immune (tumor associated macrophages, TAM) and endothelial cells (EC). TC can bidirectionally signal to each other through extracellular vesicles (EVs) production. TC can produce EVs that will regulate SCs and TAMs differentiation and activity. SCs as well as TCs can regulate ECs activity, especially in hypoxic situations. TAMs and ECs can cooperate to promote angiogenesis. TC: Tumor cells; EC: Endothelial cells; SC: Stromal cells; TAMs: Tumor associated macrophages.

growth in a paracrine manner[179].

Cancer metabolism reprogrammation

All along the natural history of cancer, malignant cells should exhibit high metabolic plasticity to adapt themselves to tumor and surrounding environment continual changes[180]. Tumor cell proliferation continuously demand the highest nutrient capacity to fulfill enhanced biosynthetic and bioenergetics requests. In normal cells, metabolism of glucose is mainly performed through cytosolic glycolysis then mitochondrial TCA and OXPHOS that produce ATP. As mitochondrial PDH is inhibited and pyruvate cannot be transformed into acetyl-coa, cancer cells enhance glycolysis to produce sufficient ATP and generate high lactate content even in aerobic conditions (the "Warburg effect"), both being hallmarks of cancer[181]. High lactate production and release induces TME acidification promoting immune surveillance escape and metastasis[182]. As lipids, amino-acids, and nucleotides are strongly required for cancer cell multiplication, either fatty acids synthesis and FAO[183], or glutamine and serine metabolisms are all increased in tumor cells. Glutamine appears as a major energy substrate in cancer cells. Glutamine could produce TCA cycle intermediates to provide an additional energy source for cancer cells[184]. It has been recently shown that TME metabolism can largely modulate cancer cells progression. CAFs can provide metabolites that will facilitate tumor cells ATP production. Lactate, exported through CAFs MCT4 lactate shuttle then up-taken through cancer cells MCT1 Lactate transporter, could be used to fuel surrounding cancer cells, a process called "reverse Warburg effect"[185-187]. TME can also induce cancer cells FAO through cancer-associated adipocytes free fatty acid (FFA) release then cancer cells FFA CD36 uptake, hereby promoting cancer progression[188]. TME associated endothelial cells that mediated tumor angiogenesis are highly glycolytic[189] while tumor-associated macrophages (TAMs) polarization to immunostimulatory M1 or immunosuppressive M2 phenotype is largely driven by metabolism, M1 cells being highly glycolytic whereas M2 cells mostly relying on FAO and OXPHOS[190]. All these TME cells can shed EVs that will modulate cancer cells metabolism and play a role in their proliferation. EVs can contain metabolites but also metabolism enzymes that can modulate cancer cells metabolism. Uptake of EVs enriched in metabolic enzymes ALDOA and ALDH3A1 accelerated glycolysis thus promoting unirradiated lung cancer cells proliferation[191]. EVs lncRNA SNHG3 sponging miR-330-5p in recipient cells positively regulated pyruvate kinase M expression inhibiting OXPHOS,

increasing glycolysis, and promoting breast cancer cells proliferation[192]. As EVs can be produced bi-directionally (Figure 3), cancer cells can also modulate TME cells fate through metabolism reprogramming. Human melanoma-associated EVs miR-210 and miR-155 can reprogram CAFs metabolism to enhance glycolytic phenotype leading to extracellular acidification that favors pre-metastatic niche formation[193]. Prostate cancer cells EVs transfer of PKM2 protein to stromal cells leads to pre-metastatic niche formation[194]. Breast cancer cells EVs were found to contain miR-122 which could remodel metabolism to exacerbate metastasis[195]. VEGF-containing EVs can enhance EC glycolytic phenotype, inducing vascular permeability and cancer cells trans-endothelial migration[196] or promoting chemoresistance[197]. By increasing glycolysis and reprograming myeloid cells to an immunosuppressive phenotype, pancreatic ductal adenocarcinoma EVs could create an immunosuppressive background favoring tumor progression[198].

Metastastic spread potentiation and secondary settlement

EVs can be involved in directional cell movement through tissues[199]. Distant spread can arise in two steps. The first one concerns local tumor cell dissemination where epithelial cell migrate through TME at the front of the tumor through generation of membrane protusions (invadopodia) and basal lamina break-in[200]. The second involves vascular disruption to allow tumor cells hematogenous spread. Once in the circulation, tumor cells migrate and must found a premetastatic niche where they can settle then proliferate.

To initiate both process, CRC cells will recruit then educate stromal cells to induce CAFs, tumor-associated macrophages with the immune-suppressive M2 phenotype, and endothelial cells that promote tumor angiogenesis[147]. CXCR4, present in HT29 EVs may also contribute to stromal cells recruitment[201]. CRC cells can induce CAF generation by EVs transfer of TGF-β[202] promoting also two CAFs distinct phenotypes, *i.e.*, proliferative or invasive, by reprogramming their proteome[171]. Concerning macrophages, mutant p53 CRC cells are able to reprogram them into M2 phenotype through EVs miR-1246 transfer[203].

In both steps, loss of epithelial characteristics in favor of mesenchymal-like phenotype through epithelial to mesenchymal transition (EMT) process is involved [140,204]. During the local movement phase, stromal cells support EMT induction in tumor cells through stromal EVs. CAFs EVs can induce EMT in CRC cells by transfer of miR-92a-3p that promotes beta-catenin ubiquitination then degradation[205]. Similarly, EVs mediated transfer of miR-21 from CAFs to CRC cells increases their metastatic potential[166]. Aside CAFs, M2 macrophages can induce CRC cell migration through EVs cotransfer of miR21-5p and miR-155-5p[206]. M2 cells can also secrete Wnt-containing EVs to induce CRC stem cell activity that is involved in metastasis development[120]. This EMT transition is largely influenced by EVs matrixins transfer. Cotransfer of claudin 7 and MMP14 induces MMP2 and MMP9 recruitment that enhance invasiveness[207].

By EVs release, tumor cells can themselves induce up-or down-regulation of EMT-related genes in neighboring tumor cells, leading to distant invasion and/or migration [208]. EVs EMT inducers such as caveolin-1, HIF1α, beta-catenin, TNFa, TGF-β transfer can result in directional tumor cell migration[199,209] by either regulating ECM composition[210] or driving fibroblast differentiation into myofibroblast[211].

An important characteristic of tumor cells relies on their capacity to colonize preferentially specific organs (organotropic metastasis) that is often determined by anatomic aspects. Indeed, an important subset of CRCs will develop through distant metastasis, mostly to the liver. CRC capacenenity to colonize liver is primarily due to the hepatic portal system that drains the colon and by the facilitating defenestrated architecture of liver sinusoid endothelium[212]. Nevertheless, a crosstalk between CRC circulating cells and hepatocytes through bidirectional EVs transfer is also mandatory. It is now well accepted that primary tumor educates metastatic microenvironment, commonly defined as the "premetastatic niche," allowing circulating tumor cells (CTC) to find a suitable environment in which they can settle then proliferate. Such niche generation is characterized by local tissue inflammation, immune suppression, stromal cell activation, and ECM remodeling[213]. EVs proteins or miRNAs have been shown to be involved in establishing this niche[167]. EVs can modify ECM to support circulating CRC cells adhesion by increasing fibronectin deposits within the liver[214]. Such ECM modifications increase CRC cell adhesion, promoting mesenchymal-to-epithelial transition (MET), and enabling liver metastasis colonization. EVs miR-25-3p promotes pre-metastatic niche formation by inducing vascular permeability and angiogenesis [169] while EVs miR-21 through toll like receptor (TLR) 7/IL-6 axis in macrophages pathway as well as EVs miR-203 seem to induce an inflammatory niche that can

potentiate liver metastasis[215,216]. EVs derived from CRC cell lines are involved in the modulation of the innate immune response, which is considered as a central step in the formation of the metastatic niche. Circulating EVs miRNAs after internalization by target cells can also act as ligands of TLRs[217].

Like in primary tumors, cancer cell EVs can reprogram resident cells to promote metastatic niche achievement and attract newly released CTCs. For example, in the niche, gastric cancer cells drive epidermal growth factor receptor (EGFR) EVs transfer to liver stromal cells that upregulate HGF expression through miR-26a/b downregulation inducing CTC attraction and further metastatic proliferation[218].

Angiogenesis induction

Angiogenesis is important for tumor proliferation and distant metastasis. Endothelial cells (ECs) can uptake *via* the endocytic pathway EVs from various origins[219]. Uptake of tumor-derived exosomes by normal endothelial cells activates angiogenic signaling pathways in endothelial cells and stimulates new vessel formation[67,68, 220]. Once internalized, EVs are immediately directed to the perinuclear zone and actin filaments enriched area. When tubules are formed, EVs move to cell periphery and enter advanced pseudopods[221]. After complete remodeling, adjacent ECs probably transport EVs to neighboring ECs and to other cells in the TME[222].

In hypoxic conditions, tumor cells can secrete angiogenic factors, such as VEGF-A, inducing ECs migration and tumor angiogenesis. Higher levels of circulating proangiogenic basic bFGF originating from CRC cells have been detected in the serum of CRC patients[223]. EVs are also released by hypoxic CRC cells. Wnt4 enriched EVs increased β-catenin nuclear translocation in ECs enhancing angiogenesis and tumor growth[224]. It holds the same for Wnt5a[225] and Wnt5b whose increased expression in CRC cells correlates with aggressiveness. Caco-2 cells, one of the mostly used human CRC cell lines, secrete Wnt5b containing EVs that stimulates cell migration and proliferation of A549 cells[210]. Mutations in adenomatous polyposis coli (APC) gene are common in CRC patients and are associated with the deregulation in Wnt signaling. Restoration of APC expression in CRC SW480 cells induces DKK4 release through EVs, a mechanism restoring Wnt signaling pathway that may be lost during CRC progression[226]. In CRC ascites, EVs released by CRC tumor cells have been shown to carry proangiogenic proteins like Plexin B2 and tetraspanin[227]. Interestingly, CRC cell lines (HCT116 and DLD-1) secrete EVS that carry high levels of tissue factor, which is involved in blood coagulation, but is also a known modulator of angiogenesis and metastasis processes[228]. Aside proteins, EVs miRs have also been involved in angiogenesis induction[229], miR-183-5p was first found to be highly expressed in CRC cell-derived EVs, which triggers a marked increase in the proliferation, migration and tube formation abilities of HMEC-1 cells by targeting FOXO1 [230]. CRC-derived miR-1229 containing EVs, by inhibiting HPIK2 expression, promote through VEGF pathway activation HUVECs tubulogenesis, transfection with exomiR-1229 inhibitor anta-miR-1229 significantly suppressing tube formation[231]. EVs from 5-FU-resistant CRC cells promoted angiogenesis through dipeptidyl peptidase IV, a potent inducer of this angiogenesis[232].

TAMs were also proven to be beneficial for angiogenesis. M2 macrophages were positively correlated with microvessel density of pancreatic ductal adenocarcinoma tissues. M2 macrophage-derived EVs could promote mouse aortic ECs angiogenesis *in vitro* and subcutaneous tumors growth *in vivo*, increasing vascular density in mice [233].

Immune escaping modulation

While tumor cell dissemination seems to be an early event of tumorigenesis, metastasis development ability is strongly associated with immune evasion. It seems that in CRC, the immune system influences tumor heterogeneity and sculpts clonal evolution. Tumor clones development is linked to the intra-metastatic immune microenvironment *via* an immune editing process[234].

CRC EVs induce recruitment to the pre-metastatic niche of suppressive immune cells, such as TAMs, tumor-associated neutrophils, Tregs leading to a strong inhibition of the antitumor response and facilitating CRC growth[235]. Specifically, it has been shown that TAMs can stimulate CRC growth by altering ECM remodeling, TME composition, tumor metabolism and angiogenesis[187]. CRC-derived EVs are involved these processes. CRC cells TGF-β EVs transfer to T cells can induce cell reprogramming toward Treg phenotype[236]. Similarly, delivery of miR-214-containing tumor cells EVs to mouse peripheral CD4+ T cells downregulates *PTEN* and promotes Treg expansion[237]. CRC CT26 cells EVs promote the proliferation of lymphatic endothelial cells and the formation of lymphatic network in sentinel lymph node

(SLN), facilitating CRC cells metastasis to SLN[238]. Cancer cell EVs miRNAs can also block the adaptive immune response by affecting natural killer (NK) cells, or by decreasing dendritic cell maturation[239]. Similarly, CRC cell EVs that contain Fas-ligand and Trail can target T cells to induce their apoptosis[240] (Figure 4). While it is well admitted that EVs from metastatic tumor cells display protumorigenic functions, it seems that, in poorly metastatic cancer, tumor cells EVs induce expansion of patrolling monocytes in bone marrow, promoting metastasis eradication *via* NK cells and macrophages recruitment[241]. Such discrepancies highlight the fact that cancer cell EVs may play heterogeneous functions in tumor immunity that remain to be elucidated.

Resistance to therapy

Despite improvement and diversification of therapeutics for CRC patients (surgery, targeted therapy, radiotherapy and chemotherapy) and the emergence of new drugs during the last years, resistance to treatment still exists and remains one of the deadlocks for patients with an advanced CRC for whom medicines no longer work [242]. Today, administration of FOLFOX, a combination of folinic acid, 5- FU and oxaliplatin (OXA), is one of the most widely used chemotherapeutic regimens for treating CRC but these treatments generate serious systemic side effects and have an impact on the patients quality of life. More recently, the use of targeted drugs (for example bevacizumab, cetuximab, regorafenib ...) allow improvement of metastatic CRC survival times but malignant tumors drug resistance still persist[243].

Resistance to conventional chemotherapy: Aside classical mechanisms of resistance to 5-FU and OXA such as impaired drug inflow or efflux, drug inactivation, or single nucleotide polymorphisms of fluoropyrimidine or platinum targets, EVs generated by CRC cells have been reported to play a critical role in resistance to treatments[244]. Cancer stemness acquisition could be a possible feature that induces chemoresistance in CRC[245]. Wnt activity may reflect stem cell features. EVs-mediated Wnt secretion by CAFs is able to induce CRC reprogramming into CSCs then potentiate CRC resistance to chemotherapy[246]. In addition, CAFs release of H19 EVs also potentiated cancer stem cell resistance to OXA. LncRNA H19 was highly expressed in CAFs and upregulated in EVs. H19 activated the Wnt/β-catenin signaling pathway and potentiated drug resistance of CSCs[247]. The role of CAFs in exporting EVs that will confer chemoresistance to CRC cells is significant as it was reported that CAFs Evs can activate CRC cells ERK/AKT pathway inducing a protective effect to OXA[162]. CAFs can export urothelial carcinoma-associated 1 (UCA1), a lncRNA with three exons that has been found to display oncogenic functions in various types of cancer [248]. In CRC, UCA1 was found to be associated with resistance to cetuximab and 5-FU[249,250]. UCA1 suppresses miRNA-204-5p expression[251] that induces drug resistance. miR-196b-5p promotes CRC cells chemoresistance to 5-FU by targeting SOCS1 and SOCS3 negative regulators of STAT3 signaling pathway, resulting in global activation of STAT3 signaling[157]. Interestingly, UCA1 and miR-196b-5p are highly expressed in CRC patients EVs as compared to healthy control subjects and may represent interesting CRC biomarkers (Figure 5).

Resistance to targeted therapies: Cetuximab or panitumumab, that target the extracellular domain of EGFR preventing downstream activation of the MAPK or mTOR pathways, increases survival times in CRC patients[252]. Nevertheless, a subset of mutations involving either *BRAF* or *PIK3* and amplifications of MET or HER2 induce resistance to these monoclonal antibodies (Mab) therapy[253]. Cetuximab CRC-resistant EVs have been shown to restrict the PI3K negative regulator PTEN in CRC cells[254] through UCA1 overexpression[250]. Aside EVs nucleic acids or proteins inhibition of EGFR-driven cellular process in the recipient cell, EGFR positive EVs could bind anti-EGFR mAbs reducing mAb bioavailability. Such mechanism has been described for anti VEGFA mAb bevacizumab in metastatic and lung cancers. VEGFA positive EVs neutralize bevacizumab inducing cancer cell chemotherapeutic escape [255].

EVs as pertinent biological markers of CRC

Being able to quantify and use EVs as relevant biological markers may improve CRC screening in the future. Nowadays, CRC is currently detected by different methods. Colonoscopy is widely used in clinical practice, which is regarded as the gold standard for detecting CRC. However, it has several limitations such as invasive nature, high cost and bothering bowel preparation[256]. Aside this invasive procedure, non-invasive screening tests such as iterative fecal occult blood testing (FOBT)[257] or
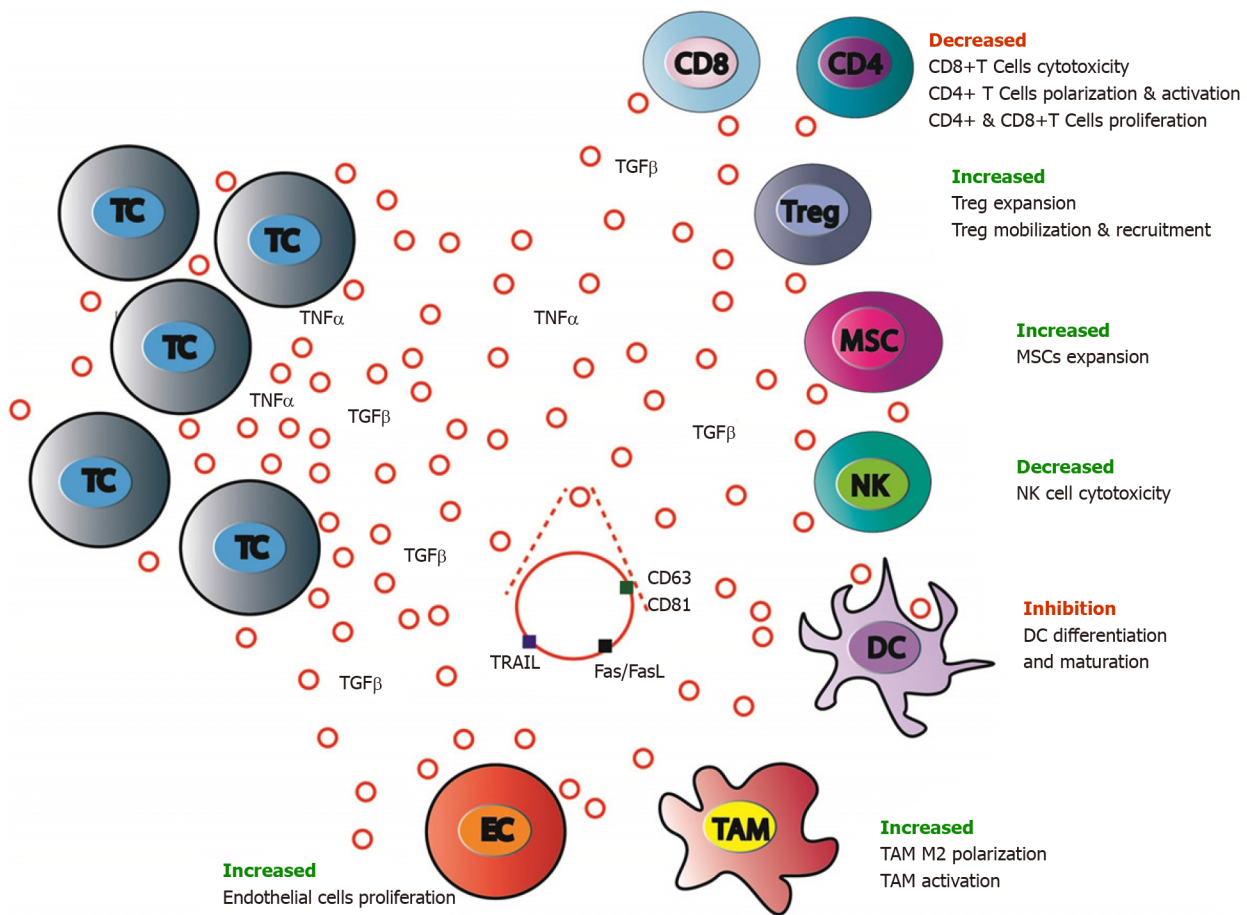
Figure 4 Antitumor immune system balance modulation by colorectal cancer cells extracellular vesicles. Antitumor immune response is largely modulated by colorectal cancer (CRC) cells through either extracellular signaling molecules (cytokines, *etc.*) secretion or extracellular vesicles (EVs) production and release. CRC cells EVs contain inhibiting or activating molecules that favor target cells expansion, mobilization, and recruitment (regulatory T cells and mesenchymal stem cells), polarization and activation (tumor associated macrophages M2) and block others (CD8+ T-cells, dendritic cells, and natural killer cells). MSC: Mesenchymal stromal cells; CD4: CD4 positive T cells; CD8: CD8 positive T cells; EC: Endothelial cells; TC: Tumor cells; TAM: Tumor associated macrophages; NK: Natural killer cells; Treg: Regulatory T cells.

plasma carcinoembryonic antigen (CEA) quantification have also been used. Unfortunately, both are of limited value mainly because poor sensitivity and specificity[258, 259] urging the need to find new methods aimed to quickly, easily and robustly diagnose and monitor CRC. This is where EVs can certainly play an important role.

EVs can be detected in many biological fluids of patients, such as blood, urine, CSF and saliva[13] and can now be easily isolated[260] even though a universal standardized and widely accepted method for isolating then analyzing EVs is still mandatory[244]. Thanks to their lipid bilayers, EVs are stable in circulation and protected from degradation of serum ribonucleases and DNases[261]. As several miRNAs, lncRNAs and proteins are differently expressed in EVs originating from tumor and normal cells, they are potential sources of biomarkers and become a promising field in CRC diagnosis (Figure 6).

EVs miRNAs as relevant CRC biological markers: EVs miRs have been regularly involved in CRC development holding promise that their quantification in plasma or serum could serve as relevant CRC biomarkers. Some of them, that have been associated to specific events in CRC natural history, have been found in blood of CRC patients[262]. Among them, miR-25-3p[169] and miR-21[216], both promoting pre-metastatic niche formation by respectively inducing vascular permeability and macrophages differentiation towards a pro-inflammatory phenotype, and miR-203 that induces TAM activation[215], have been reported to be highly expressed in plasma CRC patients EVs and related to a poor prognosis. Recently, miR-410-3p was found highly enriched in hypoxic CRC-derived EVs in a HIF1α or HIF2α-dependent manner. miR-410-3p decreases PTEN in recipient cancer cells thus activating PI3/Akt axis and leading to tumor progression. miR-410-3p levels were positively associated with poor prognosis of CRC[141]. Nevertheless, while several specific miRNAs panels
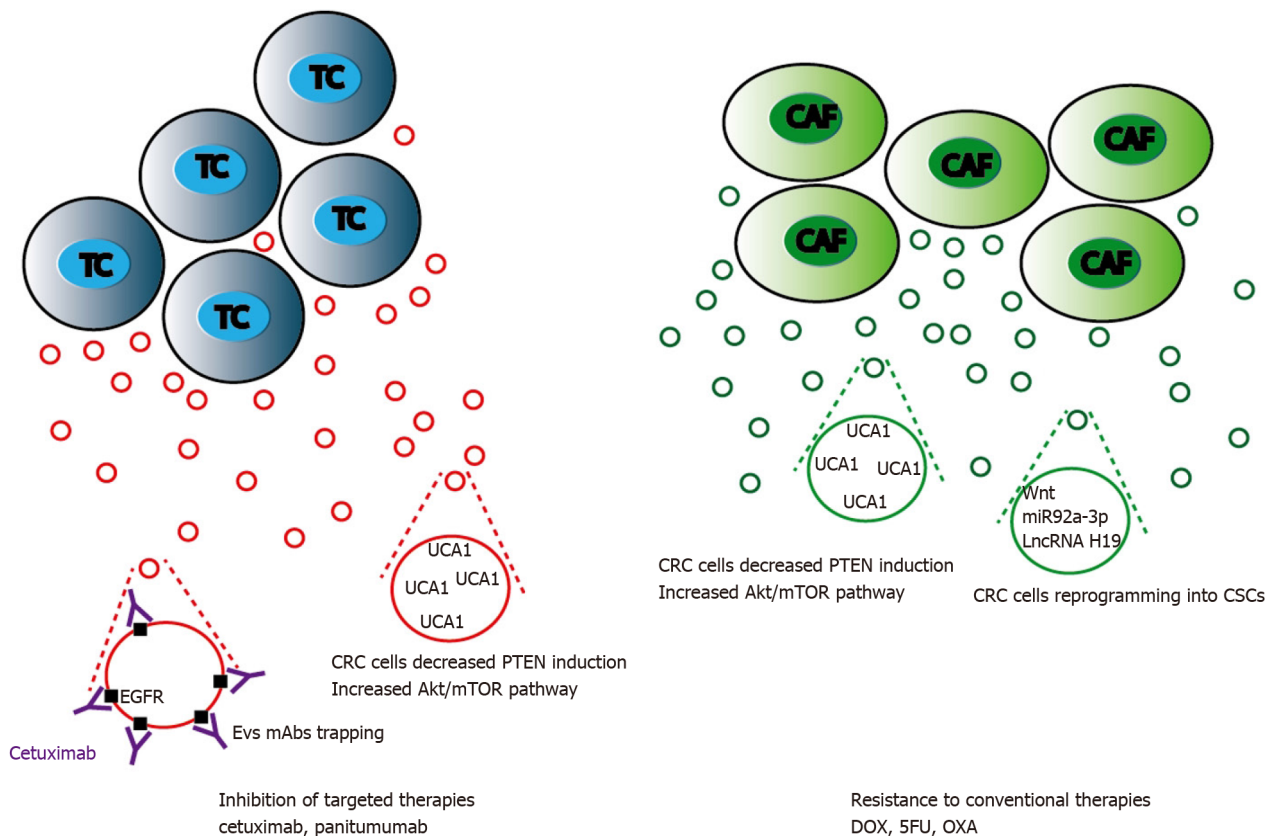
Figure 5 Mechanisms of extracellular vesicles-mediated chemoresistance in colorectal cancer treatment. Extracellular vesicles (EVs) released either by colorectal cancer (CRC) or cancer activated fibroblasts cells can cooperate to promote cytotoxic drugs or targeted therapies resistance. These processes are mainly mediated by lncRNAs such as urothelial carcinoma-associated 1 that stimulate mTOR and STAT3 signaling, and by Wnt proteins or miRNAs targeting Wnt signaling pathway leading to CRC cell acquisition of stemness features. EVs can also trap targeted anti-epidermal growth factor receptor antibodies reducing their bioavailability and further action on CRC cells. TC: Tumor cells; CAF: Cancer activated fibroblasts; DOX: Doxycycline; 5-FU: 5-fluorouracil; OXA: Oxaliplatin.

have been found in EVs from CRC patients, only a few have yet been clinically validated[263]. A panel of 7 miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) was first validated by qRT-PCR, indicating that it may be a suitable biomarker to detect CRC[264]. Among this, miR-23a, miR-1246 and miR-21 are highly interesting as all three display high specificity and sensibility[262]. If both miR-23a and miR-1246 are positive and both CA19-9 and CEA negative, one can say that it is probably an early stage CRC[265]. In addition, miR-125a-3p and miR-320c were found to be significantly increased in EVs of early-stage CRC patients, combination of miR-125a-3P and CEA improving drastically the screening power for early-stage CRCs [266]. Another interesting work showed that miR-6803-5p was significantly increased in serum samples from CRC patients and correlated to a poor prognosis as compared to healthy subjects[267]. While associated increased levels of both miR-17-5p and miR-92a-3p levels may serve as an early indicator of liver metastases[268], EVs overex-pression of miR-486-5p, miR-19a, miR-17-92a correlate with CRC recurrence[269,270]. Last, increased expression of EVs miRs that can be released by CAFs can be also an early indicator of chemotherapy resistance. High expression of miR-92a-3p activates Wnt/β-catenin pathway and inhibits mitochondrial apoptosis by directly inhibiting FBXW7 and MOAP1, contributing to cell stemness, EMT, metastasis and 5-FU resistance in CRC[205].

On the opposite, aside plasma EVs miRs increased levels, down-regulation of some miRNAs could be predictive factors of CRC. Five EVs miRNAs (miR-638, miR-5787, miR-8075, miR-6869-5p and miR-548c-5p) were decreased among CRC patients. These miRNAs may be involved in the development and progression of CRC by regulating glucose metabolism. Besides, in this study, 2 miRNAs (miR-486-5p and miR-3180-5p) have been shown to be significantly increased[271], results that were further confirmed[269]. Low levels of tumor suppressor miR-6869-5p that targets TLR4/NF-κB signaling pathway inhibiting proliferation and promoting CRC cells apoptosis have been reported in CRC patients serum EVs[272]. More recently, decreased expression of miR-1505p[273] and miR-548c-5p[274] were both associated to CRC poor prognosis.
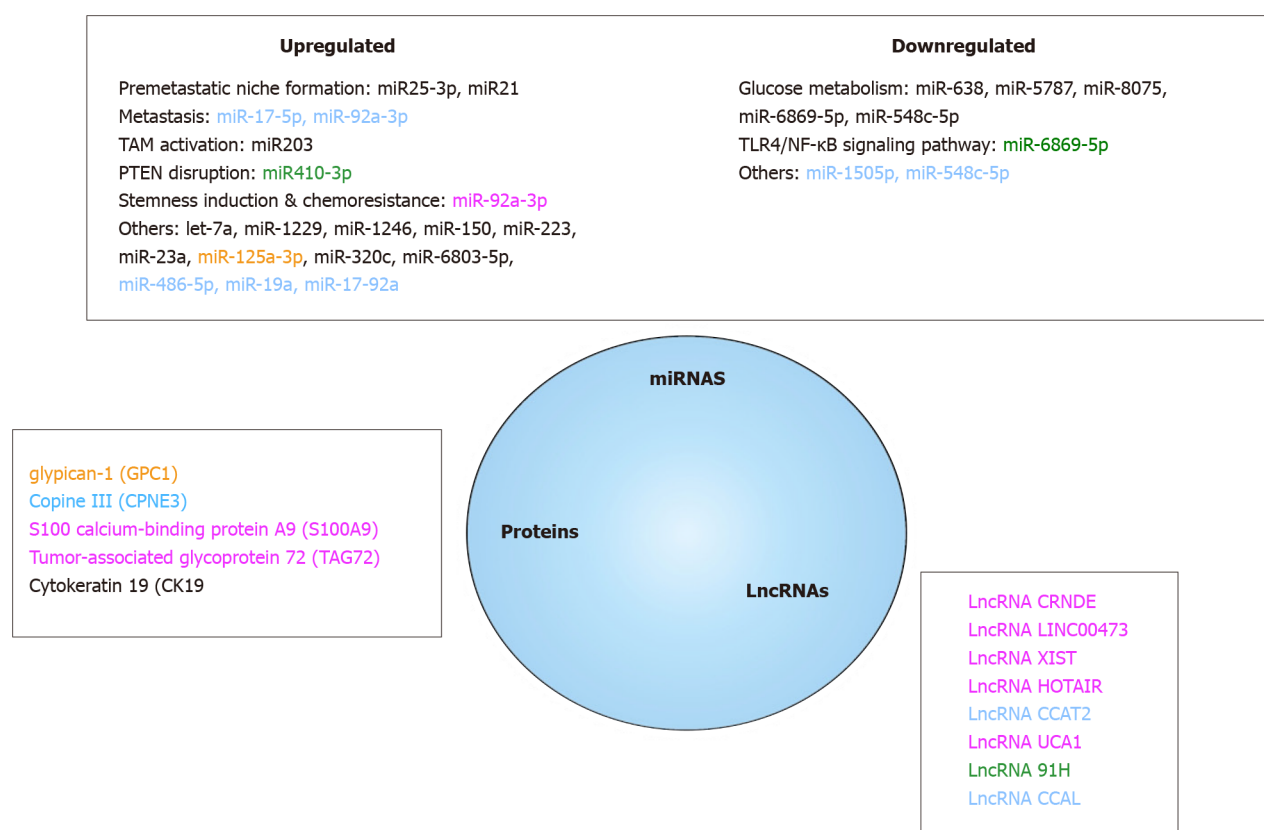
Figure 6 Colorectal cancer cells extracellular vesicles molecules as relevant cancer biomarkers. Among all the molecules present in extracellular vesicles, only a subset (proteins, miRNAs, lncRNAs) have been shown to be of potential clinical value on colorectal cancer detection, diagnosis, prognosis and treatment response evaluation. All referenced markers were found to be differentially expressed in cancer patients and in healthy people. The yellow ones were useful for diagnosis, the green ones for progression, the blue ones for prognosis and the pink ones were associated with chemoresistance. TAM: Tumor associated macrophages.

LncRNAs as interesting CRC markers: LncRNAs, non-coding RNAs greater than 200 nucleotides, were once considered as junk DNA and transcriptional noise but emerging evidences demonstrate that they are evolutionarily conserved and that their strongly regulated expression plays critical roles in regulating gene expression[275]. As they can be differentially expressed in blood EVs of CRC patients, they could be new interesting biomarkers[276]. LncRNAs have been involved in CRC initiation and progression. Colorectal cancer-associated lncRNA (CCAL) seems to be a key regulator of CRC progression[277] and it was reported that CCAL promotes OXA resistance of CRC cells[278]. It has been also demonstrated that both down-regulation of lncRNA UCA1 and up-regulation of circRNA homeodomain interacting protein kinase 3 is found in CRC patients EVs. UCA1 LncRNAs, upregulated in CRC biopsies and downregulated in serum EVs, serves as a miR143 sponge that modulate MYO6 expression[279]. Six lncRNAs (LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003, and LNCV6_98602) are significantly up-regulated in patients with CRC as compared to healthy individuals[280]. High serum EVs expression of lncRNA 91H have been associated to CRC poor prognosis[281] and an increase of growth arrest-specific 5 and colon cancer-associated transcript 2 (CCAT2) lncRNAs in CRC patients have also been reported[282]. Interestingly, CCAT2 lncRNA levels were significantly decreased after surgery and removal of the tumor[283]. Finally, several lncRNAs have been associated to treatment resistance[284]. HOTAIR [285], XIST[286] and LINC00473[287] lncRNAs have been found to confer 5-FU resistance through respective miR-218 and miR-203a-3p, miR15a and miR-152 regulations[288,289]. LncRNA CRNDE induces CRC OXA resistance *via* miR-181a-5p-mediated regulation of Wnt/beta-catenin signaling and miR 136 sponging[290,291].

EVs proteins as a source of cancer biomarkers: Finally, aside nucleic acids, EVs proteins could also be measured to diagnose CRC as they may differ between healthy and CRC individuals. A primary study has shown that 36 proteins were upregulated and 22 proteins downregulated in CRC patients EVs compared to normal volunteers

EVs. Moreover, upregulation of these proteins was associated with a pretumorigenic microenvironment for metastasis and on the opposite, downregulation was associated with tumor growth and cell survival[292]. Several studies have identified a number of proteins that can be considered as potential biomarkers. For example, among them, glypican-1[293,294] was suggested to be a specific diagnosis marker because it is highly expressed in CRC patient EVs and normalized after surgery. Identically, EVs lower expression of Copine III, a protein highly expressed in CRC tumors, was associated to better survival[295]. Additionally, S100 calcium-binding protein A9 (S100A9) levels were noticeably higher in plasma EVs of CRC relapse patients than those in tumor resection patients[296]. S100A9 has been related to CRC worsening as its overexpression could enhance TME CRC cells stemness. High levels of cytokeratin 19, CA125, and tumor-associated glycoprotein 72 (TAG72) have been quantified in CRC patients plasma EVs[297]. Interestingly, TAG72 protein overexpression was found to contribute to CRC patients chemoresistance to 5-FU.

The emergence of quantitative measurements that will be simple, inexpensive, easily performed and non-invasive for the patient is strongly mandatory. Analysis of EVs content (miRNAs, lncRNAs and proteins) may allow early diagnosing CRC and even predicting its relapse, metastasis and potential chemotherapy resistance.

EVs as potential targets to inhibit cancer

EVs have been shown to be a source of patient's resistance to chemotherapy. It is mandatory to explore new therapeutic possibilities aimed to both suppress tumor progression and reduce EVs-related drug resistance.

EVs uptake and biogenesis inhibitions: The first possibility to treat cancer would be to target EVs by inhibiting EVs uptake[298]. Indeed, EVs endocytosis is an active process but a rather complex one leading its inhibition a new therapeutic perspective but a very difficult one to achieve. Many studies have found molecules that could inhibit EVs internalization. Heparin can inhibit in a dose-dependent manner EVs absorption through direct action on heparan sulfate proteoglycans which themselves play a role EVs endocytosis[299]. Cytochalasin D that inhibits phagocytosis and other endocytosis mechanisms through an inhibitory effect of actin polymerization has been shown to inhibit EVs uptake[300]. Inhibition of EVs internalization by Methyl-β-cyclodextrin (MβCD) in glioblastoma cells has been reported[301]. MβCD depletes cholesterol from natural membranes and decreases EVs uptake by interfering with lipid rafts stability. Another molecule, dynamin, already described as an inhibitor of endocytosis, has been shown to interfere with EVs uptake in cancer[302]. Nevertheless, the large repertoire of mechanisms involved in EVs uptake in cancer impairs the overall efficiency of these molecules. A recent study showed that antibodies targeting CD9 and CD63 tetraspanins stimulate EVs macrophages phagocytose inhibiting cancer EVs-mediated communication[303]. However, such antibodies do not only target cancer EVs but also "physiological" CD9 and CD63 EVs. The role of these specific EVs being not yet known, additional studies must be carried out to know the viability of such method.

One other possibility of EVs targeting would be to inhibit EVs biogenesis. Inhibiting EVs biogenesis also involves complex issues, primarily due to the large number of proteins that are concerned in this cellular process. However many pharmacological agents have been found and seem promising. Fluidity of cell plasma membrane is fundamental during membrane lipid bilayer re-organization and thus EVs formation. During EVs biogenesis, ceramide regulate EVs production[24]. Ceramide synthesis required an ubiquitous enzyme, neutral sphingomyelinase 2 (nSMase2) that can be specifically targeted by GW4869 inhibiting cancer cells EVs release in a dose-dependent manner[304] and consequently limiting miRNAs hematogenous release [305]. On the opposite, nSMase2 overexpression increases miRNAs quantity in blood [306]. The link between nSmase2 and EVs has been shown in breast cancer aggressiveness[307]. GW4869 therapeutic effects have been observed on murine melanoma. GW4869-induced B16BL6-derived EVs secretion inhibition decreased B16BL6 cells proliferation and increased apoptosis-related proteins. Treatment of GW4869-treated cells with B16BL6-derived EVs restore their proliferation[308]. As GW4869 seems to be promising, imipramine which is a tricyclic anti-depressant is also a source of interest because of its inhibitory activity on acid sphingomyelinase (aSMase) that catalyzes sphingomyelin hydrolysis to ceramide[309]. Thus, imipramine is reported to prevent the translocation of aSMase, inhibiting EVs secretion. So, both GW4869 and imipramine can stop the production of ceramide

TSG101 is a protein involved on endosomes trafficking and exosomes biogenesis [310]. In CRC cells that express Wnt5b, knockdown of TSG-101 generates Wnt5b EVs downregulation decreasing Wnt5b-driven cell proliferation suggesting TSG101 as a potential therapeutic target in cancer[311].

EVs release inhibition: A third possibility to target EVs is to limit or inhibit their release by secreting cells.

A drug that inhibits EVs release is manumycin A, an antibiotic which is a selective and strong inhibitor of Ras farnesyltransferases. Farnesyltransferase inhibitors inhibit Ras activity and therefore EVs release[312]. Aside Ras proteins figure Rab proteins that are also modulators of EVs biogenesis[7]. Rab2b, Rab5a, Rab9a, Rab27a and Rab27b impacts in EVs release have been studied, the two latter playing also a role in EVs docking and exocytosis[29]. Knockdown of Rab27a decreased EVs-release amount[313] and Rab27a inhibition reduced tumor growth and lowered metastatic cells dissem-ination[314,315]. Gold nanoparticles conjugated with anti-sense RAB27a oligonuc-leotides to mute Rab27a generate 80% inhibition of EVs release in breast cancer[316]. Plectin enables EVs secretion in pancreatic cancer. Downregulation of plectin in pancreatic cancer cells reduced EVs release in the same way Rab27a and Rab27b knockdowns do suggesting that combining both mechanisms could be a therapeutic combination that enables greater results[317].

As plasma membrane fluidity is important for EVs shedding, drugs aimed at targeting either lipid rafts formation or cholesterol synthesis will interfere with EVs release. Lipid depletion results in EVs release reduction[318]. Pantethine, a pantothenic acid (vitamin B5) derivative is used as an intermediate in the production of co-enzyme A and it plays a role in the metabolism of lipids and reduction of total cholesterol levels. Panthetine inhibits by 80% cholesterol synthesis as well as fatty acid synthesis[319]. Panthetine has been shown to limit EVs release in systemic sclerosis [320]. Its use on chemoresistant breast cancer cells significantly reduced EVs release [321].

Actin and actin-regulating proteins are also strongly involved in EVs secretion. Invadopodia are cellular structures used by cancer cells to degrade extracellular matrix and invade. Because of high levels of actin, such structures are key sites for EVs release. Indeed, invadopodia inhibition limits EVs release[322]. Furthermore, knockdown of cortactin, that acts as an actin dynamics regulatory protein, decreased whereas its overexpression led to an increase of EVs release[323].

Rho-associated protein kinases (ROCK) are a family of serine-threonine kinases belonging to the PKA-G-C family and involved in cells shape and movement regulation, by acting on the cytoskeleton. Cytoskeleton organization as well as cellular contractility through activity on actin filaments is important features for EVs shedding. Y27632 is a commonly used ROCK competitive inhibitor which is able to compete with ATP at ROCK catalytic sites[324]. Y27632 causes a reduction in the release of EVs as well as a change in cell surface morphology[325] by sustaining activation of proteolytic enzymes, such as stathmin and calpain, that destabilized cell plasma membrane. Thus, Y27632 can be used alone or in combination with Calpeptin, the most studied calpain inhibitor[326]. Calpains, once activated through calcium binding, can activate different cellular processes including cell migration, cell invasion and EVs formation and release. Calpeptin has also been used alone to inhibit EVs release[327].

PEG-SMRwt-Clu, a drug derived from the secretion region of HIV-1 Nef protein, regulates exosomal pathway trafficking and seems promising. PEG-SMRwt-Clu was able to inhibit cell growth in breast cancer cell lines and more interesting to partially increase chemosensitivity. The use of PEG-SMRwt-Clu was also associated with a decrease in the number of released EVs[328].

Despite the current efforts and the number of EVs endocytosis, biogenesis and release inhibitors that are already available, inhibition of EVs is still a very complex issue because of the multifactorial nature of the different pathways involved in these processes. Nevertheless, EVs uptake, biogenesis or release inhibition remains a potential and interesting therapeutic cancer target in the near future.

EVs as therapeutic vectors in CRC

EVs are major players in tumor progression *via* the transfer of cargo within them. One other possible way to cure CRC would be an EVs-based therapy that uses EVs as therapeutic vectors.

In very recent years, studies have mainly focused on the idea that EVs could be natural delivery vehicles to transport therapeutic drugs, antibodies or RNA to modify gene expression[329]. In the cancer field, it would be indeed a specific and effective therapy delivery method to specifically treat cancer cells. EVs are biocompatible and

biodegradable and therefore, less toxic and immunogenic than other nanoparticular drug delivery systems such as liposomes or polymeric nanoparticles[330]. EVs have innate limited immunogenicity and cytotoxicity[331,332]. Moreover, drug stability is largely enhanced as EVs avoid drugs degradation by extracellular enzymes[333]. Thus EVs capacity to target tumor cells is 10 times higher than liposomes of a similar size. Such property is certainly linked to particular ligand-receptor interactions and to efficient endocytosis mechanisms linked to the EVs membrane lipid composition that contributes significantly to cellular adherence and internalization[334]. Last, EVs can penetrate through anatomical barriers[335,336] and their lipid composition protects them from reticuloendothelial system phagocytosis[244].

Several reports have demonstrated the potential of using EVs therapy and clinical trials are currently underway to find treatments that extend patient survival. Many kinds of EVs-based therapies have been shown to improve chemotherapy effectiveness. EVs have been used to deliver many kinds of drugs such as curcumin[337], paclitaxel[338] and doxorubicin[339]. While loading doxorubicin in EVs reduces cardiotoxicity[340], its packaging into EVs increases its efficacy when compared to free doxorubicin in cancer-bearing mice treatment. Inside EVs, doxorubicin has a better stability and will be even more collected within the tumor, significantly suppressing mice CRC growth and extending survival time[341]. EVs loaded with paclitaxel were tested in the treatment of multiple drug resistance cancers. Loaded exosomes can overcome drug efflux transporter adverse effect, decreasing metastasis growth when compared to controls[342].

EVs are also natural carriers of nucleic acids molecules and can be genetically engineered to deliver specific nucleic acid molecules such as miRNA[343], and more recently gene editing system CRISPR/Cas9[344]. EVs-based nucleic acid delivery in cancer treatment have shown promising therapeutic effects[38]. EGFR expressing cells can be targeted with GE11-positive exosomes loaded with microRNA let-7a, a tumor suppressor microRNA. The results showed an efficient delivery of exosomes cargo and consequent tumor growth inhibition[345].

EVs can also be used as a new type of tumor vaccine. Phase I clinical trials have shown that ascites EVs combination with granulocyte-macrophage colony stimulating factor induces a safe and effective response from specific anti-tumor cytotoxic T-cell in the treatment of advanced CRC[346]. EVs have also been explored as modulators of the immune response against tumor cells. Dendritic cells are antigen-presenting cells inducing immune responses. Dendritic cells have been shown to secrete antigen-presenting EVs that coexpress molecules of the major histocompatibility complex. Such exosomes activate specific cytotoxic T lymphocytes *in vivo* that can reduce or even suppress tumor growth[347]. EVs loading of anti-tumor peptides has also been used. A specific mutated form of survivin-T34A induces caspase activation leading to apoptosis. *In vitro* treatment of cancer cell lines with survivin-T34A EVs increased cell death[348].

Different cell-derived EVs may be home to specific cell types[7]. EVs derived from hypoxic tumor cells tend to be taken up by hypoxic tumor cells[349]. Different cells under different conditions determine EVs heterogeneity, generating huge and complex combinatorial possibilities. Thus, to better use EVs in cancer, engineering EVs with ligands that can specifically bind to targeted cancer cells is mandatory. Either EVs surface expression of receptor/ligand, antibody/ligand or microenvironment specific molecules can be used to specifically modify EVs. Recently, bioengineered EVs have been shown to be able to specifically bind to HER2/Neu by expressing designed ankyrin repeat proteins on their membrane surface[350]. Engineering both CD3 and EGFR expression on EVs membranes allows cross-linking of T cells with EGFR positive cancer cells enhancing antitumor immunity[351]. As hyaluronan has been evidenced in EVs[352], hyaluronidase engineered EVs have been shown to degrade tumor extracellular matrix and enhance the permeability of T cells and drugs within the tumor[353].

Using EVs as therapeutic vectors in cancer seems very promising and clinical trials are nowadays being carried out[354]. Unfortunately, no major breakthrough still occurs certainly because of the complexity to handle such new therapeutic methods *in vivo*. To accelerate their use in cancer patient treatment, there is also an urgent need to better understand both EVs biology and nature[298].

CONCLUSION

EVs exert a wide variety of biological functions, mainly *via* delivering signaling

molecules that regulate a vast repertoire of cellular processes. Their role in cancer development is central as they participate through bidirectional signaling between cancer cells and TME cells to every step of CRC carcinogenesis up to metastatic dissemination. Their detection in a large variety of biological fluids represents the future of cancer detection, an easy and reproducible mean to identify specific biomarkers of diagnostic and prognostic relevance. Moreover, they also represent new targets for treatment as their inhibition could limit or stop cancer development. Additionally, as extracellular signaling molecules, they could be used as very specific nanovectors to transport conventional or innovative therapies to cancer cells of interest.

However, although pre-clinical data appear very promising, validation from large clinical trials are needed to support EVs use as either tumor biomarkers for monitoring cancer progression and driving treatment decisions or new vectors for specifically targeted treatments. Such data are mandatory to better understand EVs function in cancer progression and translate EVs use in clinical practice.

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Radiomics in hepatocellular carcinoma: A state-of-the-art review

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Abstract

Hepatocellular carcinoma (HCC) is the most common cancer and the second major contributor to cancer-related mortality. Radiomics, a burgeoning technology that can provide invisible high-dimensional quantitative and mineable data derived from routine-acquired images, has enormous potential for HCC management from diagnosis to prognosis as well as providing contributions to the rapidly developing deep learning methodology. This article aims to review the radiomics approach and its current state-of-the-art clinical application scenario in HCC. The limitations, challenges, and thoughts on future directions are also summarized.

Key Words: Hepatocellular carcinoma; Radiomics; Deep learning; Artificial intelligence; Medical imaging; Predictive modeling

Core Tip: Medical imaging plays an indispensable role in hepatocellular carcinoma (HCC) clinical settings. Conventional imaging methods, however, provide limited and insufficient information. Recent studies have shown that radiomics and deep learning enable comprehensive insightful data mining that has achieved favorable performance in the detection and classification, diagnosis and differentiation, staging and grading, aggressive behavior, treatment responses, prognosis, and survival rates of HCC. Nevertheless, the wide implementation of radiomics and deep learning in actual routine clinical practice requires sustainable validation and optimization.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common cancer with fast rising incidence in both males and females and the second major contributor to cancer-related mortality worldwide[1,2]. Medical imaging has been playing a pivotal role in the entire diagnosis and management process of HCC, with the capacity to non-invasively provide multi-parameter, multidimensional, and multi-modality structural and functional information on lesion and peri-tissues on computed tomography (CT) and magnetic resonance imaging (MRI)[3-7].

Although the current diagnosis and treatment system continues to improve progressively, some crucial aspects such as the high heterogeneity and diverse biological behaviors of HCC tumors, which directly affect the prognosis and survival of patients, remain a concern and need to be addressed[8,9].

However, certain limitations of traditional imaging and report methods such as insufficient depth of imaging feature interpretations, the influence of subjective variability among observers, and unavailability to meet the needs of modern precise medicine may hinder comprehensive evaluations and personalized treatment of HCC.

In recent years, with rapid developments in big data mining and artificial intelligence (AI) fields, medical imaging in gastrointestinal and abdominal diseases has been empowered with more efficient combinations of data[10-12]. Radiomics, a burgeoning technology that could transform potential pathological and physiological information from routine-acquired images into high-dimensional quantitative and mineable imaging data[13-15], has been demonstrating great potential in the diagnosis, classification and staging, clinical decision assistance, and prognosis and survival predictions of HCC.

Hence, this article reviews the radiomics approach and its current state-of-the-art clinical application scenario in HCC. Additionally, the limitations, challenges, and thoughts on future directions are summarized.

RADIOMICS BASIC WORKFLOW IN HCC

Radiomics is a multi-disciplinary technology that refers to extraction and analysis of a large number of advanced and quantitative image features from medical imaging such as CT, MRI, positron emission tomography (PET), or ultrasound (US), with high fidelity and high throughput[13,15,16]. The core steps include data acquisition, image segmentation, feature extractions, analysis, and model building and validation. Most current research on radiomics in HCC was performed with the general procedure described above (Figure 1).

Image acquisition and preprocessing

At the beginning and as the basis of radiomics flow, medical images can be acquired using CT, MRI, US, or PET for single- or multi-center studies with retrospective or prospective cohorts and different task targets. CT and MRI-based, retrospective, single-center studies account for the vast majority of HCC radiomics publications. Given that the reproducibility and comparability of image characteristic analysis are influenced by facilities, platforms, parameters, and factors like those in clinical practice, there is a clear need for standardized image acquisition and reconstruction protocols[15,16]. Besides, in order to avoid bias due to inconsistent pixels, gray levels, or variable resolutions, image preprocessing mainly using resampling and normalization is indispensable to ensure a feasible and repeatable subsequent analysis[17,18].

Segmentation

Segmentation of the regions of interest (ROIs) or the volumes of interest (VOIs) is normally performed in three ways: Manual, semi-automatic, and automatic, among which the first is used most often at present. Manual segmentation relies on the radiologists to identify and annotate lesions manually. It has the advantage of higher accuracy, although it is time-consuming with low efficiency and inter-operator variability. There is a great availability of open-source software for segmentation, such as ITK-SNAP (www.itksnap.org), 3D Slicer (www.slicer.org), MIM (www.mimsoft ware.com), and ImageJ (https://imagej.nih.gov/ij/). In recent years, semi-automatic and automatic segmentations have been more developed with the assistance of a series of computer algorithms[19-23].
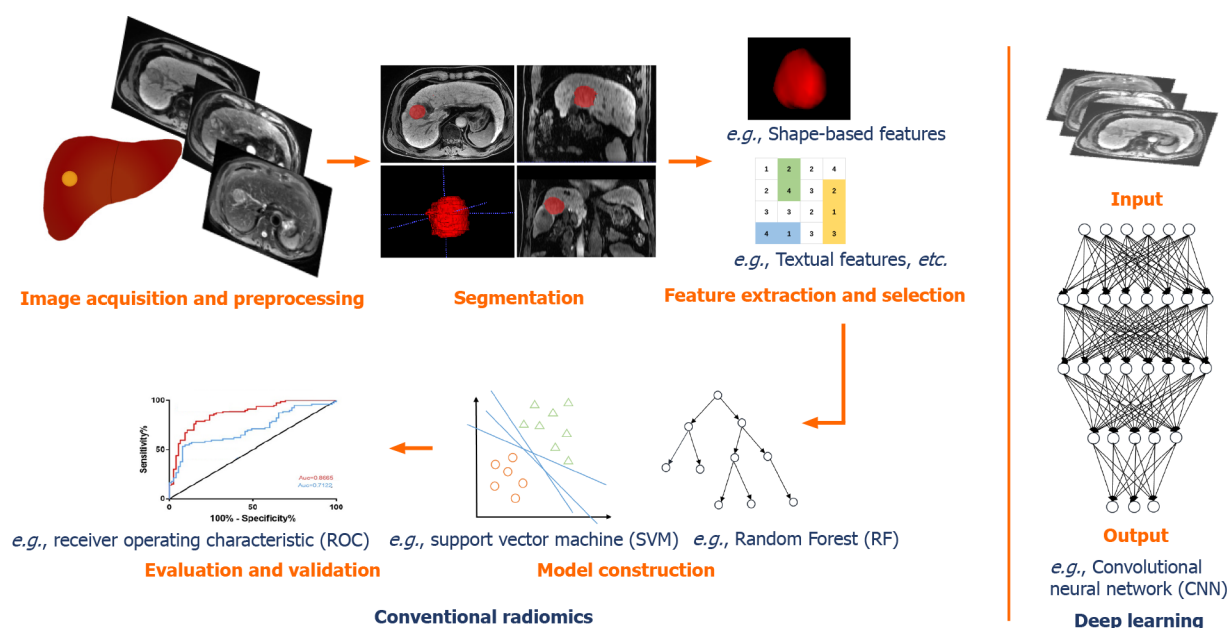
e.g., Shape-based features

e.g., Textual features, *etc.*

Image acquisition and preprocessing   **Segmentation**   **Feature extraction and selection**

Input

e.g., receiver operating characteristic (ROC)   *e.g.*, support vector machine (SVM)   *e.g.*, Random Forest (RF)

Output

e.g., Convolutional neural network (CNN)

Evaluation and validation   **Model construction**

Conventional radiomics   **Deep learning**

Figure 1 General workflow of radiomics and deep learning in hepatocellular carcinoma.

Feature extraction and selection

A number of features can be extracted from the 2D ROIs or 3D VOIs, which are attributed to the basis of radiomics analysis. Features can be divided into two types: "Semantic" and "agnostic"[15]. The "semantic" features include qualitative features like location, size, shape, and vascularity. The "agnostic" features refer to mathematically quantitative descriptions of the invisible characteristics of lesions, which can be roughly classified into four types: (1) Morphologic features that are expressed as statistical values; (2) First-order features (histogram features) reflecting the distributions of different gray levels of lesion, mainly including the standard deviation, energy, entropy, kurtosis, sharpness, skewness, and variance; (3) Second-order features (textual features) that describe the tumor heterogeneity addressing the spatial relationships of pixels or voxels, commonly using a gray-level co-occurrence matrix and gray-level run-length matrix[24,25]; and (4) Higher-order features that were extracted utilizing various filters, such as wavelet transforms, Laplacian filters, and Minkowski functionals.

However, several features are not desirable. Redundant and irrelevant features affect the accuracy and robustness of the model. In order to avoid overfitting and improve accuracy, it is necessary to select the most significant and informative features from a large number of extracted features for dimensionality reduction prior to modeling. This step has been commonly carried out in a variety of machine learning methods, such as filter-type methods like correlation or univariate regression, and embedding methods like least absolute shrinkage and the selection operator (LASSO) algorithm[26].

Model construction and validation

Clinical task-oriented models are built utilizing selected significant features, appropriately with the addition of some clinical indicators and laboratory indexes. In traditional machine learning, the commonly used methods are logistic regression, support vector machines (SVMs), decision trees, random forest (RF), K-nearest neighbor, and clustering analysis, *etc*. According to Parmar *et al*[26], the choice of modeling method has a dominant influence on the radiomics analysis results. Hence, various methods can be applied to select the model with the best performance in practice.

Taking into account the reliability and generalizability, each model must be evaluated and validated. The area under the receiver operating characteristic curve (AUC), decision curve analysis, and nomograms are commonly used for performance evaluations. Internal validation is indispensable, and external (multi-center) validation should also be conducted if conditions permit. However, most of the present studies are single-center studies with small samples, and by contrast, only a few omics models have been validated externally by multiple centers.

APPLICATIONS OF RADIOMICS IN HCC

Radiomics has been widely applied in diagnosis or differential diagnosis, pathological grading, aggressiveness evaluation, clinical treatment assistance, and recurrence and survival predictions of HCC. The tasks, methods, and results of some representative studies are listed in Table 1.

Diagnosis and differentiation of HCC

Early and accurate diagnosis of tumors is decisive for clinical decision-making and treatments. As the most common primary liver cancer, HCC can be diagnosed based on medical imaging findings without histopathological confirmation according to clinical practice guidelines[27,28].

However, some lesions with similar imaging manifestations to HCC, such as combined hepatocellular cholangiocarcinoma (cHCC-CC), intrahepatic cholangiocarcinoma (ICC), hepatic adenoma (HCA), and hepatic hemangioma (HH), are still challenging regarding diagnosis in conventional imaging. Liu *et al*[29] investigated the differentiation of HCC from non-HCC tumors (cHCC-CC and CC) with MRI and CT radiomics features using an SVM machine learning algorithm. Their results showed that contrast-enhanced MRI (CE-MRI) phases were quite useful for differentiation of HCC from non-HCC with an AUC of 0.79-0.81, as well as pre-contrast and portal phase CT with an AUC of 0.81 and 0.71, respectively. Although the study was limited by inconsistent imaging protocols and a sample size that was too small to separate into training and validation cohorts. Lewis *et al*[30] used the histogram parameters of apparent diffusion coefficient (ADC) of diffusion weighted imaging (DWI) and liver imaging reporting and data system (LI-RADS) classifications to distinguish HCC from other primary liver cancers (ICC and cHCC-ICC). The results presented that the prediction model combined with gender, ADC fifth percentile, and LI-RADS classification obtained the best predictive performance with an AUC of 0.90[30]. Regarding the distinction of HCA and HCC, Nie *et al*[31] reported that the CT-based radiomics nomogram was a potential tool to accurately differentiate HCA from HCC in the noncirrhotic liver with favorable performance (AUC of 0.96 in the training set and 0.94 in the test set). Similarly, this CT-based radiomics nomogram also achieved effective values in the preoperative differential diagnosis of FNH and HCC in the noncirrhotic liver (AUC of 0.979 in the training set and 0.917 in the test set)[32]. Another study by Wu *et al*[33] developed and validated a radiomics signature using derived features from pre-contrast MR imaging sets to distinguish HCC and HH. The results witnessed an improved diagnostic performance of combination of in-phase, out-phase, T2 weighted imaging (T2WI), and DWI with logistic regression (AUC: 0.86 in the training set and 0.89 in the test set), which outperformed the less experienced radiologist and was nearly equal to the experienced radiologist. These radiomics studies contributed potential supplements to accurate diagnosis and differentiation of HCC in medical imaging, but the results remain to be widely validated and amended in the clinical practice.

Pathological grading of HCC

The pathological grade is one of the vital factors affecting intrahepatic tumors recurrence, that is, high-grade tumors are associated with a high intrahepatic recurrence rate[34,35]. The management of HCC varies with different pathological grades, and patients with higher intrahepatic recurrence rates require special treatments for surgery and follow-up compared with the lower-risk patients[6,36]. Thus, accurate prediction of HCC pathological grade might promote clinical decision-making and formulation of the most appropriate treatment plan. Wu *et al*[37] built radiomics signatures on the basis of T1-weighted imaging (T1WI) and T2WI generated in LASSO, and assessed the predicted values of radiomics, clinical factors, and the combined models. The results showed that there were significant differences in categorization of high- and low-grade HCCs in MRI-based radiomics signatures ($P < 0.05$). The predictive value of the radiomics signature model outperformed the clinical factors-based model (AUC: 0.74 *vs* 0.60, respectively), whereas the combined model incorporating both achieved the best performance with an AUC of 0.80 [95% confidence interval (CI): 0.65-0.90][37]. Another similar study by Mao *et al*[38] aimed to predict the pathological grades of HCC preoperatively based on contrast-enhanced CT (CECT)-derived radiomics signatures. They established models using shape, first-order, second order, and higher-order features extracted from arterial phase (AP)- and venous phase-CECT images *via* recursive feature elimination and eXtreme Gradient Boosting (XGBoost). They also found that combining radiomics signatures with clinical factors

Table 1 Some representative studies of radiomics in hepatocellular carcinoma

Ref.	Application task	Study design	Imaging modality	Radiomics features	Algorithm	Sample size	Training set	Test/validation set	Performance
Liu *et al*[29], 2021	Differentiation of cHCC-CC from HCC and CC	Retrospective, single-center	CT, MRI	1419	SVM	85 patients with HCC (37), cHCC-CC (24) and CC (24)	85	NA	Excellent performance for differentiation of HCC from non-HCC (AUC: 0.79-0.81 in MRI, AUC: 0.71-0.81 in CT)
Nie *et al*[32], 2020	Differentiation of HCA from HCC	Retrospective, two-institutes	CT	3768	mRMR, LASSO	131 patients with HCC (85) and HCA (46)	93	38	Favorable performance (AUC: 0.96 in training set, AUC: 0.94 in test set)
Wu *et al*[33], 2019	Pathological grade of HCC	Retrospective, single-center	MRI	656	LASSO	170 patients with HCCs	125	45	Radiomics signature model outperformed the clinical factors-based model; the combined model achieved the best performance (AUC: 0.80)
Mao *et al*[38], 2020	Pathological grade of HCC	Retrospective, single-center	CT	3376	RFE, XGBoost	297 patients with HCCs	237	60	The radiomics signatures combined with clinical factors significantly achieved the best performance (AUC: 0.8014)
Xu *et al* [43], 2019	Preoperative prediction of MVI in HCC	Retrospective, single-center	CT	7260	Ref-SVM, Multivariable logistic regression	495 patients with HCC	300	145 (test); 50 (validation)	Good performance (AUC: 0.909 in the training/validation set, AUC: 0.889 in the test set)
Chong *et al* [47], 2021	Preoperative prediction of MVI in HCC	Retrospective, single-center	MRI	854	LASSO, RF, logistic regression	356 patients with HCCs ≤ 5 cm	250	106	AUC: 0.920 using RF; AUC: 0.879 using logistic regression (in validation set)
Fu *et al* [54], 2019	Assistant in optimal treatment choices of HCC between LR and TACE	Retrospective, multi-center (5 institutions)	MRI	708	LASSO, Akaike information criterion	520 patients with HCC	302	218	Good discrimination and calibrations for 3-year PFS (AUC: 0.80 in training set, AUC: 0.75 in validation set); threshold ≤ -5.00: suggesting LR, threshold > -5.00: suggesting TACE
Sun *et al*[56], 2020	Predicting the outcome of TACE for unresectable HCC	Retrospective, single-center	MRI	3376	LASSO, multivariable logistic regression	84 patients with BCLC B stage HCC	67	17	The radiomics signatures combined with clinical factors significantly achieved the best performance (AUC: 0.8014)
Ji *et al* [66], 2020	Predicting early recurrence after LR	Retrospective, multi-center (3 institutions)	CT	846	LASSO-Cox regression	295 patients with HCC	177 (Institution 1)	118 (Institution 2 and 3, external validation)	Better prognostic ability (C-index: 0.77, *P* < 0.05), lower prediction error (integrated brier score: 0.14), and better clinical usefulness than rival models and staging systems
Zhao *et al* [67], 2020	Predicting early recurrence after LR	Retrospective, single-center	MRI	1146	LASSO, stepwise and multivariable logistic regression	113 patients with HCC	78	35	The nomogram integrating the Rad score and clinicopathologic-radiologic risk factors

									showed better discrimination and clinical utility (AUC: 0.873)
Wang *et al* [75], 2020	Predicting 5-year survival after LR	Retrospective, multi-center (2 institutions)	MRI	3144	RF, multivariate logistic regression	201 patients with HCC	160	41 (five-fold cross-validation)	The model incorporating the radiomics signature and clinical risk factors obtained good calibration and satisfactory discrimination (AUC: 0.9804 in training set, AUC: 0.7578 in validation set)
Song *et al*[76], 2020	Predicting RFS after TACE	Retrospective, single-center	MRI	396	LASSO-Cox regression, multivariate Cox regression	184 patients with HCC	110	74	The model using the radiomics signature with the clinical-radiological risk factors showed the best performance (C-index: 0.802)

cHCC-CC: Combined hepatocellular cholangiocarcinoma; NA: Not available; HCC: Hepatocellular carcinoma; CC: Cholangiocarcinoma; CT: Computed tomography; MRI: Magnetic resonance imaging; GLCM: Gray-level co-occurrence matrix; SVM: Support vector machine; AUC: Area under the receiver operating characteristic curve; HCA: Hepatic adenoma; mRMR: Maximal relevance and minimum redundancy; LASSO: Least absolute shrinkage and the selection operator; RFE: Recursive feature elimination; XGBoost: eXtreme gradient boosting; MVI: Microvascular invasion; Ref-SVM: Recursive feature selection support vector machine; RF: Random forest; LR: Liver resection; TACE: Transarterial chemoembolization; PFS: Progression-free survival; BCLC: Barcelona clinic liver cancer; C-index: Concordance index; RFS: Recurrence free survival.

significantly improved the prediction performance at an AUC of 0.8014 (95%CI: 0.6899-0.9129)[38]. It can be known that radiomics is a powerful tool for predicting the pathological grade of HCC closely related to the follow-up management, as well as extending the predictive value of clinical factors.

Aggressiveness evaluation of HCC

The aggressive tumor behavior is strongly linked to the prognosis of HCC patients. Microvascular invasion (MVI), defined as tumor cell nest in vessels lined with the endothelium that can only be determined on the postoperative histologic examination, is one of the crucial independent predictors of early recurrence (ER) of HCC patients after surgical treatment[39-41]. So, it is of remarkable importance to accurately evaluate and predict the MVI of HCC preoperatively, so as to ensure and improve the prognosis of patients. Since Bakr *et al*[42] pointed out the potential of a CT-based radiomics signature as a surrogate for MVI in HCC (AUC: 0.76, 95%CI: 0.58-0.94) though in a small cohort, various researchers have explored an underlying association focusing on this field. Xu *et al*[43] developed a CT-based radiomics model integrating large-scale clinical factors and imaging features to predict the MVI and outcomes in surgically resected patients with HCC. The approach demonstrated good performance with an AUC of 0.909 in the training/validation set and 0.889 in the test set[43]. A radiomics nomogram based on CECT established by Ma *et al*[44] showed that portal venous phase (PVP) radiomics signatures exhibited better performance to predict MVI than AP and delay phase (DP) (AUC in validation sets: 0.793 *vs* 0.684 and 0.490, respectively). Another study performed in two independent centers by Zhang *et al*[45] shared the same goal as those above, constructing CECT-based radiomics signatures in a LASSO algorithm and multivariable logistic regression. Enrolled patients from institution 1 were divided into the training and the test set, and patients from institution 2 served as an independent validation set, of which the AUC of MVI status predictions were 0.780, 0.776, and 0.743, respectively, and the AUC of the final MVI risk classifier-integrated clinical stage reached 0.783, 0.778, and 0.740, respectively[45].

Regarding an MRI radiomics model for MVI prediction in HCC, Feng *et al*[46] first reported that the combined intratumoral and peritumoral radiomics model derived from gadolinium-ethoxybenzyl-diethylenetriamine (Gd-EOB-DTPA)-enhanced MRI showed effective value with an AUC of 0.83 (95%CI: 0.71-0.95) in the validation cohort along with a sensitivity of 90% and specificity of 75%[46]. Additionally, specific to solidary HCCs ≤ 5 cm, Chong *et al*[47] built a multi-scale and multi-parametric radiomics nomogram based on Gd-EOB-DTPA MRI, and this also yielded favorable performance for preoperative MVI predictions, of which the AUC reached up to 0.920

(95%CI: 0.861-0.979) using RF and 0.879 (95%CI: 0.820-0.938) using logistic regression in the validation set[47]. Another study by Yang *et al*[48] indicated the helpful value of hepatobiliary phase (HBP) for predicting MVI, showing that HBP T1WI images and HBP T1 maps were independent risk factors for MVI and the model incorporating the clinicoradiological factors and HBP-derived radiomic features outperformed the former only in the training cohort (AUC: 0.943 *vs* 0.850, *P* = 0.002), though there was no statistical significance in the validation set (AUC: 0.861 *vs* 0.759, *P* = 0.111)[48]. These studies provided new perspectives and approaches for aggressiveness eva-luation of HCC and might help to improve the prognosis of patients and assist in the precise treatment plan making.

Clinical treatment assistance for HCC

Caution needs to be taken comprehensively when it comes to selecting the optimal treatment for HCC patients. In addition to the patients' conditions and tumor stage, the trauma of the treatments which is associated with deterioration of liver function leading to death should be also given full consideration[49]. For example, liver resection (LR) is curative to remove the tumor completely but highly traumatic. Transarterial chemoembolization (TACE) is minimally invasive while may leave some residual tumors. And their adaptation has expanded and even overlapped with the development of medical technologies[50-53]. Focusing on this, Fu *et al*[54] proposed an individualized model to assist appropriate treatment choices for HCC patients between LR and TACE. They extracted radiomics features from CT images of HCC patients in five centers and combined them with clinical factors and radiological characteristics to construct a progression-free survival (PFS) model. The model yielded good discrimination and calibrations for 3-year PFS with an AUC of 0.80 in the training set and 0.75 in the validation set, outperforming the other four state-of-the-art models. And a nomogram was built to subdivide patients for optimal treatments by the threshold of the score difference. In the threshold ≤ -5.00 group, LR provided better PFS than TACE, which suggested LR to be a potential better option [hazard ratio (HR) = 0.50, *P* = 0.014 in the training set; HR = 0.52, *P* = 0.026 in the validation set]. For the other patients, LR and TACE had similar PFS (HR = 0.84, *P* = 0.388 in the training set; HR = 1.14, *P* = 0.614 in the validation set). TACE seemed to be a better choice as it was less invasive and helped to control unnecessary trauma and risks[54]. Moreover, for HCC patients who underwent hepatectomy, Cai *et al*[55] developed and validated a radiomics-based nomogram derived from PVP-CT images to predict posthepatectomy liver failure (PHLF) preoperatively, which exhibited superior discrimination with an AUC of 0.896 (95%CI: 0.774-1.000) in the validation set rather than other three methods [Child-Pugh, Model of End Stage Liver Disease (MELD), and albumin bilirubin]. Furthermore, another 13 patients served for a pilot prospective analysis, and the radiomics nomogram predicted PHLF effectively with an AUC of 0.833 (95%CI: 0.591-1.000)[55]. For unresectable HCC patients, Sun *et al*[56] established a radiomics model based on preoperative multiparameter MRI (mp-MRI) predicting early progression after TACE. The results identified the radiomics signature as an independent pa-rameter of progressive disease (PD), and the mp-MRI signature achieved the greatest benefit with an AUC of 0.800 compared with the single ones[56]. These studies demonstrated the guiding significance of radiomics in assisting clinical treatment selections for HCC, especially when there were more controversies, which could help patients and doctors weigh the advantages and disadvantages and choose the optimal personalized plan.

Recurrence and survival prediction in HCC

In routine clinical settings, LR is preferred as the first-line treatment option for HCC patients at an early stage and with preserved liver function, whereas liver trans-plantation (LT) is recommended for end-stage HCC patients with clinically proven portal hypertension and early-stage HCC meeting the Milan criteria. For patients who are not suitable for LR or LT (Barcelona Clinic Liver Cancer (BCLC) stage 0-A and some selected BCLC stage B), non-surgical local ablation techniques are considered as best choices[27,28,57,58]. However, post-treatment recurrence remains a thorny pro-blem that hinders clinical management progress and patient survival[59-65]. There-fore, it is of emerging significance to preoperatively predict the recurrence risk after treatments.

Several radiomics studies based on preoperative CT or MRI have yielded favorable performance in post-LR ER predictions[66-72]. In a recent multi-center study by Ji *et al* [66], recurrence-related radiomic features were extracted from preoperative CECT images of 295 surgically proven HCC patients from three independent institutions and then built with LASSO and Cox regression. The two radiomics-based models pre-

sented better prognostic ability [concordance index (C-index): 0.77, *P* < 0.05)], lower prediction error (integrated Brier score: 0.14), and better clinical usefulness than rival models and staging systems[66]. Another mp-MRI based radiomics study by Zhao *et al* [67] established radiomics models deriving from in-out-phase T1WI, T2WI, DWI, and CE-MRI images. The combined nomogram integrating the Rad score and clinicopathologic-radiologic (CPR) risk factors showed better discrimination and clinical utility than the CPR and radiomics models alone (AUC: 0.873 *vs* 0.742, respectively). For recurrence predictions for HCC after LT, Guo *et al*[73] also combined the CT-based radiomics signature and clinical risk factors to develop and validate a radiomics nomogram in LASSO and Cox regression algorithm, which achieved good predictive performance for recurrence-free survival with a C-index of 0.785 (95%CI: 0.674-0.895) in the training set and 0.789 (95%CI: 0.620-0.957) in the validation set. As for HCC patients who underwent ablation, Yuan *et al*[74] extracted radiomics features from three-phase preoperative CECT images (AP, PVP, and parenchymal phase), selected the significant features by mMRM, and then built a radiomics signature using LASSO and Cox regression. Similarly, the PVP-combined model adding the clinicopathological factors produced the best predictive performance to predict ER after curative ablation with a C-index of 0.792 (95%CI: 0.727-0.857) in the training set and 0.755 (95%CI: 0.651-0.860) in the validation set[74].

A radiomics approach has demonstrated encouraging results in survival analysis of post-treatment HCC patients[75-78]. In a recent multi-center study, Wang *et al*[75] worked on predicting the 5-year survival of HCC patients after LR using an MRI-based radiomics model. They built radiomics signatures with an RF method and developed a combined model incorporating radiomics signatures and clinical risk factors, which obtained good calibration and satisfactory discrimination for survival prediction with an AUC of 0.9804 in the training set and 0.7578 in the validation set [75]. Kim *et al*[77] predicted the overall survival (OS) of HCC patients who underwent TACE with the use of a pretreatment CT-based radiomics model. They applied LASSO-Cox regression algorithm for optimal survival-related feature selection and constructed a predictive model combining radiomics signature with clinical factors. The results suggested that the composite model can better predict the OS after TACE (HR: 19.88, 95%CI: 6.37-92.02, *P* < 0.001) compared with radiomics and clinical models only[77]. In these studies, a substantial growth was observed in the performance of the state-of-the-art conventional models when adding the radiomics signature. They demonstrated the considerable value of radiomics approach to predict the ER risk and survival conditions of post-treatment HCC patients, which may facilitate personalized risk stratification and enlighten a new way for further clinical decision-making for HCC patients.

DEEP LEARNING BASED RADIOMICS

Deep learning, a ramification of machine learning algorithms developed from neural networks with multiple layers, has been widely used in medical image analysis with promising expectations[79,80]. As a type of representation learning method, deep learning takes the strength of excellent self-taught ability which enables automatic learning and training of target-related features without manual segmentation and extraction (Figure 1). It has demonstrated deeper and more comprehensive data mining compared with radiomics based on traditional machine learning. Convolutional neural network (CNN) is the most popular model, meanwhile stacked autoencoders (SAEs), restricted Boltzmann machines (RBM), deep belief network (DBN), GAN, and U-net have been also applied[81-85]. CNNs are mainly composed of three network layers, namely, the convolutional, the sampling, and the full connection layer, of which the core mechanisms include multi-layer stacking, local connection, weight sharing, and pooling. Automatic learning of informative features of medical images is accomplished without the need for manual segmentation and feature extraction. SAE is an unsupervised learning method, which trains the models by adjusting the advantage parameters of the encoder and layer. RBM, composed of visible units and hidden units, is a kind of generative stochastic neural network that learns probability distributions from input data sets. DBN can train the weights between its multiple neurons, which enables the whole neural network to generate training data according to the maximum probability. The models are chosen for different oriented tasks in HCC, including segmentation, tumor detection or classification, diagnosis and differentiation, aggressiveness evaluation, prognosis and survival analysis, and image quality improvement. The tasks, methods, and results of some representative studies of deep

learning in HCC are presented in Table 2.

APPLICATIONS OF DEEP LEARNING-BASED RADIOMICS IN HCC

Detection and segmentation of HCC

Manual segmentation is limited as it is time-consuming with low efficiency and inter-operator variability. Thus, accurate and automatic liver and tumor segmentation methods are demanded in clinical practice. Deep learning algorithms, by contrast, enable automated segmentation and have been applied in various studies[86-91]. Bousabarah *et al*[91] trained a deep CNN (DCNN) with a U-net architecture using multiphasic CE-MRI images and the dice similarity coefficient (DSC) was used to evaluate the performance. Their approach demonstrated the feasibility of automatically detecting and segmenting the liver and HCCs, and the mean DSC between automatically detected lesions using the DCNN + RF + thresholding and corresponding manual segmentations was 0.64/0.68 (validation/test), and 0.91/0.91 for liver segmentations[91]. However, most studies investigated the whole liver, liver tissues, or malignant tumors, whereas a few focused specifically on automatic segmentation of HCC, which should be developed and validated in further studies.

Diagnosis, differentiation, and classification of HCC

Yasaka *et al*[92] utilized a deep learning algorithm with CNN to differentiate HCCs and other liver masses based on three-phase CT images (pre-contrast, AP, and DP) and obtained an accuracy of 84% in the test set. Hamm *et al*[93] designed a proof-of-concept CNN-based deep learning system (DLS) for liver tumor diagnosis on the basis of mp-MRI. The DLS achieved an accuracy of 92% and an AUC of 0.992 for HCC classification. And they further indicated an "interpretable" DLS that could identify the correct radiological features of each test lesion on MR images with a positive predictive value of 76.5% and sensitivity of 82.9%[94]. Another pilot study by Yamashita *et al*[95] developed a CNN-based model with LIRADS to diagnose and categorize HCC on CT and MRI. It exhibited that the transfer learning model outperformed the custom-made model with an overall accuracy of 60.4% and AUCs of 0.85, 0.90, 0.63, and 0.82 for LR-1/2, LR-3, LR-4, and LR-5, respectively, whereas the external validation results were not accurate enough[95]. Although the results were promising, those studies were preliminary and demonstrated the initial feasibility of deep learning in the diagnosis, differentiation, and classification of HCC.

Aggressiveness, treatment outcomes, and survival evaluation of HCC

The application of deep learning in aggressiveness behavior evaluation, treatment outcome prediction, and recurrence and survival analysis of HCC were not as sophisticated as those of conventional radiomics, but they also witnessed dramatic potential and clinical value. For MVI prediction, Wang *et al*[96] established a deep learning model with a CNN based on preoperative DWI and reported that the combination of deep features from the $b = 0$, $b = 100$, $b = 600$, and ADC images presented the best results (AUC: 0.79, $P = 0.002$)[96]. With regard to prediction of treatment responses, Peng *et al*[97] trained and validated a deep learning model using ResNet50 on preoperative CT images of HCC patients who underwent TACE from three independent institutions. This multi-center study yielded excellent predictive performance for complete response, partial response, stable disease, and PD with an accuracy of 84.3% and AUC of 0.97, 0.96, 0.95, and 0.96, respectively, in the training set, and an accuracy of 85.1% and 82.8% in the two validation sets[97]. Another multi-center study by Zhang *et al*[98] involved preoperative CECT images and they adopted a deep learning-based model utilizing DenseNet to predict OS of HCC patients treated with TACE plus sorafenib, which achieved favorable prediction performance with a C-index of 0.717 in the training set and 0.714 in the validation set.

Image quality improvement

Deep learning has been applied for image quality improvement, which helps with the diagnosis and interpretation of HCC and other liver lesions more accurately. For example, Tamada *et al*[99] indicated a CNN-based method in Gd-enhanced MR images in the AP to improve the imaging quality, and the magnitude of the artifacts and blurring induced by respiratory motion were significantly reduced. Additionally, Esses *et al*[100] described an CNN-based method in T2WI liver MRI images for automated image quality evaluation, which yielded a high negative predictive value

Table 2 Some representative studies of deep learning in hepatocellular carcinoma

Ref.	Application task	Study design	Imaging modality	Algorithm	Sample size	Training set	Test/validation set	Performance
Bousabarah *et al*[91], 2021	Automatic detection and segmentation of HCC	Retrospective, single-center	CT	DCNN, U-net	174 patients with 231 lesions	165	33 (test); 33 (validation)	Mean DSC between automatically detected lesions using the DCNN + RF + TR and corresponding manual segmentations: 0.64/0.68 (validation/test), and 0.91/0.91 for liver segmentations
Yasaka *et al* [92], 2018	Differentiation of HCC and other liver tumors	Retrospective, single-center	CT	CNN	560 patients	460	100	Accuracy: 84% in test set
Hamm *et al* [93], 2019	Diagnosis and classification of HCC	Retrospective, single-center	MRI	CNN	494 patients	434	60	Accuracy: 92%, AUC: 0.992
Yamashita *et al*[95], 2020	Diagnosis and categorization of HCC with LI-RADS	Retrospective, multi-center	CT, MRI	CNN	314 patients (163 CT, 151 MRI)	220	47 (test); 47 (internal validation); 112 (external validation)	Overall accuracy: 60.4% and AUCs: 0.85, 0.90, 0.63, and 0.82 for LR-1/2, LR-3, LR-4, and LR-5, respectively
Wang *et al* [96], 2020	Preoperative prediction of MVI in HCC	Retrospective, single-center	MRI	CNN	97 patients with 100 HCCs	60 HCCs	40 HCCs	The combination of deep features from the $b = 0$, $b = 100$, $b = 600$, and ADC images presented the best results (AUC: 0.79)
Peng *et al* [97], 2020	Prediction of treatment response of TACE	Retrospective, multi-center (3 institutions)	CT	ResNet50	789 patients with HCC	562 (Institution 1)	89 (Institution 2); 138 (Institution 3)	Excellent predictive performance for CR, PR, SD, and PD (accuracy: 84.3%; AUCs: 0.97, 0.96, 0.95, and 0.96 in training set, accuracies: 85.1% and 82.8% in the two validation sets)
Zhang *et al* [98], 2020	Predicting OS after TACE + Sorafenib	Retrospective, multi-center (3 institutions)	CT	DenseNet (CNN)	201 patients with HCC	120 (Institutions 1 and 2)	81 (Institution 3)	Favorable prediction performance (C-index: 0.717 in training set, C-index: 0.714 in validation set)
Tamada *et al* [99], 2020	Motion artifact reduction	Retrospective, single-center	MRI	CNN	34 patients with HCC	14	20	Significant reduction of the magnitude of the artifacts and blurring induced by respiratory motion
Esses *et al* [100], 2018	Automated image quality evaluation	Retrospective, single-center	MRI	CNN	522 patients with HCC	351	171	High negative predictive value (94% and 86% relative to two readers)

DCNN: Deep convolutional neural network; TR: Thresholding; DSC: Dice similarity coefficient; CNN: Convolutional neural network; LI-RADS: Liver imaging reporting and data system; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

(94% and 86% relative to two readers) for screening diagnostic and nondiagnostic liver T2WI. The applications of deep learning for medical imaging technologies will be strikingly expanded in further explorations.

LIMITATIONS, CHALLENGES, AND FUTURE DIRECTIONS

Despite the encouraging achievements and progress of radiomics and deep learning in HCC, the prior studies also highlighted the limitations and challenges that must be addressed (Figure 2). First and most critically, the majority of current studies were retrospective with a small sample size performed in single center, lacking of uniform standards and external validation. The enrolled samples, imaging acquisition protocols, facilities, platforms, segmentation methods, modeling algorithms, and radiomics tools differed in various studies, which accounted for variations and poor generalizability. The studies based on radiomics quality score and Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis
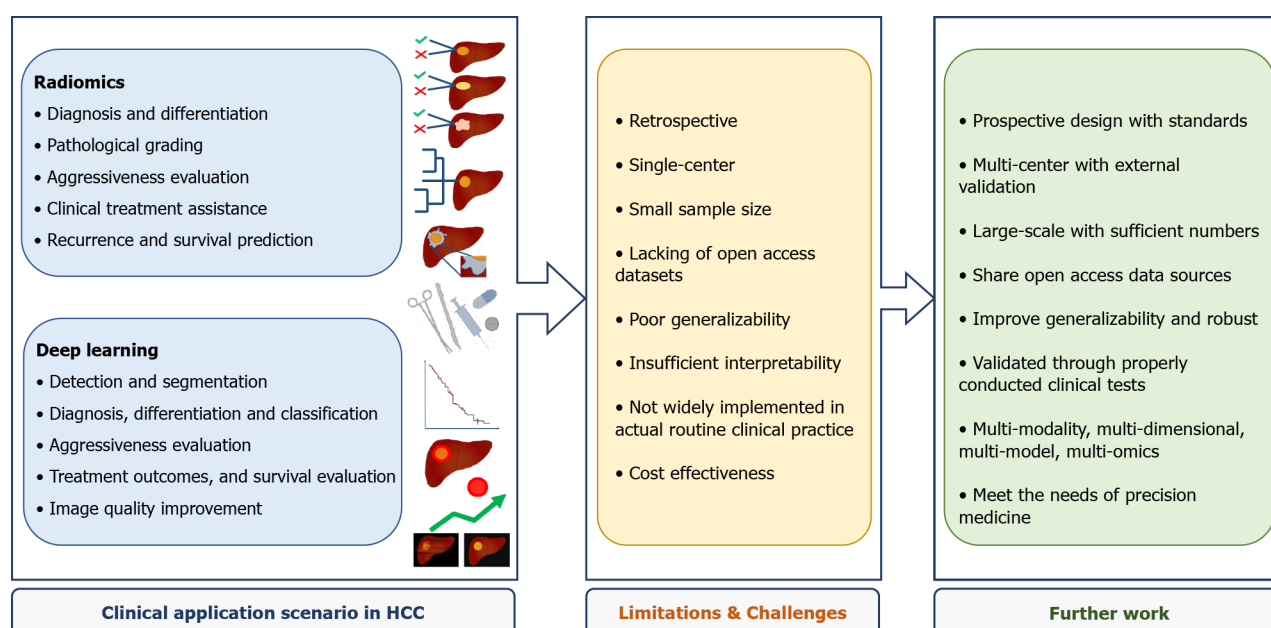
Figure 2 Summary of the clinical application scenario, limitations, challenges, and further work of state-of-the-art radiomics and deep learning in hepatocellular carcinoma.

have also emphasized these insufficiencies[101,102]. Getting with the consensus guidelines published by the image biomarker standardization initiative may help to cope with the problem[103]. More importantly, prospective-design, multi-center, large-sample studies are urgently warranted in further investigation on HCC, along with intensive and standardized quality controls throughout the entire workflow.

Deep learning has been putting a brand-new step forward in radiomics, demonstrating superior potential in HCC-oriented tasks while requiring large-scale validation and long-term justification in further studies. Besides, the insufficient interpretability of these AI-medical imaging-combined approaches remains a concern, meaning that it is still quite challenging to adequately explain the underlying associations of radiomics analysis results and tumor heterogeneity and biological behaviors of HCC.

Moreover, few valuable datasets were shared with open access, which got in the way of accumulating sufficient numbers for statistical power. Therefore, it is an expecting choice to share open access database sources across institutions to strengthen the generalization ability and establish well-curated databases and networks, as the Quantitative Imaging Network (QIN) proposing[104]. By the way, the cost-effectiveness of a radiomics or deep learning approach is also supposed to be weighted when applying it to a specific clinical situation of HCC, as it is procedure-complex, time-consuming, labor-intensive, and hardware- and software-demanding.

To date, radiomics and deep leaning have been applied in numerous HCC studies, but they have not been widely implemented into routine clinical practice, which requires to be extensively validated and optimized through further appropriate clinical trials. Radiogenomics, an encouraging field considered as a bridge connecting radiomics with genomics[105], is also of promising value in HCC whereas not in the scope of this review. Radiologists ought to get more involved to take full advantage of AI to improve the working efficiency and tackle problems driven by clinical demanding. For the foreseeable future, the multi-modality, multi-dimensional, and multi-model radiomics integrating clinical factors, laboratory information, and other omics has become the next trend of AI-driven medicine for novel evaluation and management of HCC.

CONCLUSION

To conclude, radiomics has enormous potential to become a powerful tool for HCC management covering detection and classification, diagnosis and differentiation, staging and grading, assessment of aggressive behavior and treatment responses, and prognosis and survival prediction. However, the underlying value of radiomics and

deep learning based radiomics in HCC has not been fully investigated, as well as the applicability and generalizability in routine clinical practice. In the face of great opportunities albeit with challenges, the multi-modality, multi-dimensional, multi-model radiomics and multi-omics studies will become the most appropriate clinical research approaches, so as to meet the developing needs of precision medicine and enhance precision medicine initiatives.

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Gut microbiota and immune system in liver cancer: Promising therapeutic implication from development to treatment

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Abstract

Liver cancer is a leading cause of death worldwide, and hepatocellular carcinoma (HCC) is the most frequent primary liver tumour, followed by cholangiocarcinoma. Notably, secondary tumours represent up to 90% of liver tumours. Chronic liver disease is a recognised risk factor for liver cancer development. Up to 90% of the patients with HCC and about 20% of those with cholangiocarcinoma have an underlying liver alteration. The gut microbiota-liver axis represents the bidirectional relationship between gut microbiota, its metabolites and the liver through the portal flow. The interplay between the immune system and gut microbiota is also well-known. Although primarily resulting from experiments in animal models and on HCC, growing evidence suggests a causal role for the gut microbiota in the development and progression of chronic liver pathologies and liver tumours. Despite the curative intent of "traditional" treatments, tumour recurrence remains high. Therefore, microbiota modulation is an appealing therapeutic target for liver cancer prevention and treatment. Furthermore, microbiota could represent a non-invasive biomarker for early liver cancer diagnosis. This review summarises the potential role of the microbiota and immune system in primary and secondary liver cancer development, focusing on the potential therapeutic implications.

Key Words: Gut microbiota; Immune system; Liver cancer; Primary liver cancer; Colorectal liver metastasis; Liver cancer treatment

Core Tip: Liver cancer is a worldwide leading cause of death. Growing evidence suggested a pathogenetic role of the gut microbiota and immune system in liver cancer development. Although there have been rapid developments in metagenomic science, definitive and complete knowledge of these processes is still far from being acquired. However, targeting both microbiota and the immune system could represent an appealing therapeutic option alone or as a boost of conventional treatments. Finally, the microbiota signature evaluation could represent a potential novel, non-invasive biomarker for early diagnosis.

INTRODUCTION

Primary liver cancer is a leading cause of death worldwide. Hepatocellular carcinoma (HCC) is the most common primary liver tumour, accounting for about 80% of the cases. Cholangiocarcinoma (CCA) is the second primary liver tumour, representing approximately 15% of the malignancies[1]. Finally, the secondary tumours represent up to 90% of liver tumours, and the liver is the most frequent metastatic site[2].

Chronic liver disease is a recognised risk factor for HCC. Up to 90% of the patients with HCC have an underlying liver alteration, and about 30% of the patients with cirrhosis will suffer from HCC[3]. Several pathologies may cause liver cirrhosis, including viral hepatitis, alcohol abuse, diabetes and non-alcoholic fatty liver disease (NAFLD)[4]. Although most CCAs occur without any specific predisposing factors, about 20% of the patients harbour some of the same causal pathologies as HCC[5].

The gut microbiota consists of the biological community of bacteria, Archaea, fungi and viruses harboured within a host showing a commensal, symbiotic or pathogenetic attitude[6,7]. The liver is exposed to both microbiota and microbial metabolites through the portal flow. There is a bidirectional relationship between gut microbiota and liver through signalling-sensing pathways, known as the "gut microbiota-liver axis"[3,7,8].

The intestinal barrier has a crucial role in preserving the host from the environment. Intestinal barrier and gut microbiota can influence each other in a dynamic process, and alterations in each of them may impair this balance[9].

The interplay between the immune system and gut microbiota is also well-known. The liver immune system is a dynamic microenvironment subjected to changes related to the received stimuli[10]. In the liver, there are cells of the innate immune system, including Kupffer cells, natural killer cells, natural killer T (NKT) cells and cells belonging to the adaptive immune system, including T lymphocytes[11]. The γδT cells are unconventional T lymphocytes and act as a bridge between innate and adaptive immunity. γδT cells are getting attention due to their pleiotropy and their potential causal or beneficial role in the different aspects of tumour progression[12,13].

Metagenomic analysis, polymerase chain reaction and 16S ribosomal RNA sequencing can identify bacteria and their products[14]. Although primarily resulting from experiments in animal models and on HCC, growing data suggest a causal role for the gut microbiota in the development and progression of both chronic liver pathologies and tumours[3].

Several guidelines propose the best treatment strategies for liver cancer. Traditional treatments include surgery, transplantation, locoregional therapies, chemotherapy or chemoradiotherapy[15]. Despite curative treatments, tumour recurrence remains high. Therefore, gut microbiota modulation may represent a promising therapeutic target for liver cancer prevention and therapy[7]. Since complete prevention is not achievable, as with any other cancer, an early diagnosis may allow better patient outcomes, and the gut microbiota may represent a novel, non-invasive biomarker[16-18].

This review aims to provide the actual state of the art of the potential role of microbiota-immunity in every step of both primary and secondary liver cancer,

focusing on the potential therapeutic implications.

CANCER DEVELOPMENT

Different microbial composition in health and disease

Several studies reported the progressive increase in the number of pathogenic bacteria with the decrease of those showing a healthy behaviour and the different stages of chronic liver disease and HCC development[7,19]. Furthermore, faecal biodiversity seems to decrease along with the cirrhosis progression, but it seems to increase again along with early HCC progression[20]. Tables 1 and 2 summarise the most important changes in chronic liver disease and HCC, respectively.

Studies in humans reported a significant difference between the gut microbiota of patients with chronic viral hepatitis and healthy volunteers. In particular, a significant decrease in the number of *Alistipes, Bacteroides, Asaccharobacter, Butyricimonas, Ruminococcus, Clostridium cluster IV, Parabacteroides* and *Escherichia/Shigella* was found together with a significant increase in the number of *Megamonas,* unclassified *Lachnospiraceae, Clostridium sensu stricto* and *Actinomyces* in patients with chronic hepatitis B[21].

Patients with chronic hepatitis C presented in their stool samples a lower bacterial diversity, a higher presence of *Streptococcus, Lactobacillus* and *Bacteroidetes*, and a lower presence of Clostridiales and *Bifidobacterium*[22].

Alcoholic patients showed an increased number of gram-negative bacteria[6], but also the contributing role of the *Enterococcus faecalis* in alcoholic hepatitis has been described[23].

Dysbiosis has also been found in patients with NAFLD, though different studies reported a different relative abundance of bacteria in the gut of this subgroup of patients[3]. For example, compared with healthy controls, NAFLD patients' microbiota presented enriched in *Proteobacteria* and *Fusobacteria* with higher representations of the bacteria belonging to the family *Erysipelotrichaceae*, Enterobacteriaceae, *Lachnospiraceae* and *Streptococcaceae.* An increased number of *Escherichia* and *Shigella* were also found together with a reduced number of *Prevotella*[24].

Independently from the aetiology, patients with cirrhosis presented with a progressively lower number of bacteria from the family of *Lachnospiraceae*, together with increased levels of the bacteria belonging to the families *Enterobacteriaceae, Veillonellaceae* and *Streptococcaceae*. The last two families are typically found in healthy people's oral microbiota and can colonise cirrhotic patients' guts[25,26]. In particular, the number of *Streptococcus* seems to correlate with the Child-Pugh score directly. In contrast, the number of bacteria belonging to the family of *Lachnospiraceae* appears to correlate inversely with the Child-Pugh score[26].

Patients with hepatitis B-related HCC resulted in having a higher level of pro-inflammatory bacteria, including *Escherichia, Shigella* (*Enterobacteriaceae*) and *Enterococcus*, with a reduced amount of *Faecalibacterium, Ruminococcus* and *Ruminoclostridium* compared with healthy subjects[27].

The presence of *Veillonella parvula* and *Bacteroides caecimuris* seems to allow the differentiation of patients with NAFLD-related HCC from those with NAFLD-related cirrhosis only[28].

In a murine model of non-alcoholic steatohepatitis-induced HCC, *Clostridium, Corynebacterium, Bacillus, Desulfovibrio*, and *Rhodococcus* were highly represented in male mice and were associated with a higher risk of HCC development[29].

A particular abundance of bacteria, including *Clostridium* and CF231, were uniquely observed in HCC patients, independently from the cirrhosis stage or other environmental factors[30].

Interestingly, Western and Eastern people showed different gut microbiota, but they shared similar pathogenic microbial signatures[31]. Lu *et al*[17] analyzed the tongue coating microbiota in cirrhosis-related HCC patients. They found significantly higher biodiversity and dysbiosis in patients compared with healthy controls. Epsilonproteobacteria, Actinobacteria, Clostridia and *Fusobacteria* were increased in the patients, while there was a higher presence of Gammaproteobacteria and *Bacteroidetes* in the volunteers. In particular, the number of *Fusobacterium* and *Oribacterium* seems to differentiate HCC patients from healthy people[17].

Data about CCAs have been rarely reported. *Lactobacillus, Actinomyces, Alloscardovia* and *Peptostreptococcaceae* increased in stool samples from patients with intrahepatic CCA compared to those with HCC or healthy people. Furthermore, the overgrowth of bacteria belonging to the family of the *Ruminococcaceae*, together with higher levels of interleukin-4 (IL-4) and lower levels of IL-6, correlated with vascular invasion and,

Table 1 Different microbial composition in healthy and disease — chronic liver disease			
Chronic hepatitis B[21]	**Chronic hepatitis C [22]**	**NAFLD[3,24]**	**Cirrhosis[25,26]**
↓: *Alistipes, Bacteroides, Asaccharobacter, Butyricimonas, Ruminococcus, Clostridium cluster IV, Parabacteroides, Escherichia/Shigella*	↓: *Clostridiales Bifidobacterium*	↓: *Prevotella*	↓: Lachnospiraceae
↑: *Megamonas, Lachnospiraceae, Clostridium sensustricto, Actinomyces*	↑: Bacteroidetes, *Streptococcus, Lactobacillus*	↑: *Proteobacteria, Fusobacteria, Erysipelotrichaceae, Enterobacteriaceae, Lachnospiraceae, Streptococcaceae, Escherichia, Shigella*	↑:Enterobacteriaceae, Veillonellaceae, Streptococcaceae

↓/↑: Reduced or increased in patients compared to healthy volunteers; NAFLD: Non-alcoholic fatty liver disease.

Table 2 Different microbial composition in healthy and disease — hepatocellular carcinoma		
Hepatitis B-related HCC[27]	**NASH-related HCC[29]**	**Cirrhosis-related HCC[17]**
↓: *Faecalibacterium, Ruminococcus, Ruminoclostridium*		
↑: *Escherichia, Shigella, Enterococcus*	↑: *Clostridium, Corynebacterium Bacillus, Desulfovibrio, Rhodococcus*	↑: Epsilonproteobacteria, Actinobacteria, Clostridia, *Fusobacterium, Oribacterium*

↓/↑: Reduced or increased in patients compared to healthy volunteers; HCC: Hepatocellular carcinoma; NASH: Non-alcoholic steatohepatitis.

thus, with patients' prognosis. Furthermore, *Lactobacillus* and *Alloscardovia* are directly related to the tauroursodeoxycholic acid levels, and tauroursodeoxycholic acid levels showed a negative association with survival[1].

In a small study on bile samples taken during endoscopic retrograde cholangiopancreatography of patients with extrahepatic CCA, first episodes of bile duct stones and recurrent bile duct stones, Chen *et al*[32] showed a significant increase in the presence of Gemmatimonadetes, Latescibacteria, Planctomycetes and Nitrospirae in the patients with extrahepatic CCA. At the same time, they were absent in patients with the first episode of bile duct stones.

Similarly, in another different study on bile samples of patients with gallbladder cancer and gallbladder lithiasis, Tsuchiya *et al*[33] found the predominance of *Fusobacterium nucleatum, Escherichia coli* and *Enterobacter* species in cancer patients and a predominance of *Escherichia coli, Salmonella* species and *Enterococcus gallinarum* in the patients with gallbladder lithiasis.

Despite these data, it remains challenging to assess whether these modifications in microbiota composition are related to liver disease rather than the medications used in these patients[3]. Furthermore, some results may appear conflicting. Many reasons could explain these differences, including: (1) The different study models (*in vitro*, animal, human); (2) The influence of the environment, diet, lifestyle, and, eventually, other comorbidities; (3) The different methods to take and manage the samples; (4) The potential confounding effects of known or unknown factors; and (5) The relationship between testable and untestable microbiota.

Further large-scale human studies are needed. Complete knowledge of the relationship between progressive microbiota modifications and tumour initiation/progression may represent the basis for attractive therapeutic options, mostly for tumour prevention or, at least, for early diagnosis[7].

Pathogenetic pathways
Inflammation-liver fibrosis-cirrhosis-cancer: The pathway comprehending inflammation-liver fibrosis-cirrhosis-cancer is one of the most commonly recognised for HCC development[7]. Figure 1 summarises this pathway. On the contrary, most studies reported these alterations as a protective factor for liver metastasis development. However, some papers showed similar pathogenesis for both primary and secondary liver cancers[34].

In addition, bacterial dysbiosis causes a higher release of inflammatory cytokines and an increased intestinal barrier permeability[35].
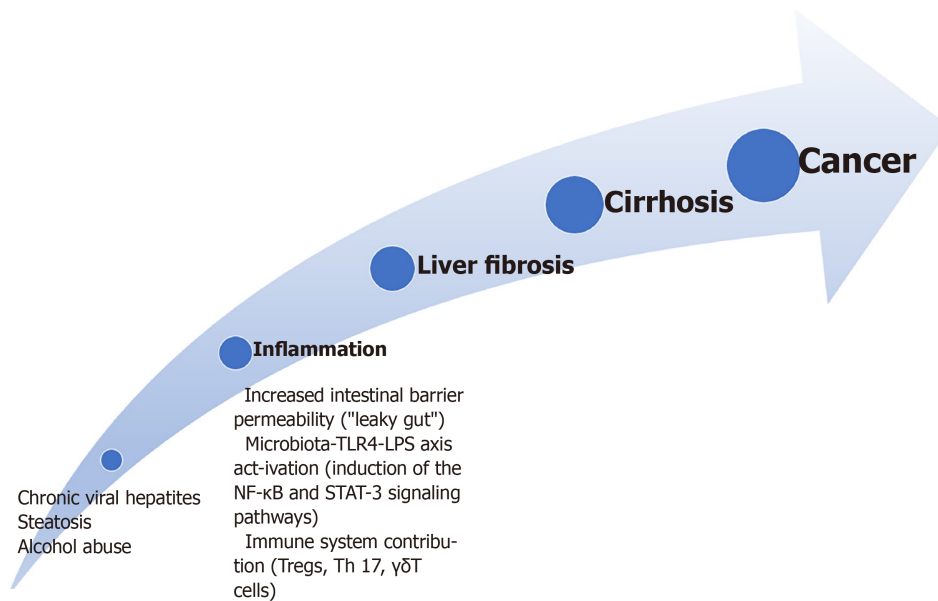
Figure 1 The inflammation-liver fibrosis-cirrhosis-cancer pathway. Dysbiosis, alcohol abuse and high-fat diet cause the alteration of the intestinal barrier permeability. With increased gut permeability, both microbiota and toxins (*e.g.*, endotoxins or flagellin) may reach the liver through the portal vein stimulating an inflammatory reaction. The toll-like receptor (TLR) 4 is expressed in the Kupffer, hepatic stellate, endothelial cells and hepatocytes. TLR4 activation causes the upregulation of the epidermal growth factor epiregulin that shows a mitogenic effect on hepatocytes causing hepatocellular carcinoma promotion. The lipopolysaccharide, a component of the gram-negative bacteria wall, binds to the transmembrane TLR4 causing the expression of the hepcidin showing an anti-apoptotic effect on the hepatocytes *via* the activation of the nuclear factor-κB and signal transducer and activator of transcription 3 signalling and the production of interleukin (IL)-17, IL-6, IL-1β, and tumour necrosis factor-α. Regulatory T cells can suppress the host antitumor immunity and cause tumour progression worsening CD8+ T cells function. T helper 17 cells showed pro-inflammatory effects through the secretion of IL-17A and IL-22 while γδT cells show pleiotropic activities. TLR: Toll-like receptor; LPS: lipopolysaccharide; NF: nuclear factor; Tregs: Regulatory T cells; Th: T helper; STAT-3: Signal transducer and activator of transcription 3.

Finally, there are growing data about the role of the microbiota toll-like receptor (TLR) 4 axis and of the lipopolysaccharide (LPS)-TRL4 axis in the development of inflammation and liver fibrosis from experimental and in clinical settings[36-38].

The TLR4 is expressed in the Kupffer, hepatic stellate, endothelial cells and hepatocytes[3]. TLR4 activation causes the upregulation of the epidermal growth factor epiregulin that shows a mitogenic effect on hepatocytes causing HCC promotion [39].

LPS, a component of the gram-negative bacteria wall, is a well-recognised inflammation inducer. It binds to the transmembrane TLR4 causing the expression of the Hepcidin (an inflammatory molecule), showing an anti-apoptotic effect on the hepatocytes *via* the activation of the nuclear factor-κB and signal transducer and activator of transcription 3 signalling and the production of IL-17, IL-6, IL-1β, and tumour necrosis factor (TNF)-α[3,40]. Furthermore, the binding between LPS and TLR4 in the Kupffer cell causes hepatocyte proliferation due to reducing TNF and IL-6 release[41].

Higher levels of LPS, together with a higher presence of bacterial unmethylated CpG DNA that binds to the TLR9, have been found in peripheral blood of patients with chronic liver disease and liver metastasis[42,43]. While there is little specific data about modification of the microbiota in the subgroup of viral hepatitis-related cirrhosis [44], a synergic action between TLR4 signalling pathway and hepatitis C infection in promoting HCC has been reported[45].

Alcohol intake may also cause increased blood LPS levels by increasing gram-negative bacteria numbers[6]. Furthermore, alcohol abuse may interfere with the tight junctions enabling intestinal translocation[46]. Similarly, a high-fat diet can increase LPS levels up to three-fold and increase intestinal barrier permeability[47].

On the other hand, mouse models of HCC demonstrated that the overexpression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) promoted by the microbiota might help reduce the inflammatory status through the modulation of the immune system. In particular, GM-CSF downregulated the pro-inflammatory cytokines IL-1β and IL-2 and TLR4 expression while increasing levels of the anti-inflammatory cytokines IL-4 and IL-10. Furthermore, mice with HCC and the overexpression of GM-CSF showed a different microbiota composition, with an increased anti-inflammatory genera *Roseburia*, *Blautia* and *Butyricimonass* and a significantly

reduced presence of *Prevotella*, *Parabacteroides*, *Anaerotruncus*, *Streptococcus*, *Clostridium* and *Mucispirillum*, together with modification in microbial metabolites. In particular, mice with HCC and GM-CSF overexpression showed higher biotin levels, reduced level of IL-2, and a low level of succinic acid levels together with an increased level of IL-4 and IL-10, thus showing a decreased intestinal barrier function and dysbiosis[48].

Alteration of the intestinal barrier has a role in the inflammation-cirrhosis-cancer pathway. The intestinal barrier is composed of a high turnover epithelium; a double layer mucus covers the epithelium and allows the microbes not to be carried away by the peristaltic movements; immunoglobulin A and defensins are secreted within the mucus layer; Paneth cells can produce antibacterial peptides; lastly, there is mucosa-associated lymphoid tissue. At the apical side of the cells, there are tight junctions that harbour signalling molecules[6,9,49].

The status of increased intestinal barrier permeability is known as "leaky gut"[8]. With increased gut permeability, microbiota and toxins, including endotoxins or flagellin, may reach the liver through the portal vein stimulating an inflammatory reaction[50]. Although the exact pathogenetic mechanism under this alteration is not yet wholly explained, both acute and chronic liver pathologies may impair the intestinal barrier function[3]. For example, excessive alcohol intake and its metabolism derive high toxic acetaldehyde levels that increase gut permeability, other than hepatocyte impairment[6]. Furthermore, mucus represents a nutrient for some bacteria, including *Akkermansia municiphila*, and, in the presence of a low-fibre diet, these species may overgrow, reducing the mucus thickness[51].

The immune system has a crucial role in cancer development, and the interplay with the gut microbiota is well-known. Regulatory T cells (Tregs) can suppress the host antitumour immunity and cause tumour progression, worsening CD8+ T cells function. High levels of Tregs have been found in the HCC patients' peripheral blood [52].

In vitro studies demonstrated that the microbiota of patients with NAFLD-related HCC, and not that of patients with NAFLD-related cirrhosis, stimulated a T cell immunosuppressive environment to reduce CD8+ T cells and an increased number of IL-10+ Tregs[53]. T helper (Th) 17 cells showed pro-inflammatory effects through the secretion of IL-17A and IL-22. Increased blood and tumour levels of Th17 have been found in HCC patients, and these levels were directly related to poor survival[54].

The γδT cells are getting attention because of their pleiotropy, with both Th1 and Th2 phenotypes, different behaviour in distinct liver pathologies and interplay with the microbiota[12,13,55]. γδT cells are scarcely represented in the peripheral blood but are highly expressed in the liver[12,56].

γδT cells are pathogenic in patients affected by hepatitis C infection and worsen the steatohepatitis in NAFLD patients[12,57]. In early-stage cirrhotic patients, γδT cells produce IL-17 causing fibrosis by stimulating the stellate and Kupffer cells[58]. On the contrary, in the late stages, γδT cells limit fibrosis and induce stellate cell apoptosis[12,59]. In vitro studies reported the cytotoxic activity of the γδT cells through the secretion of IFN-γ, TNF-α, perforin, and granzymes in the presence of HCC[12,60]. The ratio of peritumoural HSC to γδT cells resulted in a prognostic factor for resected HCC [61]. Enhancing this immunity could represent a potential therapeutic target[12].

γδT cells also play a role in intestinal barrier homeostasis and interplay with the microbiota[12,55]. Tumour-associated antigens elicit antitumour T lymphocyte response. There are many tumour-infiltrating lymphocytes in the interface between HCC and liver (CD4+ T cells) or within the tumour (CD8+ T cells), but tumour cells may induce Tregs, causing immunosuppression[62]. Interestingly, no differences in the tumour-infiltrating pattern have been found between HCC and CCA[63].

Neutrophils can induce cancer cell proliferation and remodel the extracellular matrix. High levels of neutrophils have been found in metastatic sites, including the liver[64].

The pathways involving the peroxisome proliferator-activated receptors (PPARs) could have a role in the HCC development. Published data reported their protective role in chronic liver disease development through an interplay with the microbiome and their ability to reverse leaky gut conditions and dysbiosis[65].

Li *et al*[66] reported that the tumour-released secretory protein cathepsin K (CTSK) represented a link between altered gut microbiota and metastatic behaviour of colorectal cancer. In particular, experiments *in vitro* and on mice models showed a direct correlation between *Escherichia coli*, high LPS levels, CTSK overexpression (stimulated by the LPS) and liver metastasis compared to the control group. Furthermore, the CTSK could activate an m-TOR-dependent pathway by binding to TLR4 and inducing macrophages' M2 polarisation. These macrophages could promote cancer metastasisation through the secretion of IL-10 and IL-17 and the activation of

the nuclear factor-κB pathway. The CTSK silencing or the administration of the CTSK inhibitor Odanacatib abolished colorectal cancer cell migration[66]. Consequently, CTSK could represent a therapeutic target and a biomarker for the diagnosis and prognosis of metastasis from colorectal cancer. Similarly, an engineered LPS trap protein showed the ability to reduce the chance of colorectal cancer liver metastasis development[67].

More generally, enhancing immune activity may represent a potential therapeutic target.

Microbial metabolites: Microbiota metabolites may also have a causal role in liver cancer development (Figure 2). Trimethylamine (TMA) is an example of microbial metabolites involved in the pathogenesis of NAFLD[8]. Experimental studies on mice showed that excessive intake of soluble dietary fibre is associated with excessive proliferation of fibre-fermenting bacteria, including *Clostridium* that produces short-chain fatty acids (SCFAs), which showed immunomodulatory functions[68,69]. Excessive levels of SCFAs, particularly butyrate, promote inflammation having a causal role in cholestasis, NAFLD, and HCC development, as reported in metabolomic studies[8,53,70]. Faeces and serum levels of butyrate resulted higher in patients with NAFLD-related HCC than those with NAFLD-related cirrhosis. Furthermore, butyrate can impair cytotoxic CD8^{+} T cell activity[53]. Conversely, propionate seems able to inhibit cancer progression[71]. Gut microbiota metabolises choline into several metabolites, including TMA, that the liver metabolises into TMA oxide, and TMA oxide is related to liver inflammation[72].

The intestinal microbiota also has a fundamental role in bile acids (BA) production and recycling. BAs are synthesised by the liver and metabolised by gut bacteria into secondary BAs, which are sensed by the epithelial cells' farnesoid X-activated receptor (FXR). FXR provides feedback to the liver[73]. BA excess is another recognised pathogenetic factor in carcinogenesis. Secondary BAs can cause direct DNA damage by producing reactive oxygen species, inhibiting tumour suppressor genes, and activating oncogenes[74]. Furthermore, the deoxycholic acid, a secondary BA, binding to the TLR2 in hepatic stellate cells, can induce cyclooxygenase-2 expression, enhancing the inhibition of the antitumour activity prostaglandin E2-mediated[75]. Obesity can increase BA conversions[30]. On the contrary, the inhibition of 7α-dehydroxylation responsible for secondary BA metabolization is associated with a lower incidence of HCC in mice[76]. In both animal models and humans, conversion of primary BA to secondary BA is also negatively related to NKT cell infiltration. NKT cells can control both primary and secondary cancer development[77,78].

Complete knowledge of these pathways may allow the design of further studies on several appealing preventive options, including agents able to reestablish a correct balance between the different microbial species, selective agents against pathogenic bacteria, inhibitors of bacterial pathogenic metabolites production and gut barrier improvement[3].

Specific pathways in CCA: Infection of *Opisthorchis viverrini* and *Clonorchis sinensis* is a well-known risk factor for CCA development. Besides direct mechanical damage on the biliary tract epithelium and sustained inflammation, dysbiosis in local microbiota with bacterial translocation from the duodenum may contribute to CCA development [79].

CANCER TREATMENT

Surgical resection is the elective treatment for HCC, CCA or liver metastasis from several primary cancers, mostly colorectal adenocarcinoma, whenever possible. In the setting of advanced disease, chemotherapy and novel pharmacologic treatments, including immunotherapy and targeted therapies, should be preferred.

Immunotherapy
Immune checkpoint inhibitors, including the tremelimumab, a monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4, and nivolumab or pembrolizumab that are monoclonal antibodies against programmed cell death ligand 1[7], show a response rate in HCC patients that is reported to be up to 20%[80]. Immunotherapy may also be combined with locoregional therapies, showing a synergistic effect[81]. Tremelimumab showed greater efficacy in hepatitis C-related HCC since it can enhance CD8^{+} T cell infiltration and, consequently, lower the viral load[81].
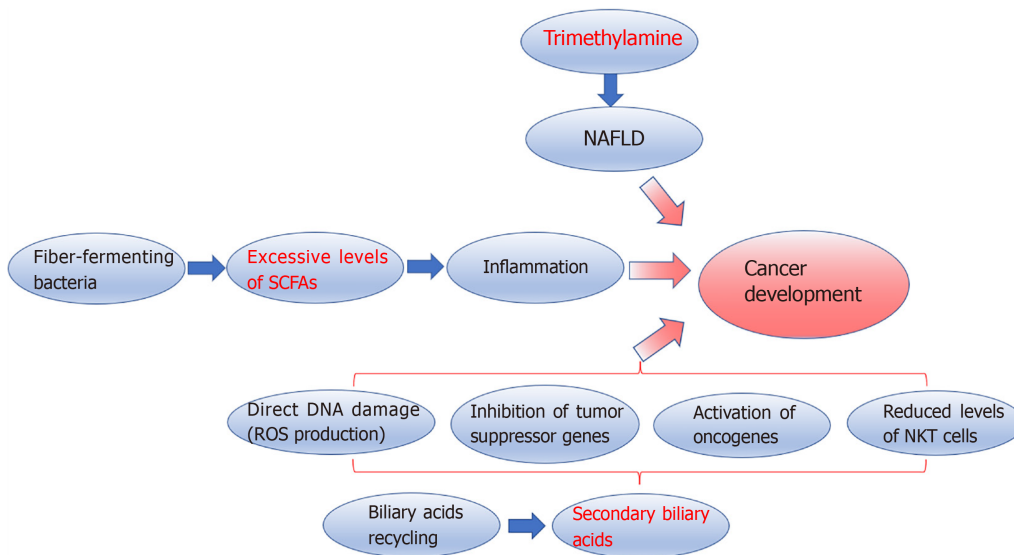
Figure 2 Microbiota metabolites causal role in liver cancer development. Gut microbiota metabolises choline into several metabolites, including trimethylamine (TMA) that the liver metabolises into TMA oxide, and TMA oxide is related to liver inflammation. Excessive intake of soluble dietary fibre is associated with excessive proliferation of fibre-fermenting bacteria, including *Clostridium* that produces short-chain fatty acids. Excessive levels of short-chain fatty acids, particularly butyrate, promote inflammation. The intestinal microbiota has a fundamental role in bile acid (BA) production and recycling. BAs are synthesised by the liver and metabolised by gut bacteria into secondary BAs, which are sensed by the farnesoid X-activated receptor of the epithelial cells. Farnesoid X-activated receptor provides feedback to the liver. Secondary BAs can cause direct DNA damage by producing reactive oxygen species, inhibiting tumour suppressor genes, activating oncogenes, and negatively affecting natural killer T cell infiltration. Natural killer T cells can control both primary and secondary cancer development. NAFLD: Non-alcoholic fatty liver disease; SCFAs: Short-chain fatty acids; NKT cell: natural killer T cell; ROS: Reactive oxygen species.

Since gut microbiota seems to impact these systemic treatments' efficacy, the microbiota's modulation to enhance treatments' response appears as a promising therapeutic target[7]. It has been reported that while antibiotics may reduce the efficacy of the checkpoint inhibitors lowering the gut microbiota biodiversity, there are specific overrepresented taxa associated with more significant responses[82].

Zheng *et al*[83] showed higher levels of *Akkermansia muciniphila* and bacteria from the family of the *Ruminococcaceae* in faecal samples of anti-programmed cell death ligand 1 immunotherapy responders. Conversely, in non-responders patients, higher *Proteobacteria* levels were found from week 3 of therapy, and a predominance of *Proteobacteria* was found at week 12[83].

The use of epigenetic drugs, including DNA methyltransferase enzymes-mediated hypermethylation and histone deacetylases-mediated histone modification, is under evaluation showing promising results in combination with conventional immunotherapy in murine models[84].

The microbiota evaluation may help in better selecting the candidate for a specific treatment hypothesising the response rate. Furthermore, the possibility to target both innate and adaptive immune systems could represent an appealing therapeutic option. In particular, actions on the innate arms may allow improvements in cytotoxic effect, stimulate the adaptive immune system and reduce the tumour-promoting effect[10].

Anticancer peptides

Antimicrobial peptides (AMPs) are constitutively or inducibly expressed in the tissues, which may be in contact with pathogens[85]. AMPs are present in the great majority of vertebrates, invertebrates and vegetables. The antimicrobial effect of the AMPs can be exerted through cellular membrane damages, inhibition of cellular replication and through their immunomodulatory abilities[85-87].

Some AMPs showed anticancer properties, also causing cancer cell apoptosis. Furthermore, the healthy or cancer cell membrane composition differs, and tumour cells are more easily damaged by the anticancer peptides (ACPs). ACPs can interact with LPS or other bacterial products resulting in an anti-inflammatory effect[50]. The use of ACPs, including TLR agonist and tumour-associated antigens-derived peptides, may represent a promising therapeutic option in HCC treatment[50,63,88]. To be effective, some ACPs would have to be delivered. Delivery systems may include peptide-derived vaccines, nanoparticles and liposomes, each related to advantages and limitations[50].

More specific details about the design and the delivery of these molecules are reviewed elsewhere[50,89].

Microbiota, immune system and treatments response

Sorafenib is a tyrosine kinase inhibitor worldwide used in advanced HCC that can suppress abnormal cell proliferation and angiogenesis. Microbiota can influence sorafenib's blood levels, affecting enterohepatic recirculation. Drug blood levels are related to the chance of suffering from the side effects[90]. Two common side effects include diarrhoea and hand-foot syndrome and require reducing the administered drug[91]. Butyric acid showed a protective action toward the inflamed intestinal mucosa by stimulating the Tregs and IL-10 secretion. Increased *Butyricimonas*, a butyric acid producer, have been found in patients not experiencing diarrhoea[91,92]. Dysbiosis and increased levels in the gut of bacteria typically found in the mouth (*Veillonella, Bacillus*, *Enterobacter*) have been found in patients not experiencing the hand-foot syndrome[91].

On the contrary, reduced Treg levels allow the achievement of better outcomes through the enhancement of the CD8^{+} T cell antitumour activity. Furthermore, the baseline CD4^{+} T effector/Tregs ratio has a prognostic value[93].

FUTURE PERSPECTIVES: OPTIONS FOR CANCER PREVENTION

Complete knowledge of the interaction between gut microbiota and liver cancer steps may help design new and tailored therapeutic options[3].

Microbiota modulation

The only actual method to prevent primary liver cancer development is to prevent and cure the underlying chronic liver disease whenever present. Although the gut microbiota role in these pathologies is still not wholly understood, microbiota modulation may be a promising target to reduce cancer. There are conditions in which microbiota modulation would have a marginal role, including perinatal viral hepatitis infections or cancers occurring on "healthy" livers[3].

The environment, diet, lifestyle, the use of antibiotics or pre/probiotics and several diseases may change the gut microbiota composition. It has been reported that a vegetable-enriched diet may lower the incidence of primary liver cancers, mainly in the male population. Conversely, a high-fat diet favours gram-negative bacteria overgrowth with increased LPS levels, and a high-fructose diet reduces the population of *Bifidobacterium* and *Lactobacillus*[94,95].

On a theoretical basis, using non-selective antibiotics may lower the entire gut microbiota population reducing the chance of bacterial translocation and the induction of a pro-inflammatory status. Treatments with selective antibiotics, if available, could reduce only those species producing cancer-promoting metabolites[3,76].

Experiments on rats demonstrated that non-absorbable antibiotic administration could positively affect steatosis and the inflammatory status. Studies on murine models showed that metronidazole administration might decrease the risk of cholestasis and HCC development by reducing the population of bacteria production of butyrate, which shows a health-promoting effect in other circumstances[70]. Similarly, vancomycin may reduce gram-positive bacteria producing secondary BAs [96]. The administration of the combination of ampicillin, neomycin, metronidazole and vancomycin showed a more powerful effect against late stages of HCC carcinogenesis compared to earlier stages[39]. On the contrary, penicillin intake was reported to be related to a higher risk of HCC development in rats[97].

The non-absorbable oral norfloxacin and rifaximin showed a good safety profile and microbiota-related positive effects in cirrhotic patients and mice with HCC[3]. An experimental study on the subcutaneous implantation model of thymoma on mice showed that an antibiotic combination of vancomycin, neomycin and primaxin reduced the chance of developing liver metastasis, though without affecting the primary tumour[77].

Despite the lack of much data on humans, it is reasonable that a long-term antibiotic assumption may be burdened with several side effects, including depletion of beneficial bacteria, kidney damages or antibiotic resistance[3]. Consequently, further studies are needed.

The use of probiotics may help resolve dysbiosis, increase the number of bacteria with favourable properties, improve the intestinal barrier functions, absorb carcinogens and interact with the immune system, causing a reduction of Th17[98]

cells. While ongoing human trials evaluate the effects of probiotic administrations in patients suffering from chronic liver diseases, evidence-based data about HCC comes only from murine models[3].

The assumption of the so-called VSL#3, a mixture of *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus delbrueckii* subspecies and *Bulgaricus* seemed to have positive effects on the pathway inflammation-fibrosis-HCC development being associated with an enriched population of *Prevotella* and *Oscillibacter* and with Th12 cell differentiation[97,99].

Similarly, prebiotics are substances able to stimulate the overgrowth of beneficial bacteria. Some examples include prebiotics of fructooligosaccharides reported to re-establish eubiosis, improve intestinal barrier function and reduce inflammation. Lactulose is related to an overgrowth of *Bifidobacterium* that shows a healthy behaviour by reducing LPS serum levels. Therapies with synbiotics are based on the combined use of probiotics and prebiotics[100].

Finally, faecal microbiota transplantation (FMT) is another treatment option that can reduce the risk of HCC development. It has been reported that FMT may reduce steatohepatitis in mice[57]. However, several concerns have been raised, including the possibility of a long-lasting efficacy and the risk of infection transmission. The opportunity to transplant only beneficial bacteria could represent an appealing option [3].

TLR4 antagonists

The LPS-TLR4 axis has a crucial role in the inflammation-fibrosis-cirrhosis-cancer pathway. Consequently, several antagonists of the TLR4 have been proposed. Some examples include polymyxin B, able to bind and sequestrate LPS; E5531 or eritoran, molecules interacting with other steps of this signalling pathway; resatorvid, able to target the TLR4; thalidomide, a TLR inhibitor[3]. Further details are not the object of this review and can be found elsewhere[3,88].

However, the primary concern is the consequent status of immunosuppression that could be detrimental in patients with chronic liver disease or HCC[3]. Furthermore, the results of published studies are sometimes controversial due to the complexity of the known and unknown interactions[88]. Consequently, further long-term studies are needed.

PPARs agonists

Several studies reported the beneficial effects of both natural and synthetic PPARs agonists in chronic liver disease development through microbiota modulation. Although specific studies on cancer progression are lacking, targeting the PPARs could represent, at least, a cancer prevention strategy. Further details can be found elsewhere[65].

Gut barrier function improvement

The integrity of the gut barrier is vital for healthy individuals. A high caloric diet seems to impair the intestinal barrier[101]. Conversely, physical exercise improves short-term and long-term gut permeability through effects on the immune system and the microbiota, increasing the *Bacteroidetes*/*Firmicutes* ratio[6,102].

Cisapride is a prokinetic medication that resulted in reducing both bacterial overgrowth and translocation, fastening the intestinal transit time[103]. Some nonselective β-adrenergic blockers showed similar properties.

BA influence the function of the gut barrier, and the FXRs are crucial in BA synthesis, other than in the regeneration of the liver and tumour growth suppression. The obeticholic acid is an FXR agonist and showed beneficial effects on damaged mucosa and reduced the gut barrier permeability, the inflammatory status, bacterial overgrowth and preventing the progression from non-alcoholic steatohepatitis to other complications, thus becoming an attractive potential treatment option[104].

Excessive TNF production is associated with increased gut barrier permeability reducing the tight junction proteins[105]. Consequently, anti-TNF-based therapies could represent potential therapeutic options, but as previously stated, the related immunosuppression may be detrimental[3]. However, n-3 polyunsaturated fatty acids (PUFA) showed anti-inflammatory properties in experimental models reducing the level of TNF and IL-1, thus resulting in an appealing option[106,107]. Furthermore, *in vitro* experiments demonstrated the ability of the n-3 PUFA to block β-catenin and cyclooxygenase-2[108]. On the contrary, n-6 PUFA seems related to a pro-inflammatory status[109].

Early diagnosis

There is a continuous search for new, non-invasive biomarkers for diagnosis, and microbiota seems promising even in this field. Since there are different microbial signatures along with disease progression, microbial samples could represent appealing non-invasive biomarkers for an early diagnosis[16].

Furthermore, Ponziani *et al*[110] demonstrated an inverse relation between *Akkermansia* and *Bifidobacterium* and the well-known inflammatory marker calprotectin. Analysis on faecal samples of patients with primary liver cancers showed a significant link between *Veillonella* and alpha-fetoprotein levels together with a negative connection between *Subdoligranulum* and alpha-fetoprotein levels[50].

Along with faecal samples, analysis of the tongue microbiota could represent another non-invasive biomarker. In particular, *Oribacterium* and *Fusobacterium* presence could differentiate HCC patients from healthy subjects[17].

Jia *et al*[1] reported that the plasma-stool ratio of two BAs, tauroursodeoxycholic and glycoursodeoxycholic acids, demonstrated the ability to identify patients with intrahepatic CCA from those with HCC or healthy people with an area under the curve of 0.801 and 0.906, respectively[1]. Although some methodological and cause-effects concerns have been raised[111], this potential biomarker is appealing. Again, further studies are needed to obtain new markers that could be used independently or within algorithms[18,111].

CONCLUSION

In conclusion, a growing body of literature demonstrates a pathogenetic role of the gut microbiota-immunity axis in liver cancer development. Although there is an ongoing rapid development of metagenomic science, definitive and complete knowledge of this process is still far from being wholly acquired. However, targeting microbiota and the immune system may represent appealing therapeutic options alone or boost conventional treatments. Finally, the gut microbiota signature evaluation could represent a potential novel, non-invasive biomarker for early diagnosis.

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Role of mammalian target of rapamycin complex 2 in primary and secondary liver cancer

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Abstract

The mammalian target of rapamycin (mTOR) acts in two structurally and functionally distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Upon deregulation, activated mTOR signaling is associated with multiple processes involved in tumor growth and metastasis. Compared with mTORC1, much less is known about mTORC2 in cancer, mainly because of the unavailability of a selective inhibitor. However, existing data suggest that mTORC2 with its two distinct subunits Rictor and mSin1 might play a more important role than assumed so far. It is one of the key effectors of the PI3K/AKT/mTOR pathway and stimulates cell growth, cell survival, metabolism, and cytoskeletal organization. It is not only implicated in tumor progression, metastasis, and the tumor microenvironment but also in resistance to therapy. Rictor, the central subunit of mTORC2, was found to be upregulated in different kinds of cancers and is associated with advanced tumor stages and a bad prognosis. Moreover, AKT, the main downstream regulator of mTORC2/Rictor, is one of the most highly activated proteins in cancer. Primary and secondary liver cancer are major problems for current cancer therapy due to the lack of specific medical treatment, emphasizing the need for further therapeutic options. This review, therefore, summarizes the role of mTORC2/Rictor in cancer, with special

focus on primary liver cancer but also on liver metastases.

Key Words: Mammalian target of rapamycin; Mammalian target of rapamycin complex 2; Rictor; Liver cancer; Liver metastases; Hepatocellular carcinoma; Cholangiocellular carcinoma

Core Tip: Mammalian target of rapamycin complex 2 (mTORC2) has recently gained importance in cancer research, as it is one of the key effectors of the PI3K/AKT/mTOR pathway and stimulates cell growth, cell survival, metabolism, and cytoskeletal organization. Rictor, the central subunit of mTORC2, was found to be upregulated in different kinds of cancers and is associated with a bad prognosis. We herein discuss the implications of mTORC2 in primary and secondary liver cancer.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is an atypical serine/threonine kinase that controls cell survival, proliferation, and metabolism through phosphorylation of its downstream targets. It acts in two structurally and functionally distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), and can be activated by several stimulating factors as hypoxia, insulin, growth factors, or dysregulation of PI3K/Akt signaling[1,2]. However, upon deregulation, activated mTOR signaling is implicated in the hallmarks of cancer and associated with increased cell survival, uncontrolled cell proliferation, metabolic reprogramming, and aberrant angiogenesis[3], which makes it a promising target in anticancer therapy.

The incidence of primary liver cancer, such as hepatocellular carcinoma (HCC) and intrahepatic cholangiocellular carcinoma (iCCC), is increasing worldwide. Systemic therapy options are limited for both cancer entities. Surgery offers the only chance for cure, although only a minority of patients with HCC or iCCC are eligible for resection. Moreover, the liver is one of the most frequent sites of metastases development. Indeed, in most cancer entities liver metastasis is associated with a dramatic decline of patient's prognosis, further emphasizing the fundamental impact of this site. A deeper understanding of processes involved in either primary or secondary liver cancer is therefore urgently needed[4-6].

Due to the availability of (more or less) selective mTORC1 targeting agents such as rapamycin, the role of mTORC1 in cancer has been extensively studied for decades. In contrast, mTORC2 was less intensively analyzed, mainly because of the lack of selective pharmacologic inhibitors. However, the last decade has brought up several studies suggesting a role for mTORC2 in cancer. For instance, Rictor (rapamycin insensitive companion of TOR), the central subunit of mTORC2, was found to be upregulated in different kinds of cancer and is associated with impaired prognosis[7-10]. Evidence from primary and secondary liver cancer is summarized in Table 1. In addition, involvement of mTORC2/Rictor in a plethora of processes implicated in tumor growth and also metastasis have been reported[11]. Therefore, the present review summarizes the current knowledge regarding the role of mTORC2 in cancer with focus on primary and secondary liver cancer.

MTOR COMPLEXES AND SIGNALING

Although mTOR acts through the different complexes mTORC1 and mTORC2, both have several subunits in common: the catalytic kinase mTOR, the scaffolding protein

Table 1 Overexpression of mammalian target of rapamycin complex 2 and its mediators determine clinical outcome

		mTORC2 mediator	Associated with	Measured by	Ref.
Primary liver cancer	HCC	p-AKT^{Ser473} overexpression	poor outcome ($P < 0.02$)	IHC	Hu *et al*[59]
		Rictor overexpression	Reduced OS ($P = 0.0029$)	mRNA expression	Xu *et al*[60]
		Rictor overexpression	Reduced RFS ($P = 0.016$)	IHC, mRNA expression	Kaibori *et al*[61]
	iCCC	p-AKT1 overexpression	Improved OS ($P = 0.0137$)	IHC	Lee *et al*[76]
	CRLM	Data only available for primary CRC			
		Rictor expression	Increasing tumor stage	mRNA expression	Gulhati *et al*[49]
		Rictor expression	Increasing tumor stage	IHC, mRNA expression	Shuhua *et al*[81]
		Rictor expression	Reduced OS ($P = 0.0004$)	IHC	Wang *et al*[10]
Secondary liver cancer	Breast cancer liver metastases	Data only available for invasive ductal breast carcinoma			
		Rictor expression	Lymph node metastasis	IHC	Zhang *et al*[90]
	Melanoma liver metastases	Rictor positivity (primary tumor)	Reduced OS ($P = 0.018$)	IHC	Liang *et al*[100]
		Rictor expression	Tumor stage/metastatic disease	mRNA expression	Schmidt *et al* [101]
	Renal cancer liver metastases	No data available			
	Gastric cancer liver metastases	Data only available for gastric cancer			
		Rictor, p-AKT^{Ser437} expression	Tumor stage, reduced RFS and OS	IHC	Bian *et al*[7]
		Rictor expression	Tumor stage, reduced RFS and OS ($P = 0.012$, $P = 0.014$)	IHC	Bian *et al*[110]
	Pancreatic cancer liver metastases	Data only available for pancreatic cancer			
		Rictor expression	Reduced OS	IHC	Schmidt *et al*[9]

CRC: Colorectal cancer; CRLM: Colorectal liver metastases; HCC: Hepatocellular carcinoma; iCCC: Intrahepatic cholangiocarcinoma; IHC: Immuno-histochemistry; mTORC2: Mammalian target of rapamycin complex 2; OS: Overall survival; RFS: Recurrence-free survival.

mLST8, the regulatory subunit DEPTOR, and the stabilizing complex Tti1/Tel2. The distinct subunits of mTORC1 are Raptor and PRAS40. While Raptor is important for mTORC1 substrate specificity, stability, and regulation, PRAS40 acts as negative regulator of mTORC1. Activation of mTORC1 depends on nutrient (*e.g.,* amino acids) and growth factor (*e.g.,* insulin) signaling through the PI3K/AKT and Ras-MAPK cascades. Phosphorylated AKT (*via* the PI3K/AKT pathway) or ERK and RSK (*via* the Ras-MAPK cascade) inhibit the TSC1/2 complex, which in turn triggers RHEB-mediated activation of mTORC1 leading to phosphorylation of its substrates 4EBP1 and S6K1. The downstream effectors act as key regulators of cap-dependent and cap-independent mTORC1 translation[3] and regulate translation and transcription of different target genes (*e.g.,* HIF1α, *etc.*) thereby being implicated in cell growth, proliferation, and metabolism[12].

mTORC2 consists of its specific subunits Rictor and mSin1. Similar to Raptor, Rictor controls mTORC2 stability, subcellular localization, and substrate identification. It is essential for mTORC2 function, as silencing Rictor leads to significant inhibition of AKT, the key substrate of mTORC2. mSin1 negatively regulates mTORC2 until PI3K-mediated growth factor signaling locates mSin1/mTORC2 to the plasma membrane and relieves its inhibition. In turn, PI3K-generated PIP3 activates mTORC2. Activated mTORC2/Rictor leads to phosphorylation of AKT at the Ser473 residue. AKT can also be phosphorylated at the Thr308 residue by PDK1, which is in turn attracted by PIP3. Importantly, AKT is one of the most frequently activated proteins in cancer, and its full activity is only achieved if both sites are phosphorylated[13,14]. On a functional

level, AKT is involved in many processes. Association with cell migration, invasion, increased tumor growth, and cell survival while inhibiting apoptosis and promoting proliferative processes including glucose uptake and glycolysis have been described [15,16]. Additional substrates of mTORC2 are the AGC kinases like the PKC (*e.g.,* PKC α, PKCδ, PKCε) family, which control tumorigenesis, cell migration, and cytoskeletal remodeling, and SGK isoforms that are implicated in cell survival and resistance to chemotherapy.

Along with the essential role of Rictor for mTORC2 functioning, it also acts independently of mTORC2. Thr1135, one of the 37 phosphorylation sites of Rictor, was shown to be stimulated directly by growth factor signaling and to be sensitive to rapamycin, as it is targeted by S6K1, one of the downstream effectors of mTORC1[17, 18]. This mechanism is assumed to represent a regulatory link between mTORC1 and mTORC2 signaling and to be part of a reciprocally influenced feedback loop mechanism[19,20]. Moreover, Rictor was also described to be associated with complexes exhibiting oncogenic and tumor suppressor properties. For example, Rictor forms a complex with ILK, which is crucial for TGFß1 mediated epithelial-to-mesenchymal transition (EMT) and cancer cell survival[16,21]. In contrast, the combination of Rictor with PCD4 acts in an anti-oncogenic manner, as renal cancer cells showed reduced metastatic ability[22].

IMPLICATIONS OF MTORC1 IN CANCER

Besides being implicated in many physiological processes such as glucose and lipid homeostasis, adipogenesis, maintaining muscle mass and function, brain and immune function, deregulation of mTORC1 signaling is not only associated with diseases such as diabetes, neurodegeneration, and cancer. As described above, mTORC1 is activated by the oncogenic pathways PI3K/Akt and Ras-MAPK cascade. However, the oncogenic pathways are frequently mutated, resulting in hyperactivation of mTORC1, which is found in many human cancers[1]. Hyperactivated mTORC1 and its association with tumorigenesis are also seen in tuberous sclerosis, a familial cancer syndrome defined by the loss of the TSC1/2 complex, a negative regulator of mTORC1. Downstream of mTORC1, its role in carcinogenesis is linked to metabolic reprogramming in cancer cells. One example is the Warburg effect; aerobic glycolysis is controlled by mTORC1 *via* increased translation of HIF1α that in turn regulates the expression of glycolytic enzymes[23]. mTORC1 is also associated with upregulation of genes involved in lipogenesis by activation of the transcription factor SREBP1 through phosphorylation of Lipin1 and S6K1[24,25]. The latter was shown to be a major mechanism to promote growth and proliferation in breast cancer cells[26]. mTORC1-mediated phosphorylation of S6K1 is not only involved in lipogenesis but also in purine and pyrimidine synthesis leading to a rapid DNA duplication in cancer cells [27,28]. Aside from its control in cancer cell metabolism, mTORC1 is also involved in the regulation of autophagy and macropinocytosis.

The implications of mTORC1 in cancer are widely studied because of the availability of rapamycin as a selective mTORC1 inhibitor. However, rapamycin analogs (rapalogs) have only shown limited efficacy in cancer therapy. While inhibition of mTORC1 by rapamycin blocks phosphorylation of S6K1, phosphorylation of 4EBP1 is not fully blocked[29]. Therefore, 4EBP1-regulated translation of proteins involved in tumorigenesis is not inhibited. Another reason for the limited efficacy of rapamycin and rapalogs is explained by compensatory upregulation of AKT through phosphorylation of its Thr308 and Ser473 residue[30,31] as inactivated S6K1 no longer prevents suppression of insulin-PI3K signaling[32,33]. Moreover, treatment with rapalogs may increase micropinocytosis and autophagy leading to enhanced cell proliferation and survival[34,35].

IMPLICATIONS OF MTORC2 IN CANCER

Compared with mTORC1, much less is known about the role of mTORC2 in cancer, although existing data suggest mTORC2 to be of importance. Particularly, it is one of the key effectors of the PI3K/AKT/mTOR pathway and stimulates cell growth, cell survival, metabolism, and cytoskeletal organization.

Tumorigenesis

As described above, AKT is the key downstream target of mTORC2 signaling and one of the most commonly activated proteins in cancer[36,37] with its isoforms AKT1 and AKT2 being the main effectors in tumorigenesis[38]. With more than 200 AKT substrates, the different isoforms seem to have unique roles in tumorigenesis. While AKT1 increases tumor development and reduces tumor invasion, the expression of AKT2 has the opposite effect[39]. Frequently, hyperactivated AKT is found in different kinds of cancers. For example, hyperactivation of AKT caused by a somatic mutation was found to induce B-cell lymphoma, in contrast to wild-type AKT[40]. The somatic mutation was also found in breast, colorectal, and ovarian cancer. In addition to somatic mutations, hyperactivation of AKT may also occur because of activating upstream mutations in PI3K or deletions in PTEN, a tumor suppressor[41]. AKT has several substrates in common with SGK family members, another downstream target of mTORC2. Hence, SGK is similarly implicated in cell growth and proliferation[42]. Its isoform SGK3 is dependent on PDK1 signaling to induce tumor growth and adopted the role of AKT in tumorigenesis in PI3K mutated cancer cells[43]. AKT and SGK, which are different isoforms of PKC, are also involved in tumorigenesis. PKC-ε, PKC-λ/ι, and PKC-β are known oncogenes with PKC-λ/ι and PKC-β for example being involved in colon carcinogenesis[44,45]. Mechanisms by which mTORC2 is involved in tumorigenesis are shown in Figure 1.

Metastasis

Cell migration and invasion are the two key components of metastasis that are affected by mTORC2 through different pathways. On the one hand, phosphorylation of AKT leads to activation of Rac1 through activation of Tiam1[46]. On the other hand, Rac1 is also upregulated by the suppression of its inhibitor RhoGD12, not only through AKT but also independent of AKT through PKCα activation[46,47] (Figure 2). Rac1 and RhoA are small GTPases known to have crucial roles in actin cytoskeletal rearrangement and cell migration, mainly by stimulating lamellipodia formation[48]. Upon mTORC2 knockdown, expression of Rac1 and RhoA is decreased, leading to a reduction of colorectal metastasis[49]. AKT1 is thus the AKT isoform implicated in metastasis. Silencing only AKT1 and not AKT2 reduces migration and invasion[50]. The gain of invasive behavior is explained by the EMT, and was reversible upon mTORC2 and mTORC1 inhibition, which was followed by an increase in cell-cell contacts and E-cadherin; vimentin, SMA, fibronectin, and MMP9 decreased[49].

Metabolic reprogramming

Metabolic reprogramming is a hallmark of cancer, and it allows tumor cells to receive and maintain their energy supply for rapid tumor growth[51]. mTORC2 was shown to control c-Myc, a regulator of the Warburg effect, by phosphorylation of class IIa HDAC and acetylation of FoxO in both an AKT dependent and independent manner, thereby increasing glycolysis[52]. Increases of glucose and acetate cause acetylation of Rictor, which in turn maintains mTORC2 signaling[53]. Besides glycolysis, mTORC2 also controls cystine uptake and glutathione metabolism. Phosphorylation of SLC7A11 thereby allows tumor cells to focus mainly on survival rather than proliferation if the extracellular environment changes[54]. In addition to energy supply, metabolic reprogramming can also be involved in drug resistance, as mTORC2 was shown to act as a central link between glucose metabolism and resistance to EGFR tyrosine kinase inhibitors[55].

Drug resistance

As mentioned above, metabolic reprogramming is one of the mechanisms of mTORC2-mediated drug resistance in cancer cells. Glucose metabolism has been linked not only to resistance to EGFR tyrosine kinase inhibitors[55] but also has caused Rictor acetylation that can be achieved by either glucose or acetate. Rictor acetylation induces auto-activation of mTORC2 signaling despite the absence of upstream growth factor signaling leading to resistance to EGFR-, PI3K- and AKT-targeted therapies[56]. Furthermore, Rictor utilizes inhibition of apoptosis by activation of NF-κB as another mechanism to develop resistance to chemotherapy[57]. Interestingly, the process could be overcome only by Rictor and not by AKT inhibition, suggesting that NF-κB is an AKT-independent mTORC2 downstream effector. In contrast, the positive feedback mechanism between amplified Rictor, known to occur in many cancers, and AKT leads to constant AKT activation, inducing not only tumor progression but also drug resistance[11].
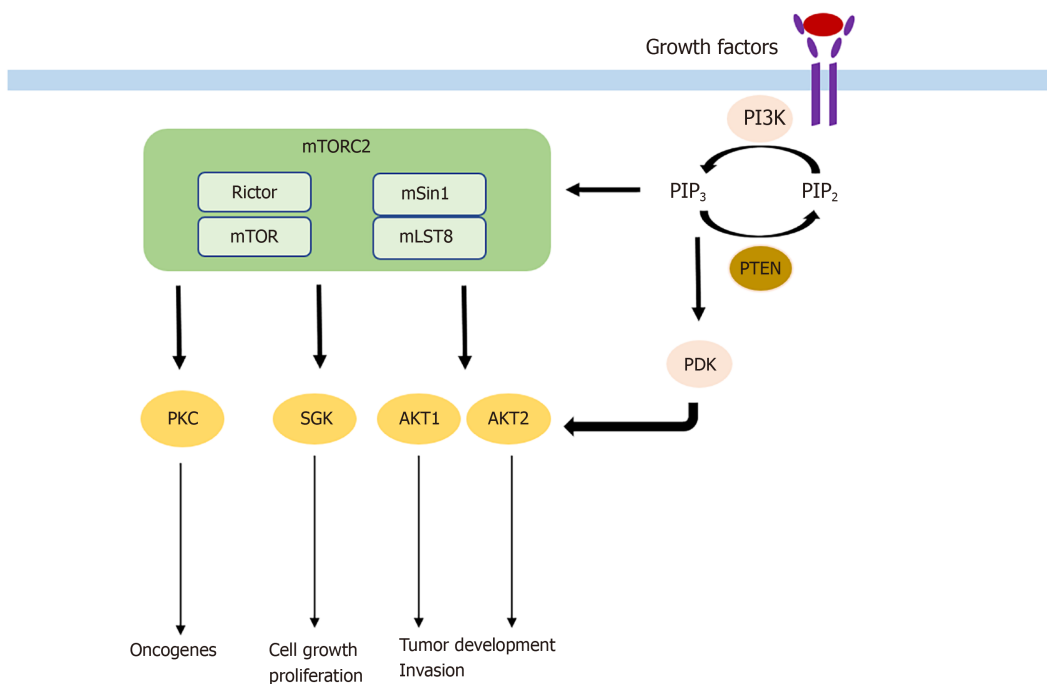
Figure 1 Mechanism by which mammalian target of rapamycin complex 2 participates in tumorigenesis. mTOR: Mammalian target of rapamycin; mTORC2: mTOR complex 2.
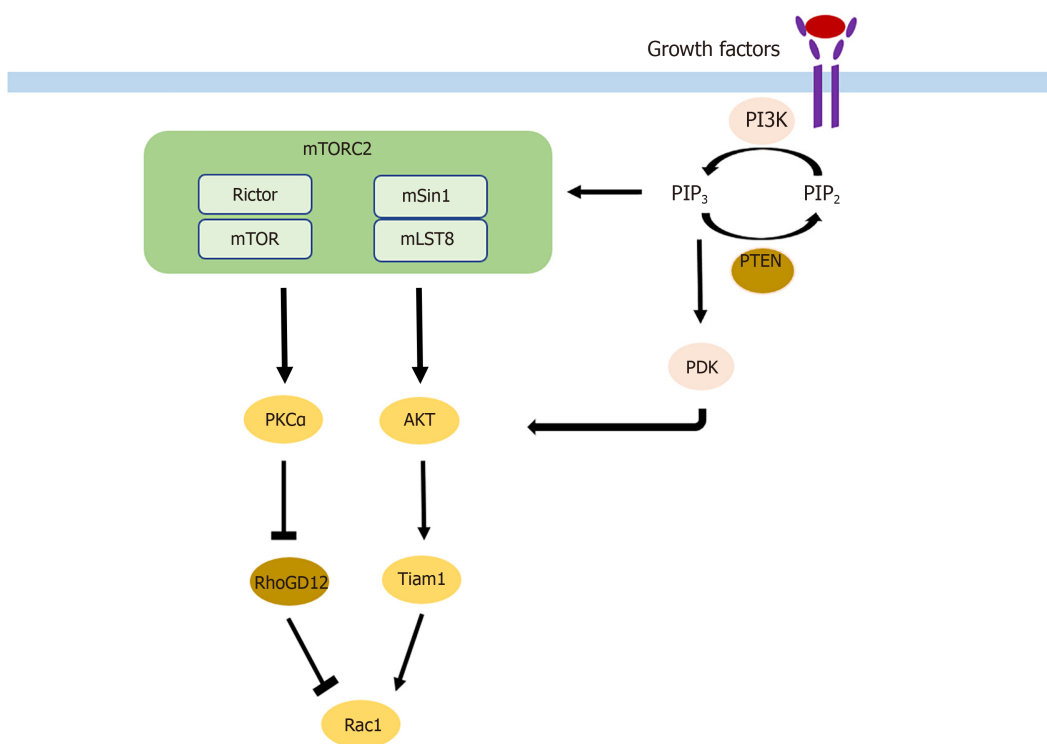

Figure 2 Mechanism by which mammalian target of rapamycin complex 2 participates in metastasis. mTOR: Mammalian target of rapamycin; mTORC2: mTOR complex 2.

MTORC2 IN PRIMARY LIVER CANCER

Hepatocellular carcinoma

HCC is the most common primary liver cancer and one of the main cancer-related deaths worldwide, with increasing incidence in recent years[58]. Systemic treatment options, with the multikinase inhibitors sorafenib and lenvatinib as the only approved drugs, are very limited in case of unresectability or unavailability to local treatment

options. Activation of mTORC2 as determined by immunohistochemistry of phospho-AKT was detectable in 60% of HCCs[59]. Chromosomal gain of Rictor was described in 25% of HCCs, and its high expression was associated with a poor prognosis in HCC patients[60]. Similarly, Kaibori *et al*[61] found high expression of Rictor mRNA and protein and association with Rictor/Raptor ≥ 0,3 was a prognostic factor indicating poor recurrence-free survival. Rictor knockdown was shown to inhibit HCC cell growth *in vitro*[62], and AKT overexpression *in vivo* led to increased HCC development[63]. In the liver, there are two AKT isoforms, AKT1 and AKT2. Only AKT1 is phosphorylated and is thus activated by mTORC2 in c-Myc-induced HCC. AKT1 was the main driver of HCC formation, as AKT1 inhibition completely abolished c-Myc-induced tumor development[60]. Silencing of Rictor also led to inhibition of c-Myc-induced HCC formation[60]. In contrast, inhibition of AKT2 significantly reduced loss of PTEN-induced HCC formation[64]. Moreover, it was found that loss of Rictor completely inhibited sgPTEN/c-Met HCC formation, leading to the assumption that mTORC2 regulates different AKT isoforms in HCC tumor development[65]. In contrast, the role of the other mTORC2 substrates, PKC and SGK in HCC, remains poorly characterized. However, mTORC2 also impacts HCC tumorigenesis through its role in metabolic reprogramming. Fatty acid and lipid synthesis are triggered by mTORC2 thereby leading to hepatic steatosis and tumor development[66]. The causal context was proven, as HCC development was completely abolished upon inhibition of fatty acid or sphingolipid synthesis[66]. Not only mTORC2-associated lipid synthesis but also gluconeogenesis impacts HCC cell survival. Khan *et al*[67] showed that blocking mTORC2 led to increased gluconeogenesis and decreased HCC cell proliferation and survival. In addition to the important role of mTORC2 in hepatocarcinogenesis, it also seems to be involved in HCC metastasis and drug resistance. CHKA is an enzyme known to be associated with HCC metastasis and EGFR-resistance. Inhibition of Rictor completely abolished CHKA-enhanced cell migration and invasion[8]. In line with that, pharmacologic mTORC1/mTORC2 inhibition reduced tumor cell metastasis, while there was no effect shown if the mTORC1 inhibitor rapamycin was used[8]. Moreover, following Rictor knockdown, CHKA-mediated resistance of HCC cells to EGFR-inhibitors decreased, and sensitivity to the drugs increased[8]. Similarly, the dual mTORC1/mTORC2 inhibitor OSI-027 reversed high MDR1 expression in HCC induced by doxorubicin and therefore increased chemosensitivity of doxorubicin. Combining both drugs led to inhibition of tumor growth *in vitro* and *in vivo*[68]. In that context, the investigators concluded that mTORC2 was the component responsible for the effect, as inhibition of mTORC1 alone resulted in a modest decrease of MDR1 expression.

While these data show an important role of mTORC2/Rictor in the tumorigenesis and tumor progression of HCC, it is also involved in pre-tumor conditions. For example, Reyes-Gordillo *et al*[69] showed that the AKT isoforms were activated in an *in vivo* two-hit model of alcoholic liver disease, leading to an increase of mTORC2 and inflammatory, proliferative, and fibrogenic genes. In line with the results, blocking of AKT1 and AKT2 led to a decrease in progression of liver fibrosis. In addition, mTORC2 was involved in the progression of nonalcoholic fatty liver disease (NAFLD) by dysregulation of white adipose tissue. Thereby, *de novo* lipogenesis, lipolysis, glycolysis, and increased glucose uptake by GLUT4 are the mechanisms by which mTORC2 regulates adiposity and NAFLD[70]. Besides alcoholic and nonalcoholic liver disease, viral hepatitis is one of the main risk factors for the development of HCC. In that context, increased AKT activity was demonstrated for hepatitis B and C. In hepatitis B, activation of AKT by the hepatitis B virus protein HBx leads to a persistent, noncytopathic virus replication[71]. In hepatitis C, its NS3/4A protease increases AKT activity by enhancing EGF-induced signal transduction[72].

Intahepatic cholangiocarcinoma

iCCC is a highly aggressive tumor entity with increasing incidence in recent years[73]. As systemic treatment only has partial benefits in advanced stages of iCCC[74], surgical resection remains the only curative option. Only a few studies examining the role of mTORC2 in iCCC exist. mTORC2 was found to be activated in almost 70% of iCCCs as determined by immunohistochemistry of phospho-AKT[75]. When examining the AKT isoforms, protein expression of phospho-AKT1 was shown in 34% of patients with iCCC and was associated with a favorable prognosis[76]. This unexpected result might be dependent on the mechanism of AKT activation triggering different downstream targets or a potential distinct role of AKT1 in iCCC. However, after Rictor knockdown, growth of iCCC cells *in vitro* was impaired and activated AKT was shown to cooperate with YAP to induce iCCC in mice[75]. Moreover, in liver-specific Rictor knockout mice, cholangiocarcinogenesis induced by AKT/YapS127A

was completely abolished, while wild-type mice had a lethal tumor burden at the same time point[75]. Therefore, Zhang *et al*[77] used the pan-mTOR inhibitor MLN0128 and noticed significantly increased apoptosis but only slight effects on proliferation in iCCC *in vitro* and *in vivo*. Significantly enhanced apoptosis and consequently impaired cell proliferation in iCCC was also found after siRNA-mediated Rictor knockdown and simultaneous treatment with sorafenib *via* increase of FoxO1. Wang *et al*[78] reported another mechanism of tumorigenesis, which supported the oncogenic potential of mTORC2 signaling in iCCC. Briefly, activated AKT in combination with downregulation of the tumor suppressor FXBW7, increased cholangiocarcinogenesis. Interestingly, silencing cMyc in AKT/Fbxw7ΔF mice completely impaired iCCC growth[78]. Furthermore, the results of a study by Yang *et al*[79] examining the impact of FXBW7 on EMT and metastasis of iCCC and perihilar CCC (pCCC) is also interesting even though it did not directly connect FXBW7 to mTORC2. In that study, silencing of FXBW7 lead to promotion of EMT, stem-like property, and metastasis of iCCC and pCCC.

While mTORC2 seems to be also involved in the pre-tumoral conditions of HCC including (non) alcoholic liver disease and viral hepatitis, no data exist on the role of mTORC2 chronic cholangitis, primary or secondary biliary cirrhosis as risk factors for the development of iCCC. In summary, mTORC2/Rictor seems to play a role in the development and progression of HCC and iCCC *via* different mechanisms (Figure 3). However, more research is necessary to determine its exact role and to define potential targets for antineoplastic therapy.

MTORC2 IN SECONDARY LIVER CANCER

Colorectal cancer liver metastasis
Colorectal cancer (CRC) is the second leading cause of cancer-related deaths[80], with liver metastases being one of the most important predictors of poor long-term outcome. While it was shown that Rictor mRNA and protein are overexpressed in CRC[49] and expression is correlated with tumor progression, Dukes stage, lymph node metastasis, and impaired overall survival[10,81], there was no difference in Rictor expression between primary tumors and metastatic liver lesions[49]. However, Rictor expression in primary tumors with metastatic liver lesions was significantly higher than it was in primary tumors without metastatic disease[49]. Further, not only Rictor but also Raptor seems to be involved in colorectal liver metastases (CRLM), as knockdown of Rictor, as well as knockdown of Raptor, led to decreased migration and invasion of colorectal cancer cells *in vitro*[49]. In addition, *in vivo* knockdown of Raptor and Rictor in CRC cell lines impaired the formation of even micrometastases[49]. A study by Gulhati *et al*[49] did not focus on the development of liver metastases, but they showed that mTORC2 *via* Rictor regulated actin cytoskeleton reorganization and cell migration through Rac1 and RhoA signaling. However, that is not the only mTORC2-associated mechanism involved in the formation of CRLM. TELO2, known to be essential for mTOR complex integrity, was found to be associated with colorectal tumorigenesis, migration, and invasion, as Rictor knockdown led to reduced TELO2-induced migratory and invasive behavior of colorectal cancer cells[82]. Moreover, colorectal metastasis is not controlled only *via* Rictor but also by mSin1. Wang *et al*[83] showed that the tumor suppressor Pdcd4 inhibited Sin1 translation leading to reduced mTORC2 activation and inhibited invasion of CRC cells. While the studies support the important oncogenic and metastatic potential of mTORC2 in CRC, it is also involved in resistance to systemic chemotherapeutic agents[81]. In particular, resistance of CRC cells to irinotecan, one of the three drugs of FOLFIRI, was resolved by treatment with mTORC1/2 inhibitors. Reita *et al*[84] demonstrated that the combination of irinotecan and a mTORC1/2 blocker reduced migration and invasion *in vitro* as well as the development of liver metastases *in vivo* more effectively than irinotecan alone. Consistently, the knockdown of Rictor increased the sensitivity to irinotecan in SMAD4-negative colon cancer cells[85].

Breast cancer liver metastasis
Breast cancer accounts for almost one in four cancer cases among women, thereby representing the leading cause of cancer in over 100 countries worldwide[80]. Ma *et al*[86] recently reviewed the evidence that after the bony skeleton and the lung, breast cancer metastasizes most often to the liver, leading to very limited survival if untreated. Although many systemic therapies exist for metastatic breast cancer, overactive PI3K/AKT/mTOR signaling was shown to be associated with resistance to
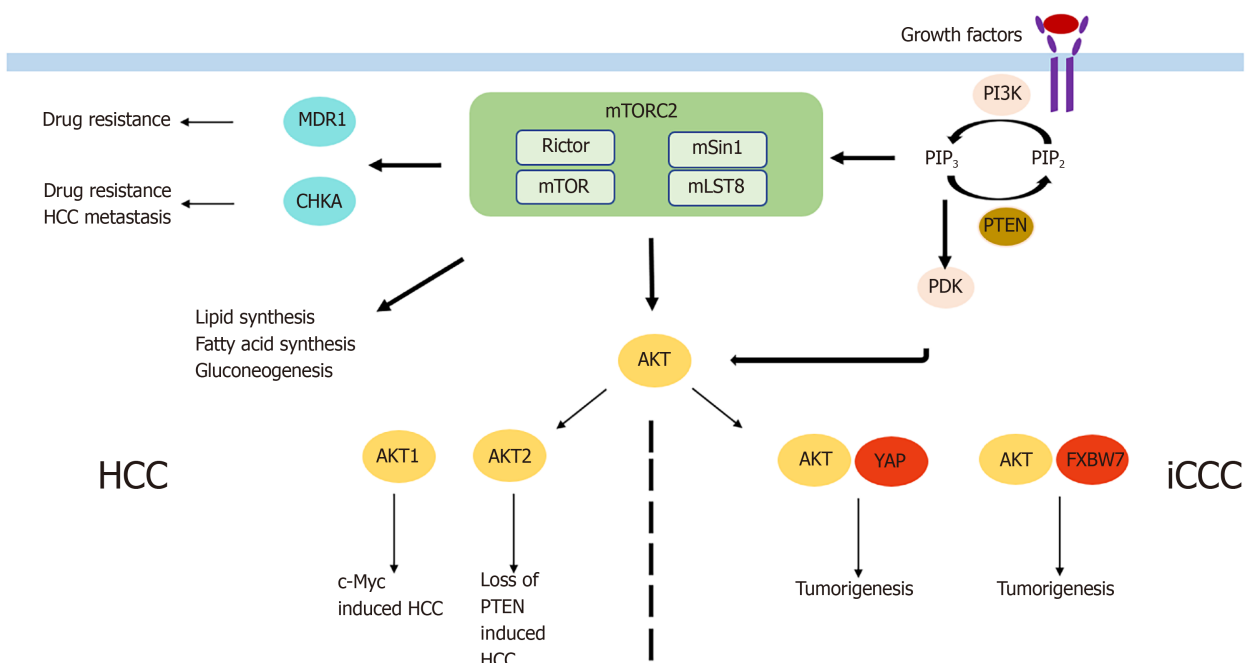
Figure 3 Mechanism by which mammalian target of rapamycin complex 2 is involved in tumorigenesis of primary liver cancer. HCC: Hepatocellular carcinoma; iCCC: Intrahepatic cholangiocellular carcinoma; mTOR: Mammalian target of rapamycin; mTORC2: mTOR complex 2.

therapy and with tumor progression[87-89]. The findings revealed 92% Rictor positivity in breast cancer lymph node metastases[90] as well as decreased tumor growth and migration but increased apoptosis upon Rictor knockdown[91]. Functionally, different pathways of mTORC2/Rictor involvement in breast cancer metastasis have been described. mTORC2 activates Rac1 through AKT phosph-orylation and PKC-dependent downregulation of RhoGD12 leading to increased invasion and migration[46]. Rac1 is also activated by IBP, which was shown to regulate migration and invasion as well as actin cytoskeleton rearrangement and matrix metalloprotease production in breast cancer cells[92] by activation of the mTORC2/AKT/FoxO3a signaling pathway[93]. Moreover, interactions between mTORC2 and PRICKLE1 were shown to control cancer cell dissemination and motility [94]. Similarly, Rictor interacts with PKC-ζ to regulate breast cancer metastasis[90]. mTORC2 is further implicated in EMT in breast cancer by regulation of Snail and TGFß to control migration and invasion[21,95].

Melanoma liver metastasis
Melanoma liver metastasis occurs in up to 20% of patients with cutaneous melanoma and is one of the main prognostic factors of poor survival[96,97]. mTORC2/Rictor is not only involved in PI3K dependent melanoma development[98] and metabolic reprogramming[99] but also in melanoma liver metastases. Rictor mRNA and protein were shown to be overexpressed in invasive melanoma[100] and to be significantly enhanced in metastatic compared with nonmetastatic melanoma[101]. Consistent with those findings, siRNA-mediated Rictor knockdown as well as pharmacological inhibition of mTORC2 not only led to reduced tumor cell motility, migration, and invasion *in vitro*[100,101] but also reduced melanoma liver metastasis *in vivo*[101,102]. Rictor depletion was shown to reduce AKT phosphorylation at the Ser473 and Thr308 residues and to inhibit the expression of MMP-2 and MMP-9[100,102]. Moreover, upon Rictor inhibition, interaction with stromal components such as hepatic stellate cells and HGF-induced melanoma cell activation/motility was impaired[101].

Renal cancer liver metastasis
Liver metastases occur in about one-fifth of patients with metastatic renal cancer[103], and surgical therapy remains the only strategy to improve survival (see Pinotti *et al* [104] for review). However, mTORC2 signaling might be a potential therapeutic target, as it is involved in the formation of renal cancer liver metastasis. Sun *et al*[105] showed that the proinflammatory cytokines TNFα and IL-6 increased upregulation of Rictor through the NF-κB pathway, thereby enhancing chemotaxis, invasion, and migration

of renal cancer cells. Upon Rictor knockdown, the formation of renal cancer liver and lung metastases was significantly reduced[105]. Increased migration and invasion were also associated with activation of mTORC2/Akt/GSK3β/β-catenin signaling through TCTP overexpression[106]. Furthermore, pharmacological mTORC2 inhibition led to reduced migration by regulation of HIF2α and increase of cell-cell-junctions *via* E-cadherin[107].

Gastric and pancreatic cancer liver metastasis

Gastric cancer is the third leading cause of cancer-related deaths worldwide[80] with the liver being the most common side of gastric cancer metastasis[108]. Similarly, pancreatic cancer is one of the most fatal diseases with a 5-year survival rate of only 7%[109]. Rictor expression in gastric tumor samples was shown to correlate with TNM stage, lymph node metastasis, and poor long-term outcome. Positive staining of Akt at the Ser473 residue was associated with distant metastasis[7,110]. Also, Wang *et al*[111] reported the role of mTORC2 in gastric cancer metastasis, as DDR2 was found to enhance invasion and EMT through mTORC2 activation and AKT phosphorylation. Upon Rictor knockdown, proliferation, migration, and invasion of gastric cancer cells were significantly reduced while apoptosis was enhanced[110]. Regarding pancreatic cancer, Rictor protein expression was associated with overall survival after surgical resection. Patients with high or medium Rictor expression had significantly shorter survival compared with those with low expression[9]. Upon siRNA-mediated Rictor knockdown, pancreatic tumor cell proliferation and vascularization were significantly impaired and a trend toward fewer liver metastases was observed[9].

CONCLUSION

Compared with mTORC1, little is known about the role of mTORC2 and its distinct subunit Rictor, in cancer. However, the present review underlines the importance and high relevance of mTORC2 not only in tumorigenesis of primary liver cancer but also in the formation of metastatic liver lesions with different primaries. Thereby, mTORC2/Rictor and AKT, its main downstream effector, are associated with various steps of the metastatic cascade, including EMT, migration and invasion, and angiogenesis, and tumor cell proliferation through different signaling pathways. However, a more refined understanding of the implications of mTORC2 in primary and secondary liver cancer is essential to convert this knowledge into the development of specific mTORC2 targeting therapies.

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REVIEW

Regulatory role of the transforming growth factor-β signaling pathway in the drug resistance of gastrointestinal cancers

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Abstract

Gastrointestinal (GI) cancer, including esophageal, gastric, and colorectal cancer, is one of the most prevalent types of malignant carcinoma and the leading cause of cancer-related deaths. Despite significant advances in therapeutic strategies for GI cancers in recent decades, drug resistance with various mechanisms remains the prevailing cause of therapy failure in GI cancers. Accumulating evidence has demonstrated that the transforming growth factor (TGF)-β signaling pathway has crucial, complex roles in many cellular functions related to drug resistance. This review summarizes current knowledge regarding the role of the TGF-β signaling pathway in the resistance of GI cancers to conventional chemotherapy, targeted therapy, immunotherapy, and traditional medicine. Various processes, including epithelial-mesenchymal transition, cancer stem cell development, tumor microenvironment alteration, and microRNA biogenesis, are proposed as the main mechanisms of TGF-β-mediated drug resistance in GI cancers. Several studies have already indicated the benefit of combining antitumor drugs with agents that suppress the TGF-β signaling pathway, but this approach needs to be verified in additional clinical studies. Moreover, the identification of potential biological markers that can be used to predict the response to TGF-β signaling pathway inhibitors during anticancer treatments will have important clinical implications in the future.

Key Words: Drug resistance; Gastrointestinal cancer; Transforming growth factor-β; Epithelial-mesenchymal transition; Cancer stem cells; MicroRNAs

Core Tip: The transforming growth factor (TGF)-β signaling pathway is involved in the drug resistance of gastrointestinal (GI) cancers. This review summarizes the current understanding of the roles played by the TGF-β signaling pathway in resistance to conventional chemotherapy, targeted therapy, immunotherapy, and traditional medicine in GI cancers as well as the various processes by which this occurs, including epithelial-mesenchymal transition, cancer stem cell development, tumor microenvironment alteration, and microRNA biogenesis.

INTRODUCTION

Gastrointestinal (GI) cancer, including esophageal cancer (EC), gastric cancer (GC), and colorectal cancer (CRC), is one of the most prevalent types of malignant carcinoma, falling within the top six in mortality according to global cancer statistics in 2018. In both sexes, CRC is the second leading cause of cancer death (9.2% of total cancer deaths), closely followed by GC (8.2%), and EC as the sixth leading cause of mortality (5.3%)[1]. CRC is also the second most common cause of cancer death in the United States[2]. Despite improvements in current therapeutic strategies, including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, clinical prognoses and therapeutic responses of GI cancer patients are far from satisfactory because of delayed diagnosis, recurrence, poor clinical response, high cost, and medication side effects[3,4].

Chemotherapy is the most commonly used treatment for patients with advanced GI cancer. The most widely used chemotherapeutic regimens for GI cancer are fluorouracil and platinum[5-7]. Despite the continual development of new chemotherapeutic strategies, resistance to anticancer drugs remains a significant problem that is responsible for unfavorable clinical outcomes and treatment failures. Chemoresistance, including intrinsic and acquired drug resistance, is defined as the resistance of cancer cells to various structurally and functionally unrelated anti-cancer drugs[8]. The mechanisms of drug resistance are complex and closely related to various signaling pathways that are activated by many stimuli to promote chemoresistance[9].

The transforming growth factor (TGF)-β signaling pathway is deregulated in cancer and can have tumor-suppressive or tumor-promoting roles, depending on the molecular and cellular context[10,11]. In the GI tract, TGF-β has crucial and complex roles in many cellular functions related to drug resistance, such as maintaining stem cell homeostasis, regulating epithelial to mesenchymal transition, modulating immunity, and promoting fibrosis[12,13]. In this review, we discuss the role of the TGF-β signaling pathway in regulating chemoresistance in GI cancers.

MECHANISMS OF CHEMORESISTANCE IN CANCER

Molecular investigations have revealed several mechanisms underlying chemoresistance, including the epithelial-mesenchymal transition (EMT), the efflux of intracellular chemotherapeutic drugs, noncoding RNAs, stem cell development, and the tumor microenvironment[14-17]. EMT is a complex and important cellular program in which epithelial cells shed their differentiated characteristics and acquire mesenchymal phenotypes, including motility, invasiveness, and resistance to apoptosis. Cells undergoing EMT become more invasive and exhibit increased resistance to anticancer drugs[18,19]. In addition, EMT has been found to result in stem cell-like characteristics and is positively correlated with the expression of ATP-

binding cassette (ABC) transporters[18,20,21]. Different stimulus-induced EMT may contribute to chemoresistance *via* the upregulation of distinct transcription factors[19].

Failure of cancer chemotherapy can also be caused by changes in the expression or activity of membrane transporters, primarily those belonging to the ABC transporter family. ABC transporters can export chemotherapeutic agents out of the cell, thereby reducing intracellular drug levels and drug sensitivity and ultimately contributing to cancer chemoresistance[22,23]. In addition, ABC proteins transport signaling molecules that contribute to tumorigenesis[24].

Increasing evidence shows that non-coding RNAs, especially microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), can affect chemoresistance by forming a competing endogenous RNA regulatory network with mRNAs[25]. MiRNAs can play roles in drug resistance by targeting hundreds of tumor-related gene transcripts and affecting complex molecular pathways[14,26]. Specific miRNAs may be used as potential predictive biomarkers to guide individualized chemotherapy by reversing drug resistance[14].

Cancer stem cells (CSCs), which make up a distinct population within the tumor mass, possess unique self-renewal, multilineage differentiation, and potent tumorigenic abilities[27,28]. These cells acquire chemoresistance through various pathways involving apoptosis and DNA repair mechanisms[29]. In addition, upon exposure to cytotoxic therapies, CSCs can convert non-CSCs to CSC-like cells that may persist after treatment and serve as a mechanism for relapse. In GI malignancies, CSCs are abundant and contribute to chemotherapeutic resistance[15].

Interactions of tumor cells with alterations of the microenvironment, such as energy deprivation, hypoxia, and inflammation, give rise to heterogeneity and chemoresistance. Most tumor cells display deviations from the normal energy metabolism, allowing them to survive in hypoxic and low nutrient microenvironments[30,31]. Mitochondrial dysfunction and fatty acid (FA) metabolism are associated with chemotherapeutic resistance[31,32]. Hypoxia can also drive tumor resistance to chemotherapy by upregulating hypoxia-inducible factor-1 (HIF-1) and its downstream genes[33]. Inflammation and inflammatory mediators, including TGF-β, have been shown to contribute to the development, progression, metastasis, and chemoresistance of cancer[34,35]. In addition, the gut microbiota, which is linked to chronic inflammation and carcinogenesis[36], has an important role in the modulation of the host response to antitumor treatments, especially chemotherapy and immunotherapy[37]. Moreover, emerging evidence has demonstrated that cancer-associated fibroblasts (CAFs), one of the critical components of the tumor microenvironment, confer substantial resistance to chemotherapy and influence tumor cell responsiveness to immune checkpoint inhibitors[38].

ROLE AND ALTERATIONS OF THE TGF-β SIGNALING PATHWAY IN GI CANCER

The TGF-β signaling pathway can be subdivided into canonical Smad-dependent and noncanonical Smad-independent pathways. In the canonical pathway, TGF-β initially binds to the TGF-β type 2 receptor (TβRII), which recruits and phosphorylates the kinase domain of TGF-β type 1 receptor (TβRI), leading to the activation and phosphorylation of Smad2 and Smad3. Then, phosphorylated Smad2 and Smad3 bind to Smad4, allowing the entire complex to translocate into the nucleus. In the nucleus, the Smad complex regulates transcriptional activity by interacting with Smad binding elements within downstream target genes[39-41]. Smad7 negatively regulates the TGF-β signaling pathway by blocking the interaction between Smads and receptors and inhibiting the phosphorylation of Smad2 and Smad3[42,43]. In addition to the Smad-dependent pathway, the binding of the TGF-β ligand to its receptors also activates several Smad-independent signaling pathways, including the mitogen-activated protein kinase, phosphoinositide 3-kinase (PI3K)/AKT, and Rho-associated protein kinase pathways[44,45].

The TGF-β signaling pathway has an important role in controlling tissue development, proliferation, apoptosis, differentiation, and homeostasis[46]. Disruption of this signaling pathway leads to various diseases, including some cancers. In cancer cells, TGF-β signaling causes EMT and CSC-like traits, resulting in an aggressive phenotype and a poor prognosis[47,48]. In addition to its direct effect on epithelial tumor cells, TGF-β controls tumor development by regulating the tumor microenvironment and growth factors from the surrounding stroma[13,49]. Furthermore, TGF-β signaling activation in the tumor microenvironment suppresses antitumor immune

responses and supports cancer cell survival[50]. TGF-β has been found to inhibit multiple components of the immune system, including natural killer cells, CD8+ cytotoxic T lymphocytes, B-cell proliferation, and immunoglobulin A secretion[51]. Therefore, the TGF-β signaling pathway is associated with drug resistance and immune system escape.

In CRC, TGF-β1 expression is markedly increased and is correlated with poor clinical outcomes and a high risk of relapse[52,53]. TGF-β1 expression is also increased in GC mucosa and precancerous gastric cells[54,55]. However, active TGF-β1 is expressed most highly in smooth muscle actin-positive fibroblasts rather than in the malignant epithelial cells of gastric tumors[56]. In GC patients, high serum and tissue TGF-β1 levels are associated with lymph node involvement and poor prognosis[57]. Moreover, increased expression of TGF-β is found in EC[58]. In sum, serum and tissue TGF-β levels are upregulated in GI cancers and are associated with metastases and poor prognoses. Alterations in the TGF-β signaling pathway, especially receptor and *Smad* gene mutations, are commonly observed in GI cancers where they lead to tumor formation and metastasis[13]. Mutations in the TGF-β signaling pathway are found in 80% of CRC cell lines and approximately one-third of CRC tumors[46]. A decreased or complete loss of TGF-β receptor expression is common in patients with esophageal adenocarcinoma, primary gastric tumors, and CRC[49]. TβRII mutations frequently occur in the advanced stages of the colon[59] and gastric tumors along with progressive microsatellite instability (MSI-H)[49,60]. The overall incidence of TβRII mutations is approximately 30% in CRC, while frameshift mutations can be found in approximately 80% of MSI-H CRC[60,61]. TβRII mutations in CRC cells can contribute to the malignant phenotype *via* multiple pathways, regulate the components secreted by cancer cells, and directly promote inflammation in the tumor microenvironment [50]. Compared with TβRII, mutations in its counterpart TβRI are less frequent in both CRC and GC[13,60].

A study of over 700 cases of sporadic CRCs reveals that the prevalence of Smad4, Smad2, and Smad3 mutations was 8.6%, 3.4%, and 4.3%, respectively, with a combined prevalence of 14.8%[62]. Both Smad2 and Smad4 are located on chromosome 18q, which is commonly deleted in CRC[63]. However, Smad2 and Smad4 mutations tend to occur in the early and advanced stages of CRC, respectively[13,61,64]. Loss of Smad4 contributes to colorectal carcinogenesis[46] and may be a predictive biomarker of the response to 5-fluorouracil (5-FU)-based chemotherapy[65]. In GC, the expression of Smad3 is low or even undetectable in 40% of tissues, so mutations in Smad2 and Smad3 have not been described[13].

TGF-β SIGNALING AND DRUG RESISTANCE IN GI CANCER

Accumulating evidence suggests that the expression levels of components of the TGF-β signaling pathway are closely associated with response to chemotherapy. Immuno-histochemical analysis of 78 patient biopsies reveals that p-Smad2/3 expression was elevated in C-type CRC tumors, which benefit the least from chemotherapy[66]. Mediator Complex Subunit 12 (MED12) negatively regulates TβRII through physical interactions; therefore, its suppression induces the activation of TGF-β signaling[67]. In CRC cells, both MED12 knockdown and recombinant TGF-β treatment result in resistance to cisplatin, oxaliplatin (OXA), and 5-FU[66,67]. However, another study shows that TGF-β2 suppression was associated with recurrence in patients with colorectal adenocarcinomas. In addition, disease-free survival (DFS) and overall survival (OS) are significantly longer in patients with tumors expressing TGF-β2[68]. Additionally, in esophageal squamous cell carcinoma (ESCC) patients, TGF-β 1–509C/T polymorphisms benefit from radiochemotherapy and therefore might be useful genetic markers for predicting radiochemotherapy response[69]. In GI cancers, the TGF-β pathway is correlated with resistance to antitumor agents, including conventional chemotherapy, targeted therapy, immunotherapy, and traditional medicine. In Table 1, we provide a summary of the relationships between the TGF-β signaling pathway and drug resistance in GI cancers.

Conventional chemotherapy
Fluorouracil: 5-FU belongs to the antimetabolite family[70] and is a commonly used chemotherapeutic regimen for CRC and GC. 5-FU, a pyrimidine analog and an inhibitor of thymidylate synthase, is incorporated into RNA or DNA in the place of uracil or thymine and leads to the prevention of DNA replication and cell death[71]. Unfortunately, the treatment effectiveness of 5-FU is reduced, and its clinical

Table 1 Studies of the transforming growth factor-β signaling pathway in drug resistance in gastrointestinal cancer

Cancer type	*In vivo/In vitro*	Upstream regulator	Alteration of TGF-β signaling	Effect	Downstream antitumor drug	Ref.
CRC	SK-CO-1 cells	MED12 knockdown	The activation of TGF-β signaling or TGF-β treatment	Resistance	DDP, OXA, and 5-FU	Brunen *et al* [66], 2013
CRC	HCT116/HCT116p53KO chemoresistant cell lines	-	TGF-β1 treatment/TβRI inhibition	Resistance/sensitivity	5-FU	Romano *et al* [82], 2016
CRC	HCT116 cells	-	Smad4 knockdown	Sensitivity	Dox	Li *et al*[103], 2015
CRC	*in vivo*, CRC animal model, stable OXA-resistant cell line HCT116/OXA	Curcumin	Inhibition of p-Smad2 and p-Smad3	Sensitivity	OXA	Yin *et al*[90], 2019
CRC	The resistant cell model HCT-8/5-FU cell line	*Hedyotis diffusa* Willd	Inhibition of TGF-β signaling	Antimetastasis in 5-FU-resistant cells	5-FU	Lai *et al*[116], 2017
CRC	HCT116 and DLD1 CRC cell lines	-	siRNA-mediated knockdown of SMAD2/3, TGF-β inhibitor SB431542	Sensitivity	OXA	Kim *et al*[89], 2019
CRC	RKO cells	-	Silencing of TβRII expression, TβRI inhibitor LY2157299	Sensitivity	BETi	Shi *et al*[112], 2016
CRC	HCT116 cells	-	TGF-β inhibitor LY2157299	Sensitivity	5-FU	Quan *et al* [81], 2019
CRC	CT26 cells	Chemokine C-C motif ligand-1 secreted by Snail-expression fibroblasts	Phosphorylated Smad2	Resistance	5-FU or paclitaxel	Li *et al*[143], 2018
CRC	5-FU resistant cell line(HCT-8/5-FU)	Pien Tze Huang (PZH)	Suppression of TGF-β and Smad4	Overcome MDR and inhibit EMT	-	Shen *et al* [117], 2014
CRC	Patients	-	P-Smad3 overexpression	Resistance	5-FU and leucovorin, capecitabine	Huang *et al* [78], 2015
CRC	HCT116 *Smad4*^{+/+} and *Smad4*^{-/-} cell lines	-	Smad4 defect	Resistance	5-FU	Papageorgis *et al*[76], 2011
CRC	*in vivo*, colorectal tumor biopsies	-	Normal SMAD4 diploidy	Sensitivity	5-FU and mitomycin	Boulay *et al* [73], 2002
CRC	Dukes CRC patients	-	Low SMAD4 mRNA and protein levels	Resistance	5-FU-based adjuvant chemotherapy	Alhopuro *et al*[75], 2005
CRC	Colorectal tumor biopsies	-	The amplification of *STRAP*, an inhibitor of TGF-β signaling	Resistance	5-FU /mitomycin C adjuvant chemotherapy	Buess *et al* [74], 2004
CRC	Colo205 and RKO cells	-	TGF-β1 treatment	Resistance	5-FU, etoposide	Moon *et al* [80], 2019
CRC	Mouse models	-	Blockade of TGF-β signaling	Sensitivity	Anti-PD-1-PD-L1 checkpoint therapy	Tauriello *et al* [115], 2018
CRC	Mice models of MC38-derived tumors	-	1D11 antibody anti-TGF-β mAb	Sensitivity	Anti-PD1 plus anti-CD137 mAb	Rodríguez-Ruiz *et al* [114], 2019
CRC	SNU-C5/5-FU -resistant cells.	(1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide (LS-1) from *Lobophytum* sp	The increase of Smad-3 phosphorylation and the nuclear localization of p-Smad3 and Smad4	Sensitivity	5-FU	Kim *et al* [118], 2015
CRC	The early stages of colorectal carcinogenesis in rats	5-FU/thymoquinone (TQ) combination therapy	Upregulation of the TGF-β1, TβRII, Smad4	Sensitivity	5-FU	Kensara *et al* [122], 2016
CRC	Azoxymethane (AOM) rat model	Vitamin D3/5-FU co-therapy	Upregulation of the TGF-β1, TβRII, smad4	Sensitivity	5-FU	Refaat *et al* [77], 2015

CRC	RKO cells	Oxymatrine	Inhibition of the Smad2 phosphorylation and the formation of Smad2/3/4	Sensitivity	-	Wang *et al* [119], 2017
EC	Paclitaxel-resistant EC109 cells	-	BMP-4 and p-Smad1/5 overexpression	Resistance	Paclitaxel	Zhou *et al* [100], 2017
ESCC	KYSE-150 and KYSE-180 cells, xenograft tumors in nude mice	-	TβRI inhibitor LY2157299	Sensitivity	DDP and taxol	Zhang *et al* [142], 2017
ESCC	Xenotransplanted tumor mice model	-	Dual PD-1/PD-L1 and TGF-β blockades	Sensitivity	PD-1/PD-L1 blockade	Chen *et al* [139], 2018
EC and GC	EC cells T.T, GC cells MKN28 and MKN45	-	Pretreatment with TGF-β	Sensitivity	Adriamycin	Izutani *et al* [104], 2002
EAC	EAC cells, EAC patient-derived xenograft tumors	-	TβR inhibitor and trastuzumab, pertuzumab	Sensitivity	Trastuzumab and Pertuzumab	Ebbing *et al* [106], 2017
EC	KYSE150 andKYSE450 cells	Garcinol	Inhibition of the p300/CBP and p-Smad2/3 expression	Sensitivity	-	Wang *et al* [120], 2020
ESCC	Patients	-	High serum levels of VEGF-A and TGF-β1	Resistance	Taxane-based/5-FU -based chemotherapy	Cheng *et al* [79], 2014
ESCC	TE1	-	Anti-TGF-β2 neutralizing mAb and SB-431542	Sensitivity	Trastuzumab	Mimura *et al* [110], 2005
ESCC	TE1/TE5	-	Anti-TGF-β2 neutralizing mAb/exogenous addition of TGF-β2	Sensitivity/resistance	Cetuximab	Kawaguchi *et al*[109], 2007
ESCC	ECA109 and TE1 cells	Overexpression of LEF1	Upregulation of p-Smad2, p-Smad3, and TGF-β	Resistance	DDP	Zhao *et al* [130], 2019
GC	AGS cells	Glycoprotein from the *Capsosiphon fulvescens*	Inhibition of TGF-β1-activated FAK/PI3K/AKT pathways	Sensitivity	-	Kim *et al* [121], 2013
GC	SGC7901 and BGC823 cells	HMMR	Upregulation of p-Smad2 level and the nuclearaccumulation of Smad2	Resistance	5-FU	Zhang *et al* [84], 2019
GC	A peritoneal-metastatic cell line, 60As6	-	TGF-β treatment	Sensitivity	Docetaxel	Fujita *et al* [99], 2015
GC	MKN-45 cells	Eribulin	Inhibition of the TGF-β /Smad pathway	Sensitivity	-	Kurata *et al* [126], 2018
GC	Peritoneal mesothelial cells (HPMCs)	Paclitaxel	Inhibition of phosphorylation of Smad2	Reduce stromal fibrosis	-	Tsukada *et al* [98], 2013
GC	NCI-N87 cells	-	TGF-β treatment	Resistance	Trastuzumab	Zhou *et al* [107], 2018
	AGS and MKN45 cells	MSCs	Activated TGF-β signaling	Resistance	5-FU and OXA	He *et al*[146], 2019
CRC	Patients	-	TGF-β2 expression	Sensitivity	Fluoropyrimidine	Kim *et al*[68], 2009

5-FU: 5-fluorouracil; BETi: Bromodomain and extraterminal domain protein inhibitors; BMP-4: Bone morphogenetic protein 4; CRC: Colorectal cancer; DDP: Cisplatin; Dox: Doxorubicin; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; GC: Gastric cancer; MDR: Multidrug resistance; MSCs: Mesenchymal stem cells; TGF-β: Transforming growth factor-β; TβRI: Type 1 TGF-β receptor; TβRII: Type 2 TGF-β receptor.

application is limited by the emergence of drug resistance. The response rate to 5-FU is limited to 10%–15% in CRC. Various strategies have been used to improve the efficacy of 5-FU, resulting in the extension of the median survival to 30 mo[72].

A study of colorectal tumor biopsies shows that CRC patients with normal Smad4 diploidy experienced a threefold higher benefit from postoperative 5-FU-based adjuvant chemotherapy than those with Smad4 deficiency[73]. Another study of the same collection of tumor specimens reveals that serine-threonine receptor-associated

protein, a TGF-β-signaling inhibitor that acts at the receptor level, was a predictor of unfavorable responses to 5-FU-based adjuvant chemotherapy[74]. Similarly, CRC patients treated with surgery and 5-FU-based adjuvant therapy and followed for over 6 years to evaluate the prognostic value of Smad4 expression, demonstrate that patients with a low level of Smad4 expression had shortened DFS and OS compared with those with a high level of Smad4 expression[75]. In HCT116 colon cancer cells, Smad4 deficiency is found to be responsible for 5-FU resistance[76]. Moreover, in an azoxymethane rat model of colon cancer, vitamin D3 supplementation promotes the efficacy of 5-FU through multiple mechanisms including increased expression of TGF-β1, TβRII, and Smad4[77]. In brief, the results indicate that the effectiveness of adjuvant 5-FU-based chemotherapy might depend on TGF-β signaling in CRC.

As the TGF-β signaling pathway appears to have both suppressive and promoting effects in cancer, other studies have suggested that activation of the TGF-β signaling pathway might induce resistance to 5-FU in GI cancers. Immunohistochemical staining in patients with stage II-III advanced rectal cancer showed that p-Smad3 overexpression was associated with poor preoperative responses to fluoropyrimidine-based chemoradiotherapy. Therefore, p-Smad3 could be a potential predictor of a poor response to radiochemotherapy[78]. Moreover, pre-CCRT serum TGF-β1 levels were found to be negatively correlated with DFS in patients with ESCC receiving concurrent neoadjuvant chemoradiotherapy with taxane-based/5-FU-based chemotherapy followed by esophagectomy[79]. In CRC cells, TGF-β1 treatment was found to increase apoptotic resistance in cells exposed to therapeutics including 5-FU and etoposide[80]. TGF-β inhibition was found to sensitize HCT116 cells to 5-FU treatment and suppress cell migration[81]. Likewise, TβRI inhibition reduced proliferation and increased cell death in chemoresistant cancer cells[82]. Furthermore, Moon *et al*[83] found that Smad3/4 acted as a drug sensitivity regulator in TGF-β-mediated chemoresistant CRC cells, and knockdown of Smad3/4 significantly decreased tumor propagation and migration in the presence of 5-FU[83]. In GC, hyaluronan-mediated motility receptor is a key regulator of chemoresistance, and its upregulation was found to promote EMT and CSC properties by activating the TGF-β/Smad2 signaling pathway, ultimately leading to 5-FU resistance[84].

Platinum compounds: Platinum compounds are used as single agents or in combinatorial regimens for the treatment of GI cancers. The molecular mechanism of platinum compound-induced apoptosis involves the inhibition of DNA synthesis and repair, resulting in cell cycle arrest. This effect is mediated by the activation of various signal transduction pathways[85]. OXA is an important platinum-based option for the treatment of CRC[86]. In two multicenter trials in which single-agent OXA was administered as first-line treatment of advanced CRC, response rates were 12% and 24%, progression-free survival was 4 mo, and median survival was 14.5 mo and 13 mo, respectively[87].

In CRC cells, TGF-β1 contributes to OXA resistance primarily through EMT, which leads to antiapoptotic effects and the attenuation of DNA damage[88]. Furthermore, both siRNA-mediated knockdown of Smad2/3 and treatment with the potent TGF-β inhibitor SB43154225 suppress migration and invasion and increase therapeutic sensitivity to OXA in HCT116 and DLD1 CRC cell lines[89]. Curcumin, a naturally occurring polyphenolic substance extracted from the Curcaceae plant *Curcuma longa,* sensitizes CRC to OXA treatment by inhibiting the TGF-β/Smad2/3 pathway in the OXA-resistant cell line HCT116/OXA and in an *in vivo* animal model of CRC[90]. In EC, TGF-β secreted from CAF-like fibroblasts induces chemoresistance to cisplatin, which is reversed after administration of TGF-β neutralizing antibodies[91].

Taxoid compounds: Paclitaxel (PTX) is an antineoplastic agent derived from the bark of the Pacific yew *Taxus brevifolia*[92]. Docetaxel is a semi-synthetic taxane that primarily acts to promote microtubule assembly and prevents the depolymerization of assembled microtubules[93]. Both PTX and docetaxel exert potent antitumor effects by stabilizing microtubules, resulting in cell cycle arrest and apoptosis[94]. The results of a multicenter trial in patients with advanced or recurrent GC showed that the response rate to PTX as a second-line monotherapy was 17.5%[95]. The median duration of response to PTX monotherapy was 2.8 mo in patients with advanced gastric or gastroesophageal junction adenocarcinoma, and the patients eventually developed resistance to PTX[96]. The results of a phase II study in previously-untreated GC patients reported overall response rates to single-agent docetaxel in the range of 17% to 24%[97].

Peritoneal dissemination is the most common mode of metastasis in GC. Low-dose PTX can significantly inhibit Smad2 phosphorylation in human peritoneal mesothelial

cells, leading to a decrease in stromal fibrosis[98]. The results of a microarray analysis showed that C-X-C chemokine receptor type 4 (CXCR4) was a novel marker for highly metastatic CSCs. Treatment with TGF-β enhanced the anticancer effect of docetaxel *via* the induction of cell differentiation/asymmetric cell division within the CXCR4-positive gastric CSC population, even when the cells were in a dormant state[99]. Bone morphogenetic protein 4 (BMP-4), which is involved in TGF-β signaling, is upregulated in PTX-resistant human esophageal carcinoma EC109 cells and docetaxel-resistant human GC MGC803 cells. p-Smad1/5, which is also involved in the TGF-β /Smad pathway, is also overexpressed in EC109/Taxol cells[100].

Doxorubicin: Doxorubicin (Dox), a chemotherapeutic agent extensively used to treat a wide range of cancers, exerts cytotoxic and DNA damaging effects through interference with nucleoside metabolism, but is less efficacious in GI cancers relative to other cancer types[101]. The antineoplastic activity of Dox is attributed to its intercalation into the DNA helix and its ability to generate free radicals[102]. In HCT116 colon cancer cells, long-term administration of low concentrations of Dox may promote resistance partly *via* the activation of TGF-β signaling. Moreover, knockdown of Smad4 significantly increases the sensitivity of HCT116 cells to Dox, in part *via* the inhibition of multidrug-resistant plasma membrane glycoprotein expression and reversal of the EMT process[103]. Therefore, the combination of Dox treatment and TGF-β downregulation might be a potential therapeutic strategy to overcome chemoresistance.

Adriamycin: Adriamycin (ADM) generates superoxide radicals that kill tumor cells by damaging DNA, directly intercalating into DNA, and preventing DNA replication. In human EC cells (T.T) and GC cells (MKN28 and MKN45), pretreatment with TGF-β1 results in increased sensitivity to ADM. *In vivo*, the combined administration of TGF-β 1 and ADM delayed tumor growth better than either treatment alone and further exhibited synergistic antitumor effects[104].

Targeted therapy
Knockdown of MED12 in the CRC cell lines SK-CO-1 (KRASV12) and SW1417 (BRAFV600E) resulted in the activation of MEK/ERK and induced resistance to the MEK inhibitor AZD6244 (selumetinib). Moreover, TGF-β-induced resistance to AZD6244 and the BRAF inhibitor PLX4032 (vemurafenib) have also been observed in CRC cells[67]. However, another study demonstrated that vemurafenib downregulated the expression of TGF-β and p-Smad3 in HT29 CRC cells[105]. Trastuzumab, a human epidermal growth factor receptor (HER)2-targeting antibody, is the only available targeted agent for first-line palliative systemic treatment of HER2-positive esophagogastric adenocarcinoma (EAC). EAC cells become resistant to trastuzumab and the HER2-HER3 signaling inhibitor pertuzumab by activating TGF-β signaling, which subsequently induces EMT. TGF-β receptor inhibitors were shown to increase the antitumor efficacy of trastuzumab and pertuzumab in EAC cells and EAC patient-derived xenograft tumors[106]. Sensitivity of the GC cell line NCI-N87 to trastuzumab was significantly decreased after treatment with TGF-β. Moreover, TGF-β was upregulated in trastuzumab-resistant NCI-N87/TR cells[107]. Cetuximab and trastuzumab, humanized antibodies against the HER family, exert antitumor effects by directly inhibiting epidermal growth factor receptor (EGFR) tyrosine kinase activity, inhibiting cell cycle progression, and activating proapoptotic molecules[108]. In addition, an anti-TGF-β2 neutralizing mAb enhances cetuximab-mediated and trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC) in TE1 TGF-β -producing ESCC cells. The TGF-β signaling inhibitor SB-431542 was found to enhance trastuzumab-mediated ADCC of TE1 cells. Furthermore, the exogenous addition of TGF-β2 significantly decreased cetuximab-mediated ADCC in non-TGF-β2-producing TE5 cells, and TGF-β2 inhibited the activity of trastuzumab-mediated ADCC in TE1 cells[109,110]. TGF-β expression is upregulated in three FGFR2-amplified SNU-16 GC cell lines that are resistant to AZD4547, BGJ398, and PD173074. However, parental SNU-16 cells treated with TGF-β1 did not undergo EMT, and inhibition of TβRI was not sufficient to reverse EMT in the resistant cells[111]. Bromodomain and extraterminal domain protein inhibitors (BETis) are in clinical trials as a novel class of cancer therapeutics. Both TβRII knockdown and treatment with the small-molecule Tβ RI inhibitor LY2157299 (galunisertib) were reported to increase the sensitivity of RKO colon carcinoma cells to BETis[112].

Immunotherapy
Treatment with the TGF-β inhibitors P144 and P17 may be able to enhance the efficacy

of immunotherapies by increasing antitumor immune responses[113]. Moreover, treatment with the TGF-β-neutralizing mAb 1D11 enhanced the abscopal effect of radiotherapy as well as overall treatment efficacy in subcutaneous large MC38 colorectal tumors in conjunction with anti-programmed cell death protein 1 (PD-1) plus anti-CD137 mAb[114]. In mice with progressive metastatic liver disease, enabling immune infiltration using TGF-β inhibitors render tumors susceptible to anti-PD-1/Programmed cell death ligand 1 (PD-L1) checkpoint-based therapies[115]. Immuno-therapies directed against TGF-β signaling may have broad applications in treating patients with advanced CRC.

Traditional medicine

Traditional herbal medicine has an important role in reversing the resistance of CRC cells to 5-FU. *Hedyotis diffusa* Willd, a traditional Chinese herbal medicine in the family of Rubiaceae, may exert its antimetastatic activity by suppressing TGF-β/Smad4 signaling pathway-mediated EMT in 5-FU-resistant CRC cells[116]. Similarly, the traditional Chinese medicine formula Pien Tze Huang can effectively overcome multidrug resistance and inhibit EMT *via* suppression of the TGF-β pathway in the 5-FU-resistant CRC cell line HCT-8/5-FU[117]. Moreover, (1*S*,2*S*,3*E*,7*E*,11*E*)-3,7,11,15-Cembratetraen-17,2-olide (LS-1), a marine cembrenolide diterpene from *Lobophytum* sp., can restore TGF-β signaling pathway activity and induce apoptosis in fluorouracil-resistant human colon cancer SNU-C5/5-FU cells[118].

Various other Chinese herbs have been reported to exert antitumor or synergistic antitumor effects *via* TGF-β signaling pathway-mediated mechanisms. Oxymatrine, an alkaloid extracted from the Chinese herb *Sophora flavescens* Ait, can exert antimetastatic and anti-invasive effects through the inhibition of Smad2 phosphorylation and the formation of Smad2/3/4 in colorectal carcinoma RKO cells[119]. Garcinol, a natural compound extracted from *Gambogic genera*, can inhibit EC metastasis in vitro and *in vivo* by dose-dependent suppression of p-Smad2/3 expression in the nucleus[120]. In addition, a glycoprotein from the green alga *Capsosiphon fulvescens* was shown to suppress the proliferation and migration of AGS GC cells by downregulating integrin expression *via* inhibition of the TGF-β1-activated FAK/PI3K/AKT pathways[121]. However, combination therapy with 5-FU and thymoquinone, which is the main bioactive compound derived from *Nigella sativa*, enhanced antitumor effects in a preclinical rat model of colorectal tumorigenesis partly by upregulating the expression of TGF-β1, TβRII, and Smad4[122].

TGF-β SIGNALING AND EMT IN GI CANCER DRUG RESISTANCE

TGF-β secreted from tumor cells is involved in paracrine signaling cascades that promote EMT and activate CAFs. CAFs, in turn, secrete more TGF-β that further drives EMT. Extracellular TGF-β binds to its receptor, resulting in the expression of key EMT genes. Furthermore, TGF-β can promote non-Smad pathways to accelerate EMT progression[16]. It has been reported that fibronectin, a marker of EMT progression, induced EMT through Smad3/4-mediated TGF-β signaling[123]. Therefore, TGF-β is an important inducer of EMT. SW837 rectal cancer cells treated with a TβR inhibitor or transfected with TβRII siRNA exhibited downregulation of mesenchymal markers, such as N-cadherin and vimentin and EMT regulators, including Snail, Twist, Slug, and Zeb1[124]. Ginsenoside Rb2, the bioactive component in ginseng, inhibited EMT in CRC cells by inhibiting the expression of Smad4 and p-Smad2/3[125]. Similarly, eribulin significantly inhibited EMT by downregulating the TGF-β/Smad pathway in GC[126]. The EMT phenotype has been observed in GC cell lines resistant to 5-FU and AZD4547 and CRC cell lines resistant to BGJ398, PD173074, and OXA[84,111,127]. Anticancer drugs can activate the TGF-β signaling pathway and further induce EMT, which is closely associated with chemotherapy resistance and evasion of immune surveillance[10,128]. Dox treatment of HCT116 colon cancer cells was found to increase TGF-β1 and p-Smad2/3 expression and induce an EMT phenotype, exemplified by a reduction in E-cadherin and the upregulation of vimentin and N-cadherin. The changes ultimately resulted in the acquisition of Dox resistance. Furthermore, silencing Smad4 by stable RNA interference reversed the EMT process and increased the sensitivity of HCT116 cells to Dox[103]. In EAC cells, EMT has been identified as a chemoradiation resistance mechanism in which EMT is mediated by the autocrine production of TGF-β in response to chemoradiation. Neutralization of TGF-β ligands effectively counteracted chemoradiation-induced EMT by reversing the mesenchymal phenotype[129]. EAC cells incubated with trastuzumab and

pertuzumab can secrete ligands for the TGF-β receptor and induce EMT-related changes, including reduced expression of epithelial markers (CD24, CD29, and CDH1) and increased expression of mesenchymal markers (CXCR4, VIM, ZEB1, SNAI2, and CDH2), resulting in drug resistance. However, combining the drugs with a TGF-β receptor inhibitor caused the cells to regain an epithelial phenotype[106].

TGF-β SIGNALING AND CSC IN GI CANCER DRUG RESISTANCE

Emerging evidence indicates that CSCs are the main factor underlying therapeutic failure, and chemotherapeutic resistance. The TGF-β pathway has been identified as a major stem cell-associated signaling pathway. ESCC has been found to arise from CSCs. Zhao *et al*[130] showed that the TGF-β signaling pathway contributed to the lymphoid enhancer-binding factor 1-mediated CSC-like phenotype in ESCC cells. In EC, the TGF-β1 inhibitor SB525334 significantly suppressed the migration and invasion of sphere-forming stem-like cells, which possess key traits of CSCs, including chemoresistance[131]. EMT is a critical process for the generation and maintenance of CSCs and the invasive front of ESCC. Moreover, the EGFR inhibitors erlotinib and cetuximab can both markedly suppress CSCs enrichments *via* TGF-β1-mediated EMT in ESCC[132]. In mouse GC cells, activation of the TGF-β pathway downregulated the expression of Sca-1, which has been identified as a potential CSC enrichment marker. High expression of Sca-1 was related to increased resistance to cisplatin/fluorouracil-based chemotherapy[133]. In addition, TGF-β enhanced the anticancer effect of docetaxel by inducing the differentiation of gastric CSCs[99].

TGF-β SIGNALING AND TUMOR MICROENVIRONMENT IN GI CANCER DRUG RESISTANCE

TGF-β is a pleiotropic cytokine with potent immunosuppressive effects. TGF-β downregulates CD8+ and CD4+ T cell activation and stimulates the differentiation of immune-suppressive regulatory T (Treg) cells[10,114]. CRC cells secrete anti-inflammatory cytokines, including TGF-β, which can affect the dendritic cell (DC) phenotype and support tumor escape from immune surveillance[134]. However, the TGF-β receptor inhibitor SB-431542 can induce potent phenotypic and functional maturation of DCs and trigger an antitumor immune response[135]. In ESCC, TGF-β1 was shown to partially contribute to the downregulation of CD16 on natural killer (NK) cells, resulting in NK cell dysfunction[136].

TGF-β signaling pathway activation plays an important role in immune evasion and contributes to immune checkpoint therapy failure[137,138]. Enabling immune infiltration by blocking TGF-β signaling renders tumors susceptible to anti-PD-1-PD-L1 checkpoint-based therapy[115]. Moreover, the TGF-β neutralizing monoclonal antibody 1D11 markedly enhanced the abscopal effects and the overall treatment efficacy in conjunction with an anti-PD-1 plus anti-CD137 mAb combination in large MC38 colorectal tumors[114]. In ESCC, myeloid-derived suppressor cell-derived TGF-β increased PD-1 expression on CD8+ T cells, which led to resistance to PD-1/PD-L1 blockade in the tumor microenvironment. Dual PD-1/PD-L1 and TGF-β pathway blockades restored the function and antitumor ability of CD8+ T cells[139]. Furthermore, combined treatment with cyclophosphamide and interleukin (IL)-12-expressing adenovirus, which might be a valid immunotherapeutic strategy for advanced GI cancer, was shown to revert the Treg immunosuppressive phenotype by blocking the secretion of IL-10 and TGF-β, resulting in loss of their DC inhibitory activity[140].

CAFs are the most abundant cell type in the tumor microenvironment. One of the main sources of CAFs is endothelial cells undergoing EMT, which is mainly promoted by TGF-β[141]. CAFs can confer TGF-β1-mediated ESCC cell resistance to several chemotherapeutic drugs, including cisplatin, taxol, irinotecan, 5-FU, carboplatin, docetaxel, pharmorubicin, and vincristine. Inhibition of CAF-secreted TGF-β1 signaling *via* treatment with the TβRI inhibitor LY2157299 significantly enhanced chemosensitivity[142]. Moreover, TGF-β secreted by miR-27-induced CAFs induced chemoresistance to cisplatin in EC[91]. In CRC, Snail-expressing 3T3 fibroblasts exhibit CAF properties that support 5-FU and PTX chemoresistance *via* TGF-β/NF-κB-mediated CCL1 secretion[143]. Tang *et al*[144] found that, in CRC, hypoxia-inducible factor 1α (HIF-1α) and CAF-secreted TGF-β2 synergistically induced the expression of

Table 2 Studies evaluating the relationship between miRNAs and drug resistance related to the transforming growth factor-β signaling pathway in gastrointestinal cancer

miRNA	Tumor type	Target	Effect on drug resistance	Ref.
miR-21	CRC cell line HCT-116	Downregulation of TβRII	Induction of stemness	Yu *et al*[148], 2012
miR-552	CRC tissues of patients, CRC cell lines SW-480 and SW-620	The 3'-UTR of Smad2	Reduction 5-FU resistance	Zhao *et al*[150], 2019
miR-34a	CRC cell line HT29	Downregulation of the TGF-β/Smad4 signaling pathway	Acquired chemoresistance to oxaliplatin	Sun *et al*[149], 2017
miR-455-3p	ESCC cell lines Eca109 and Kyse30	Enhanced expression level of p-Smad2	Resistance to DDP and docetaxel	Liu *et al*[151], 2017
miR-27	ESCC cell line TE10	TGF-β secreted from CAF-like fibroblasts	Resistance to DDP	Tanaka *et al*[91], 2015
miR-187	DDP-resistant GC cells SGC7901/DDP	Downregulated TGF-β1 and p-Smad4	Alleviates DDP-resistance	Zhu *et al*[153], 2019
miR-204	GC cell lines AGS and SGC-7901	Target TβRII	Sensitizes GC cells to 5-FU	Li *et al*[154], 2018

5-FU: 5-fluorouracil; CAF: Cancer-associated fibroblast; CRC: Colorectal cancer; DDP: Cisplatin; ESCC: Esophageal squamous cell carcinoma; GC: Gastric cancer; miRNA: Microribonucleic acid; TGF-β: Transforming growth factor-β.

GLI2, which promoted chemoresistance.

Mesenchymal stem cells (MSCs), an important part of the tumor environment, contribute to the development of drug resistance[145]. In GC cells, TGF-β1 secretion by MSCs activated Smad2/3 and induced expression of the lncRNA MACC1-AS1 that promoted FA oxidation-dependent stemness and chemoresistance to 5-FU and OXA [146].

TGF-β SIGNALING AND MIRNA IN GI CANCER DRUG RESISTANCE

Emerging evidence indicates that some miRNAs can regulate the resistance of GI cancers to a variety of chemotherapeutic drugs through the TGF-β signaling pathway, as summarized in Table 2. In HT-29 colon cancer cells, overexpression of miR-146a was found to be associated with various processes in the cancer microenvironment, including enhancement of 5-FU and irinotecan resistance and promotion of TGF-β secretion[147]. MiR-21 was shown to increase both stemness and the overall proportion of CSCs in colon cancer cells by downregulating TβRII, a direct target of miR-21, and by activating the Wnt/β-catenin pathway[148]. MiR-34a was found to mediate OXA resistance in CRC cells by inhibiting macroautophagy *via* regulation of the TGF-β/Smad4 pathway[149]. However, the expression levels of miR-552 were negatively correlated with resistance to 5-FU-based chemotherapy in CRC cells. Mechanically, miR-552 directly targeted the 3'-UTR of Smad2, and stable knockdown of Smad2 reversed miR-552 deficiency-induced 5-FU resistance[150]. Overexpression of miR-455–3p conferred resistance to cisplatin and docetaxel in ESCC cells, whereas miR-455–3p antagonism reversed chemoresistance and reduced the number of CD90^{+} and CD271^{+} tumor-initiating cells *via* the suppression of multiple stemness-associated pathways, including TGF-β signaling[151]. Moreover, miR-27 has shown to play a role in cisplatin resistance in EC through the transformation of normal fibroblasts into CAFs and the induction of TGF-β secretion from the CAFs[91]. In GC, overexpression of miR-577 contributed to TGF-β-mediated EMT and stemness by forming a positive feedback loop, resulting in chemoresistance to OXA[152]. However, overexpression of miR-187 in GC cells alleviated cisplatin resistance by inhibiting the TGF-β/Smad signaling pathway[153]. Furthermore, overexpression of miR-204 was found to sensitize 5-FU-resistant GC cells through the suppression of TβRII-mediated EMT [154].

CONCLUSION

Drug resistance, which leads to unfavorable clinical outcomes and treatment failure, remains a considerable challenge in the treatment of GI cancers. The TGF-β signaling
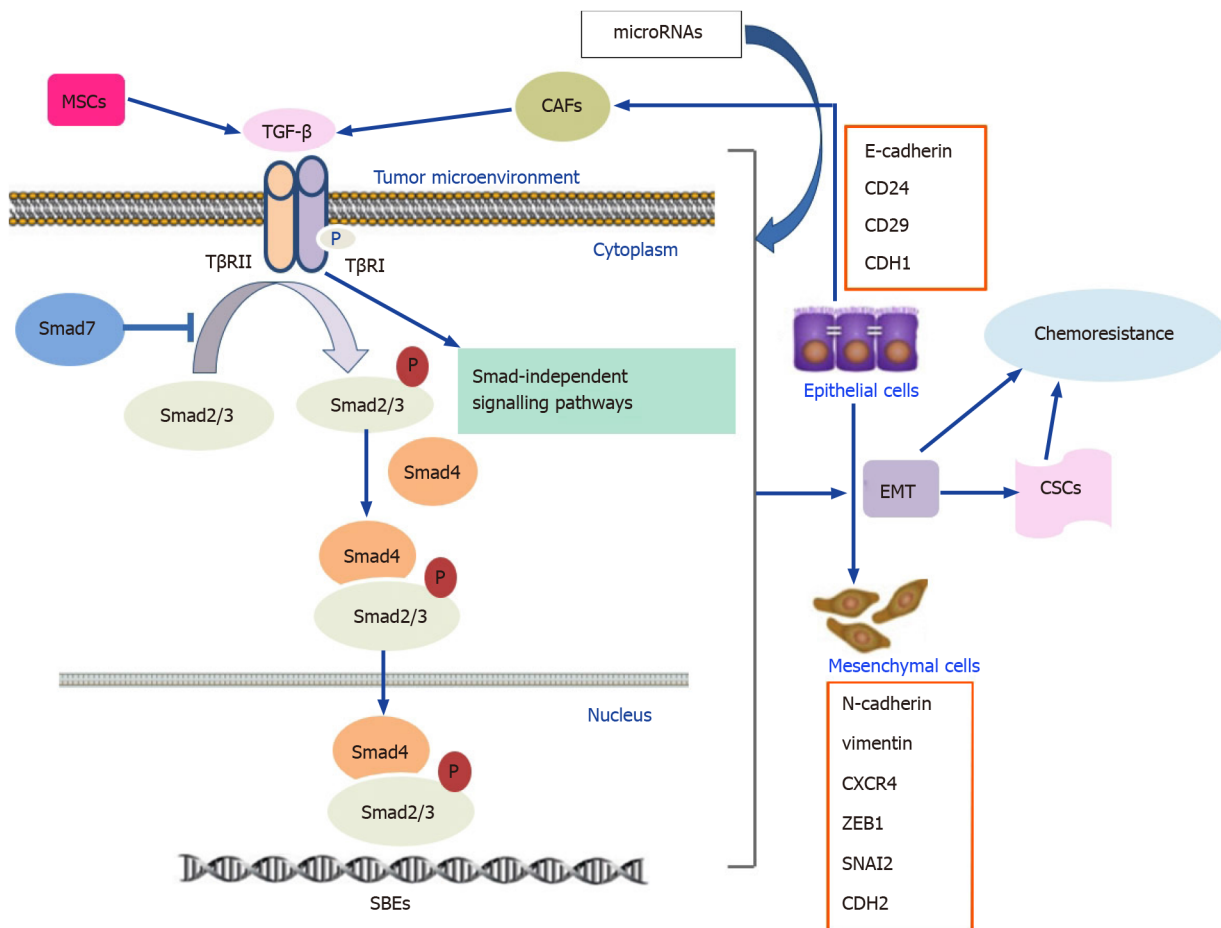
Figure 1 Mechanisms of transforming growth factor-β signaling and involvement in gastrointestinal cancer chemoresistance. CAFs: Cancer-associated fibroblasts; CSCs: Cancer stem cells; EMT: Epithelial-mesenchymal transition; MSCs: Mesenchymal stem cells; TβRI: TGF-β Type 1 receptor; TβRII: TGF-β Type 2 receptor.

pathway plays an important role in the regulation of the drug responses to conventional chemotherapy, targeted therapy, immunotherapy, and traditional medicine. Furthermore, TGF-β-mediated drug resistance in GI cancers is closely associated with several processes, including EMT, CSC development, alteration of the tumor microenvironment, and miRNA biogenesis (Figure 1).

Despite improvements in treatment strategies, EC, GC, and metastatic CRC have a poor prognosis, with 5-year OS rates of 15%–25%, 29.3%, and 14%, respectively[2,155, 156]. The key obstacle to therapeutic success is the development of drug resistance, highlighting the urgency driving the development of alternative treatments for GI cancers. Many reports indicate the benefits of combining antitumor agents with agents that suppress TGF-β signaling. However, the findings require further verification by additional clinical studies. The use of some small-molecule inhibitors of TGF-β signaling is currently being investigated in both preclinical and clinical trials[60,157]. As TGF-β possesses paradoxical activities, the identification of potential biological markers related to the response to TGF-β inhibitors would have important clinical implications and would help select patients most likely to benefit from their use.

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MINIREVIEWS

Novel perspective in pancreatic cancer therapy: Targeting ferroptosis pathway

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Abstract

Pancreatic cancer is a highly lethal malignancy with low resection and survival rates and is not sensitive to radiotherapy and chemotherapy. Ferroptosis is a novel form of nonapoptotic regulated cell death characterized by the accumulation of lipid peroxides and reactive oxygen species involved in iron metabolism. Ferroptosis has a significant role in the occurrence and development of various tumors. Previous studies have shown that regulating ferroptosis-induced cell death inhibited tumor growth in pancreatic cancer and was synergistic with other antitumor drugs to improve treatment sensitivity. Herein, we discuss the mechanism, inducers, and developments of ferroptosis in pancreatic cancer to provide new strategies for the treatment of the malignancy.

Key Words: Pancreatic cancer; Ferroptosis; Reactive oxygen species; Iron metabolism; Lipid peroxides

Core Tip: Many studies have confirmed that ferroptosis is closely related to the occurrence and development of pancreatic cancer, but there are few systematic reviews on the mechanism and treatment of ferroptosis in pancreatic cancer. This review focuses on the research progress of the mechanism of ferroptosis in pancreatic cancer, and summarizes feasible treatment from the perspective of the processes leading to the occurrence of ferroptosis.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) accounts for approximately 90% of all pancreatic malignancies and is commonly known as pancreatic cancer. PDAC is a highly lethal malignancy, wherein the number of deaths in 2018 was almost the same as the number of new cases (432,242 and 458,918, respectively; GLOBOCAN database) [1]. The clinical features of PDAC include a short course, rapid progression, and high probability of malignancy. Surgery is the only option to cure PDAC; however, most patients are diagnosed at advanced stages because of the absence of distinctive clinical symptoms, they lose the opportunity for radical surgery. The postoperative 5-year survival rate of patients with PDAC is 12%-27%[2]. Currently, adjuvant gemcitabine chemotherapy is commonly performed after surgical resection; however, the 5-year survival rate is only 22.5%-26.0%[3-5]. Moreover, the high resistance of PDAC cells to gemcitabine limits its efficacy. Novel adjuvant chemotherapy drugs, such as modified FOLFIRINOX and a 5-fluorouracil derivative (S-1), have been approved in recent years for patients who have undergone resection of PDAC. A study reported that the 3-year survival rate of patients was 63.4% in the modified FOLFIRINOX group; however, the outcome was associated with an increased risk of toxic effects[6]. The 3-year survival rate of the patients in the S-1 group was 59.0%. However, the data were limited by the fact that all the patients were East-Asian residents of Japan[7]. The global incidence of PDAC has increased over the past few decades, significantly affecting the health of the patients and causing a heavy social burden. Therefore, the need of the hour is to explore new, targeted therapies for PDAC.

Ferroptosis is a novel form of nonapoptotic regulated cell death (RCD)[8] characterized by the accumulation of lipid peroxides and reactive oxygen species (ROS) involved in iron metabolism[9]. ROS react with polyunsaturated fatty acids (PUFAs) in the lipid membrane to generate excessive amounts of lipid peroxides, resulting in cell membrane damage and eventually ferroptosis. Studies have shown that ferroptosis is involved in the occurrence and development of various diseases, such as neuropathy [10], ischemia-reperfusion injury[11], acute renal failure[12], and cancer. A study reported that ferroptosis might be a common and dynamic form of RCD in the treatment of cancer[13].

More than 90% of PDAC patients have mutations in the *KRAS* gene that promotes proliferation, alters cellular metabolism, and affects invasion and autophagy[14]. Mutations in *KRAS* lead to a significant increase in intracellular ROS[15]. To avoid cell death, cancer cells must promptly remove intracellular ROS during rapid division. A study reported that PDAC cells transport a large amount of cystine/cysteine to synthesize glutathione (GSH) as a compensatory mechanism, thereby eliminating excess intracellular ROS[16]. Ferroptosis is closely related to the production of cystine/cysteine and ROS and thus can be considered a critical form of RCD in PDAC and might be selectively targeted as an anticancer therapy. In this review, we briefly describe the mechanism of ferroptosis, its research status, and prospects for use in treating PDAC.

PROFILE AND RESEARCH PERSPECTIVE OF FERROPTOSIS

Origin

In 2003, Dolma *et al*[17] discovered an antitumor drug named erastin that induced cell death without causing changes in nuclear morphology, DNA fragmentation, and caspase 3 activation. Moreover, caspase inhibitors did not reverse the process. Subsequently, the group identified RAS-selective lethal small molecule 3 (RSL3), which induced cell death similar to that caused by erastin[18]. In 2012, Dixon *et al*[8] found that erastin inhibited the cystine/glutamate antiporter (system XC-), causing excessive accumulation of lipid ROS, ultimately leading to an iron-dependent oxidative death known as ferroptosis.

Characteristics

Compared with other RCDs such as necrosis, apoptosis, and autophagy[19] (Table 1),

Table 1 Features of ferroptosis and other forms of regulated cell death

	Ferroptosis	Necrosis	Apoptosis	Autophagy
Morphological features	Condensed mitochondrial membrane densities, reduction or vanishing of mitochondria crista, and outer mitochondrial membrane rupture	Organelle swelling, plasma membrane damage, cell disruption	Cell membrane foaming, cell shrinkage and the formation of apoptotic bodies	Cytoplasm vacuolization, formation of autophagosomes and removal of substances through lysosomes
Biochemical features	Iron accumulation; lipid peroxidation; glutaminolysis	Activation of RIPK1, RIPK3, and MLKL; activation of inflammasome and release of pro-inflammatory cytokines	DNA fragmentation; Caspases cascade activation; Ca2+/mg2+ -dependent endogenous nuclease and calpain activation	MAP1LC3B-I to MAP1LC3B-II conversion; increased autophagic flux and lysosomal

ferroptosis is characterized by the maintenance of an intact nucleus, nonaggregation of chromatin, nonrupture and foaming of the protoplast membrane, condensed mitochondrial membrane densities, reduction or loss of mitochondrial crista, and outer mitochondrial membrane rupture[9]. The biochemical characteristics of ferroptosis are increased concentration of lipid hydrogen peroxide (H_2O_2) and ferrous iron (Fe^{2+}). Intracellular lipid oxides are abnormally metabolized by the catalysis of iron ions, and the increased lipids production affects the original redox balance. Thus, the biological macromolecules are attacked, leading to cell death manifested by the inactivation of glutathione peroxidase 4 (GPX4) and deposition of lipid peroxide[20].

Main metabolic process of ferroptosis

Currently, the metabolic mechanism of ferroptosis is known to include three processes. (1) Iron metabolism includes participation of iron ions in the formation of ROS through enzymatic or non-enzymatic reactions to mediate ferroptosis; (2) Amino acid metabolism includes GSH, which is a substrate of GPX4. GSH is the most important intracellular antilipid oxidation molecule. Cysteine is the raw material required for its synthesis, and an abundance of intracellular cysteine determines the synthesis of GSH and the process of cellular resistance to lipid oxidation, ultimately affecting ferroptosis [21,22]; and (3) Lipid metabolism is involved. The accumulation of lipid peroxides, especially phospholipid peroxides, is considered a landmark of ferroptosis[23]. A recent study reported that ferroptosis suppressor protein 1 exists as an independent parallel system that cooperates with GPX4 and GSH to suppress phospholipid peroxidation and ferroptosis[24]. Furthermore, induction of ferroptosis occurs by regulating the tumor microenvironment (Figure 1).

CURRENT STATUS OF FERROPTOSIS IN PDAC

In recent years, systemic treatment of PDAC has mainly relied on 5-fluorouracil and gemcitabine-based therapy. However, because of rapid and widespread development of chemical resistance, the prognosis remains poor. Recent studies have demonstrated that ferroptosis is associated with PDAC (Figure 2). Therefore, inducing ferroptosis is a new strategy to combat PDAC.

Ferroptosis regulated by iron metabolism in PDAC

Extracellular ferric ions (Fe^{3+}) form a conjugate with transferrin and are transported *via* the transferrin receptor 1 on the surface of the cell membrane. First, the conjugate enters the cell by endocytosis. Subsequently, Fe^{3+} are reduced by the six-transmembrane epithelial antigen of prostate 3 to Fe^{2+} and enter the cytoplasm from the endosome *via* the divalent metal ion transporter 1[25]. Fe^{2+} can be stored as ferritin or in the free form. Meanwhile, ferritin, as a downstream regulatory gene of nuclear factor erythroid 2-related factor 2 (NRF2), is regulated by the p62-KEAP1-NRF2 signaling pathway[26] (Figure 2).

Excess intracellular Fe^{2+} catalyzes the Fenton reaction, in which Fe^{2+} reacts with H_2O_2 to produce Fe^{3+} and hydroxyl radicals. The hydroxyl radical is a type of ROS that can damage proteins, lipids, and DNA, affect the function of cell membranes, and lead to cell death[26]. From the perspective of chemical reactions of intracellular Fe^{2+}, the Fenton reaction may be considered one of the important processes involved in ferroptosis. The reaction between H_2O_2 and Fe^{2+} generates Fe^{3+} along with OH^- and hydroxyl radicals (Formula 1). The hydroxyl radical is one of the most active ROS. In addition, the Fenton reaction can generate peroxy free radicals[27] (Formulas 2 and 3).
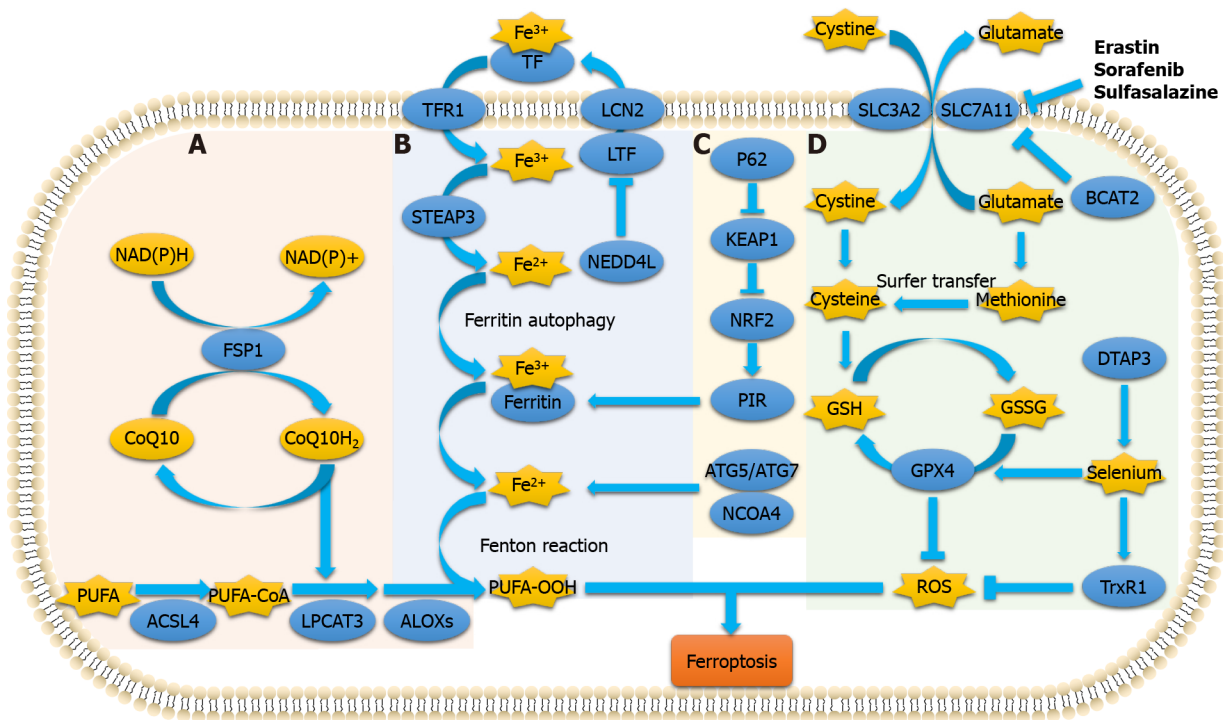
Figure 1 Metabolic mechanisms of ferroptosis in pancreatic ductal adenocarcinoma. A: Lipid metabolism; B: Iron metabolism[25]; C: Autophagy[26-28]; D: Amino acid metabolism[29-31]. ACSL4: Long-chain acyl-CoA synthetase 4; ALOXs: Arachidonate lipoxygenases; ATG5: Autophagy-related 5; ATG7: Autophagy-related 7; CoQ10: Coenzyme Q10; CoQ10H$_2$: Ubiquinol-10; DIAPH3: Diaphanous homolog 3; FSP1: Ferroptosis suppressor protein 1; GPX4: Glutathione peroxidase 4; GSH/GSSG: Glutathione; KEAP1: Kelch-like ECH-associated protein 1; LPCAT3: Lysophosphatidylcholine acyltransferase 3; LTF: Lactotransferrin; NAD(P)H: Nicotinamide adenine dinucleotide phosphate; NCOA4: Nuclear receptor coactivator 4; NEDD4L: Neural precursor cell-expressed developmentally downregulated 4-like; NRF2: Nuclear factor erythroid 2-related factor 2; P62/SQSTM1: Sequestosome 1; PIR: Pirin; PUFA: Polyunsaturated fatty acids; ROS: Reactive oxygen species; SLC3A2: Solute carrier family 3 member 2; SLC7A11: Solute carrier family 7 member 11; STEAP3: Six-transmembrane epithelial antigen of prostate 3; TF: Transferrin; TrxR1: Thioredoxin reductase 1.
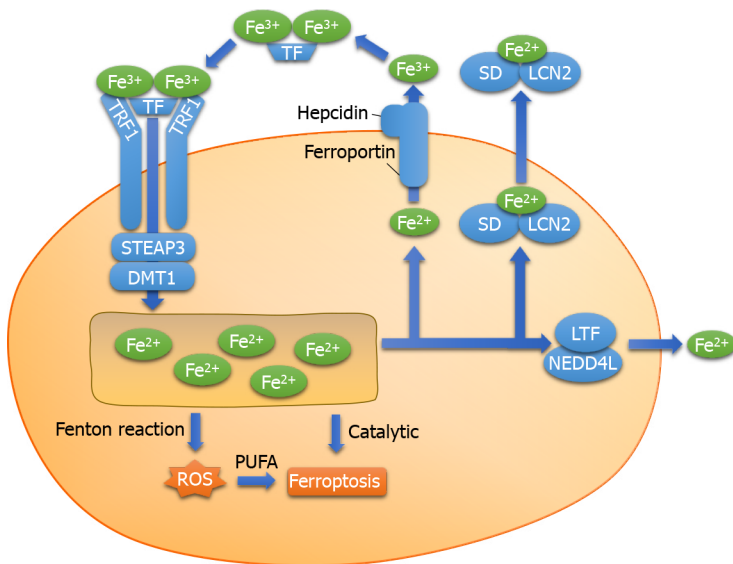
Figure 2 Iron transport. DMT1: Divalent metal transporter 1; LCN2: Lipocalin-2; LTF: Lactotransferrin; NEDD4L: Neural precursor cell-expressed developmentally downregulated 4-like. SD: Siderophore; STEAP3: Six-transmembrane epithelial antigen of the prostate 3; TFR1: Transferrin receptor 1; TFR2: Transferrin receptor 2.

The series of reactions suggest that iron ions act as a catalyst to promote the production of ROS in cells, especially in tumor cells[28]. Therefore, the Fenton reaction not only provides Fe^{2+} but also continuously catalyzes the production of ROS, both of which are essential conditions for ferroptosis. Formulas: (1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + (OH-) + OH$ (Formula 1); (2) $O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ (Formula 2); and (3) $O_2 + Fe^{2+} \rightarrow Fe^{3+} + O_2^-$ (Formula 3).

Ferritin is composed of two subunits, namely ferritin heavy chain (FHC) and ferritin light chain (FLC). A study reported that iron-responsive element-binding protein 2 increased the expression of FHC and FLC to inhibit ferroptosis[29]. Lactotransferrin (LTF) is a member of the transferrin family that is associated with increased intracellular iron during inflammatory injury and is by neural precursor cell-expressed developmentally downregulated 4-like (NEDD4L). Wang *et al*[30] reported that NEDD4L-mediated LTF protein degradation inhibited intracellular iron accumulation and subsequent oxidative damage-mediated ferroptosis in PDAC. Lipocalin-2 (LCN2) interacts with siderophores (iron-binding proteins) and acts as an iron carrier to intracellular and extracellular iron levels. Another study reported that LCN2 inhibited invasion and angiogenesis in PDAC[31].

Autophagy-dependent ferroptosis in PDAC

The autophagic degradation of ferritin to release Fe^{2+} is known as ferritinophagy, which is mediated by nuclear receptor coactivator 4 (NCOA4)[32]. Ferritinophagy is closely associated with the physiological and pathological processes of cell growth, proliferation, differentiation, apoptosis, and carcinogenesis. Under physiological conditions, ferritinophagy is tightly regulated by the iron-dependent protein network to maintain the balance of iron in cells and perform its functions. However, excessive activation of ferritinophagy leads to intracellular iron overload and accumulation of a large amount of ROS in a short period, resulting in ferroptosis. Therefore, it has been proposed that ferroptosis is a type of autophagy-dependent cell death[33].

Overexpression of NCOA4 enhances the degradation of ferritin, increases intracellular free iron levels, and promotes ferroptosis. Knockout or knockdown of autophagy-related 5 (ATG5) and ATG7-limited erastin-induced ferroptosis are associated with decreased intracellular Fe^{2+} levels and lipid peroxidation. Hou *et al*[32] further found that activating the ATG5/7-NCOA4 axis inhibited the expression of FHC and degraded ferritin, leading to an increase in intracellular Fe^{2+} and lipid ROS, thereby promoting ferroptosis in PDAC. Zhu *et al*[34] reported that heat shock protein 5 (HSPA5) is closely related to the prognosis of PDAC patients treated with gemcitabine. Activation of the HSPA5-GPX4 pathway led to the resistance of PDAC cells to gemcitabine. Inhibition of *HSPA5* or *GPX4* gene expression reversed the resistance and ferroptosis played an important role in the process. NRF2 is a transcription factor that regulates heme and iron metabolism. Pirin (PIR), an iron-binding nuclear protein, is a nuclear redox sensor and regulator. Overexpression of PIR limits oxidative damage to DNA, subsequent cytoplasmic transport, and extracellular release of high mobility group box protein 1, which is released by ferroptotic cells and subsequently triggers an inflammatory response in peripheral macrophages. NRF2 mediates the upregulation of PIR leading to autophagy-dependent ferroptosis[35,36]. In addition, the ferroptosis inducers erastin, sorafenib[37], and sulfasalazine[38], (Table 2) have been shown to activate the adenosine monophosphate-activated protein kinase/sterol regulatory element-binding protein 2 signaling pathway through iron-dependent ferritinophagy [39]. Furthermore, a phase I study revealed that the combination of sorafenib and gemcitabine demonstrated promising antitumor activity in patients with advanced PDAC[40]. However, the combination therapy did not improve recurrence-free and overall survival of patients with PDAC with postsurgical R1 residual status. However, a subgroup analysis revealed significantly improved disease-free and overall survival of patients who underwent more than six cycles of chemotherapy. Twelve cycles of additive chemotherapy with gemcitabine may be considered for patients in poor general health[41]. Therefore, research on the relationship between autophagy and ferroptosis may provide new ideas for the treatment of PDAC.

Ferroptosis is regulated by amino acid and GSH metabolism in PDAC

Cellular entry and exit of cysteine and glutamic acid require a specific transporter, system XC-, which is a heterodimer formed by the glycosylated heavy chain CD98hc, which is also called solute carrier family 3 member 2 (SLC3A2), and nonglycosylated xCT (SLC7A11) joined by disulfide bonds[42]. Cystine is reduced to cysteine to synthesize GSH and regulate downstream lipid peroxidation. As an electron donor, GSH converts toxic phospholipid peroxides into nontoxic phospholipid alcohols and oxidized glutathione under the action of GPX4[43]. In addition to system XC-, cysteine can be transported directly into the cell by the alanine-serine-cysteine system, which is also known to inhibit ferroptosis[44]. Furthermore, cystine can be synthesized from methionine *via* the transsulfuration pathway.

Many cells rely on system XC- for cystine uptake, which is the rate-limiting step for cysteine synthesis. Blocking or inhibiting this step leads to a decrease in intracellular cysteine, inhibits the lipid repair function of GPX4, and ultimately induces ferroptosis.

Table 2 Ferroptosis inducers in pancreatic ductal adenocarcinoma

Inducers	Target	Inhibited by	Ref.
Erastin	System XC-/GPX4	CPX	Yang *et al*[18]
Sulfasalazine	System XC-	β-ME, CHX, DFO, Fer-1, NAC, or Trolox	Kim *et al*[38,68]
Sorafenib	System XC-	DFO, Fer-1, Trolox, or VE	Lachaier *et al*[37]
Artesunate	System XC-	DFO or Fer-1	Xie *et al*[9]
RSL3	GPX4	CPX, DFO, Ebs, Fer-1, Lip-1, Trolox, or U0126	Yang *et al*[18]
Rapamycin	GPX4	Lip-1	Liu *et al*[60]
FIN56	GPX4	DFO, BSO and α-Toc	Liang *et al*[68]
FINO2	GPX4/Iron	β-ME or Fer-1	Liang *et al*[68]
Piperlongumine	GPX4	Fer-1, lip-1, CPX and DFO	Yamaguchi *et al*[63]
Ruscogenin	Iron	DFO, FAC	Song *et al*[59]
Irisin	Iron, ROS, and glutathione depletion	Not mentioned	Yang *et al*[64]

α-Toc: α-tocopherol; β-ME: β-mercaptoethanol; BSO: Buthionine sulfoximine; CHX: Cycloheximide; CPX: Ciclopirox olamine; DFO: Iron chelator deferoxamine; Ebs: Ebselen; FAC: Ferric ammonium citrate. Fer-1: Ferrostatin-1; Lip-1: Liproxstatin-1; NAC: N-acetylcysteine; VE: Vitamin E.

It has been reported that erastin and its analogs (*e.g.*, sulfasalazine and sorafenib) can block the transport function of system XC- and induce ferroptosis. Wang *et al*[39] studied the effect of system XC- on ferroptosis and found that branched-chain amino acid transaminase 2 (BCAT2) was the key enzyme mediating the metabolism of sulfur amino acids. BCAT2 was found to regulate intracellular glutamate concentration and its activation by ectopic expression specifically antagonized the inhibition of system XC- and protected PDAC cells from ferroptosis *in vitro* and *in vivo*. Furthermore, BCAT2 participates in the synergistic mechanisms of sulfasalazine and sorafenib to induce ferroptosis. Therefore, BCAT2 may be considered a suppressor of ferroptosis, and inhibiting intracellular glutamate synthesis might be effective in inducing ferroptosis.

Another small molecule, RSL3 directly inhibits GPX4, leading to the accumulation of lipid ROS and ferroptosis. Selenium increases the antiferroptotic activity of GPX4 through a selenocysteine residue at 46. In addition, selenium is incorporated during the synthesis of selenoproteins such as thioredoxin reductase 1 (TrxR1; direct reduction of hydroperoxides). Rong *et al*[45] reported that diaphanous homolog 3 (DIAPH3) was highly expressed in the tissues of patients with PDAC, wherein it promoted an increase of selenium content and interacted with the selenoprotein, ribosomal protein L6. DIAPH3 downregulated cellular ROS levels by upregulating the expression of TrxR1.

Ferroptosis regulated by lipid metabolism in PDAC

Fatty acids are substrates of lipid peroxidation reactions, and are esterified to form membrane phospholipids. PUFAs are more prone to oxidation than either saturated or monounsaturated fatty acids (MUFAs). Membrane phospholipids react with oxygen and adjacent lipids to generate phospholipid hydroperoxide (PL-OOH). The reaction product of Fe^{2+} and PL-OOH continues to react with lipids to generate phospholipid radicals for a new round of lipid peroxidation[46]. The degradation products of PL-OOH damage the cell membrane. Extensive lipid peroxidation affects the fluidity and structure of the cell membrane, increases its permeability, and leads to cell death. Lipid peroxidation is catalyzed by long-chain acyl-CoA synthetase 4 (ACSL4), lyso-phosphatidylcholine acyltransferase 3 (LPCAT3), and arachidonate lipoxygenase (ALOX). ACSL4 catalyzes lipid reactions and tends to esterify the acyl group of arachidonic acid, while LPCAT3 aids in the insertion of PUFA into membrane phospholipids. Subsequently, free PUFAs are catalyzed by ALOXs to produce various lipid hydrogen peroxides[47,48].

Lipid peroxides cause cellular damage through several mechanisms. The first is by the decomposition of lipid peroxides into ROS, which further amplifies the lipid peroxidation process. Second, lipid peroxides alter the physical structure of the membrane, with changes in thickness, the degree of curvature, and pore formation

that results in the release of harmful substances and disrupting intracellular metabolism. The third is *via* by-products such as malondialdehyde and 4-hydroxy-2-nonenal produced by lipid peroxidation, which can damage the cells[48]. ADP ribosylation factor 6 (ARF6) is a member of the RAS superfamily and regulates vesicular trafficking, remodeling of membrane lipids, and signaling pathways. A study reported that ARF6 regulated the sensitivity to RSL3-induced ferroptosis and enhanced RSL3-induced lipid peroxidation by affecting the level of ACSL4 protein [49]. ALOX5 is the functional subtype of the ALOX family. It catalyzes the peroxidation of PUFAs such as arachidonic acid and is a key mediator of lipid peroxidation [50]. Kuang *et al*[51] observed that NRF2 mediated the upregulation of microsomal glutathione transferase 1, which by binding to ALOX5, limited lipid peroxidation during ferroptosis in PDAC. p53 protein is a transcription factor that has an important role in preventing the development of PDAC. In addition to being regulated by a variety of cellular stressors and as a master regulator, p53 is involved in the arrest of cell growth, apoptosis, and senescence. Recently, p53 has been found to regulate a variety of cellular metabolic functions and the stress response to ROS[52]. Ou *et al*[53] discovered that p53 stimulated ferroptosis by directly activating expression of its target gene spermidine/spermine N1-acetyltransferase 1, which triggers ferroptosis upon stress from ROS. The activity of PUFAs in ferroptosis is competitively affected by MUFAs, indicating that exogenous MUFAs cause resistance to ferroptosis. The resistance depends on ACSL3 or stearoyl-CoA desaturase (SCD/SCD1), an enzyme involved in fatty acid biosynthesis, primarily the synthesis of oleic acid[35]. Ye *et al*[54] found that F-box and WD repeat domain-containing 7 promoted both ferroptosis and apoptosis in PDAC by downregulating SCD1 and inhibited the transcription of SCD1 by reducing the binding of NR4A1 to the SCD1 promoter.

Ferroptosis is regulated by the tumor microenvironment in PDAC

The tumor microenvironment, including the tumor cells, vascular system, extracellular matrix, and immune cells, is an important factor affecting the outcomes of therapy. It has been reported that nano-inducers of ferroptosis attract iron from the extracellular environment to increase the intracellular content. It has also been shown that simultaneous upregulation of FHC and downregulation of GSH that increased the levels of intracellular ROS led to ferroptosis in tumor cells[55]. Another inducer is a near-infrared photosensitizer, IR780, which can be loaded into perfluorocarbon nanodroplets. The function of the inducer functions depends on differences of the microenvironments of normal and tumor tissue such as oxygen level, pH, and the immune system, among other factors. Photodynamic therapy activated oxygen enriched with perfluorocarbon generated ROS in the tumor tissue to kill the tumor cells [56]. Traditionally, CD8+ T cells in the tumor microenvironment induce cell death through perforin, granzyme, and Fas/Fas ligand pathways. However, a recent study demonstrated that immunotherapy-activated CD8+ T cells enhanced ferroptosis-specific lipid peroxidation in tumor cells. Interferon-gamma released from CD8+ T cells was found to downregulate the expression of SLC3A2 and SLC7A11, restrain cystine uptake in tumor cells, and promote lipid peroxidation and ferroptosis[57]. In addition, various macrophage subsets have different sensitivities to ferroptosis. Resting macrophages can be polarized to form antitumor M1 and procarcinogenic M2 subtypes. Dai *et al*[58] reported that KRAS^{G12D} caused macrophages to switch to an M2-like protumor phenotype *via* signal transducer and activator of transcription 3-dependent fatty acid oxidation, which can be considered a key mediator of cancer cell-macrophage communication in PDAC. Furthermore, oxidative stress induced the release of KRAS^{G12D} protein from cancer cells undergoing ferroptosis. Targeting the tumor microenvironment to promote ferroptosis of PDAC cells could be a new strategy for cancer therapy (Figure 3).

INDUCING FERROPTOSIS TO TREAT PDAC

Ferroptosis has an important role in tumor cell death and inhibition of tumor growth; therefore, inducing ferroptosis in PDAC is expected to become a new therapeutic strategy. Inducers of ferroptosis can be divided into several categories based on the regulatory mechanism.

Iron metabolism

Song *et al*[59] reported that ruscogenin induced ferroptosis by regulating the levels of transferrin and ferroportin. Ruscogenin increased the concentration of intracellular
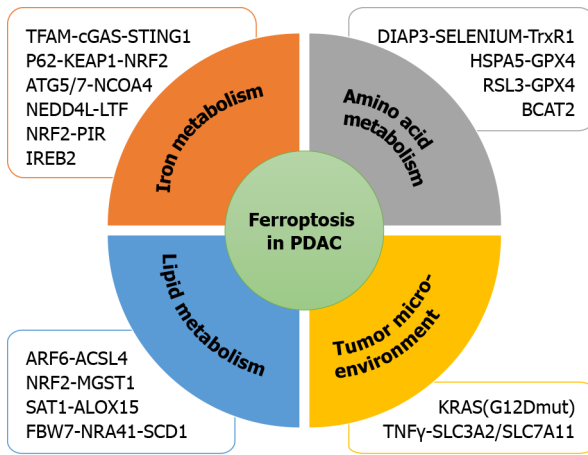
Figure 3 Regulatory molecules of ferroptosis in pancreatic ductal adenocarcinoma. ARF6: ADP ribosylation factor 6; ATG5/7: Autophagy-related 5/7; BCAT2: Branched-chain amino acid transaminase 2; cGAS: Cyclic GMP-AMP synthase; FBW7: F-box and WD repeat domain-containing 7;IREB2: Iron-responsive element-binding protein 2; HSPA5: Heat shock 70-kDa protein 5; LTF: Lactotransferrin; MGST: Microsomal glutathione transferase 1; NCOA4: Nuclear receptor coactivator 4; NEDD4L: Neural precursor cell-expressed developmentally downregulated 4-like; NR4A1: Nuclear receptor subfamily 4 group A member 1; PIR: Pirin; RSL3: RAS-selective lethal small molecule 3; SAT1: Spermidine/spermine N1-acetyltransferase 1; SCD1: Stearoyl-CoA desaturase-1. STING1: Stimulator of interferon genes; TFAM: Transcription factor A, mitochondrial.

Fe^{2+} and the production of ROS, which was inhibited by deferoxamine.

Ferritinophagy

Liu *et al*[60] observed that rapamycin caused autophagy-dependent ferroptosis by inducing the degradation of GPX4 protein but did not inhibit GPX4 gene transcription. In animal studies, the researchers observed that GPX4 depletion in PDAC cells enhanced the anticancer activity of rapamycin *in vivo*. Li *et al*[61] proposed a new model of cell death, wherein mitochondrial DNA stress triggered autophagy-dependent ferroptosis. Degradation of zalcitabine-induced transcription factor A, mitochondrial triggered oxidative DNA damage, the release of mitochondrial DNA into the cytosol, and subsequent activation of the cyclic GMP-AMP synthase-stimulator of interferon genes pathway. Zalcitabine suppressed pancreatic tumor growth *via* the autophagy-dependent ferroptosis.

Lipid metabolism

Several key enzymes (ACSL4, LPCAT3, and ALOXs) are involved in lipid oxidation and can be regulated to induce ferroptosis. Studies have reported that erastin and RSL3 induced ferroptosis in PDAC[62], and ALOXs enhanced the sensitivity of *RAS*-mutated tumor cells to erastin and RSL3[50].

Amino acid metabolism

Piperlongumine (PL) is a natural product with cytotoxic properties restricted to cancer cells. PL acts by significantly increasing ROS levels in an iron-dependent manner. Yamaguchi *et al*[63] found that PL rapidly induced the death of human PDAC cells chiefly through the inhibition of GPX4, and sulfasalazine enhanced cell death. Moreover, sulfasalazine enhanced the cancer cell-killing ability of the combination of PL and cotylenin A, which is a plant growth regulator with potent antitumor activity.

Comprehensive regulation

Bao *et al*[64] investigated the effects of irisin on the expression of the ROS-related protein NRF2 and the autophagy-related protein, microtubule-associated protein 1A/1B-light chain 3 during ferroptosis. They observed that irisin promoted the up-regulation of erastin-induced free iron, lipid ROS, and GSH depletion and positively regulated ferroptosis in PDAC. Eling *et al*[65] reported that artesunate (ART) was a specific activator of ferroptosis in PDAC cells and that erastin and ART activated ferroptosis in PDAC cell lines in an iron- and ROS-dependent manner. ART-induced ferroptosis was most effective in mutationally-active *KRAS* expressing PDAC cell lines. Subsequently, Wang *et al*[66] showed that inhibition of 78-kDa glucose-regulated protein 78 reversed the resistance of PDAC cells to ferroptosis and increased tumor sensitivity to ART.

CONCLUSION

Ferroptosis is a new model of cell death induced by small molecules such as erastin and RSL3, which are regulated at multiple levels. In this review, we briefly described the mechanism of ferroptosis, which includes iron, amino acid, and lipid metabolism, and summarized the regulatory pathways of ferroptosis in PDAC. The occurrence and development of ferroptosis are accompanied by the accumulation of ROS, resulting in lipid peroxidation of the cell membrane. Inducing ferroptosis can cause the death of PDAC cells, and can have a synergistic role with anticancer drugs to improve the sensitivity of PDAC to the existing treatment modalities. In addition, the level of ferroptosis inducer is associated with the prognosis of the disease. Therefore, induction of ferroptosis may have potential as a treatment of PDAC. However, ferroptosis has not been studied extensively in PDAC. A study reported that knockout of the *GPX4* gene in B1 and marginal zone B cells triggered ferroptosis by inducing lipid peroxidation, thus affecting the immune response of B cells[67]. However, there are no studies of B cells and ferroptosis in PDAC. Therefore, clarification of the molecular mechanism of ferroptosis and exploration of its role in the development and treatment of PDAC will help explain not only the mechanism of cell death and escape of PDAC cells, but also to develop novel effective therapeutic targets.

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Liver tumors in children with chronic liver diseases

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Abstract

Liver tumors are rare in children, but the incidence may increase in some circumstances and particularly in chronic liver diseases. Most liver tumors consequent to chronic liver diseases are malignant hepatocellular carcinoma. Other liver tumors include hepatoblastoma, focal nodular hyperplasia, adenoma, pseudotumor, and nodular regenerative hyperplasia. Screening of suspected cases is beneficial. Imaging and surrogate markers of alpha-fetoprotein are used initially as noninvasive tools for surveillance. However, liver biopsy for histopathology evaluation might be necessary for patients with inconclusive findings. Once the malignant liver tumor is detected in children with cirrhosis, liver transplantation is currently considered the preferred option and achieves favorable outcomes. Based on the current evidence, this review focuses on liver tumors with underlying chronic liver disease, their epidemiology, pathogenesis, early recognition, and effective management.

Key Words: Liver tumor; Chronic liver disease; Children; Hepatocellular carcinoma; Liver cancer; Liver neoplasm

Core Tip: Liver tumors in children are rare, although children with underlying chronic

liver diseases may present a higher risk. Early detection and timely management lead to a good prognosis and outcome. Recently, contrast enhanced ultrasound has been the preferred modality for surveillance to identify and classify the etiology of liver tumors. As the more frequent liver tumors in children with chronic liver diseases are mainly malignant, liver transplant should be considered as the first option to achieve favorable results. In addition, regular assessment is necessary in asymptomatic benign liver tumors with the potential for malignant transformation.

INTRODUCTION

Liver tumors are rare[1] compared to other neoplasms in the pediatric population, and are mostly asymptomatic. However, most liver tumors are malignant neoplasms that necessitate timely management—especially in children who present with predisposing factors, including chronic liver diseases from genetic and metabolic origins. The most frequently described liver neoplasms in children with chronic liver diseases are hepatocellular carcinoma (HCC), hepatic adenoma (HA), focal nodular hyperplasia (FNH), hepatoblastoma (HB), pseudotumor, and nodular regenerative hyperplasia (NRH). The key preventative approach for liver tumors is not only specific management for the chronic liver disease itself but also tumor surveillance. As different tumors require different management approaches, tumor type identification is crucial. Collaboration among multicenter study groups, including the Children's Oncology Group, the International Childhood Liver Tumor Study Group, the German Society for Pediatric Oncology and Hematology, and the Japanese Study Group for Pediatric Liver Tumors, are necessary to obtain meaningful data regarding natural history, management, and long-term prognosis of these tumors in children[2].

EPIDEMIOLOGY

Liver tumors are rare and account for approximately 1%–4% of tumors in children[3] or 0.5–2.5 cases per million children per year[4-7]. The incidence rates of liver tumors in children regardless of the presence of underlying chronic liver diseases are HB (37%), HCC (21%), benign vascular tumors (15%), sarcoma (8%), mesenchymal hamartoma (7%), FNH (5%), HA (2.5%), and other forms (4%)[8]. In children with chronic liver diseases, the most common primary tumor is HCC. The incidence of HCC increases to 30%, as HCC may develop in children with a background of chronic liver diseases[8]. Mother-to-child transmission of hepatitis B virus (HBV) infection and tyrosinemia are significant predisposing factors associated with HCC. The lifetime risk of developing HCC from chronic HBV infection is estimated to be 10%–25% or 100 times that of the normal population[9,10] whereas the incidence of HCC from tyrosinemia is approximately 14%–75%[11-13]. The prevalence of HCC associated with different liver diseases has been reported, and includes biliary atresia (BA) (1.3%)[11], progressive familial intrahepatic cholestasis (PFIC) type 2 or bile-salt excretory protein deficiency (5%–15%)[14], congenital portosystemic shunt (2.5%)[15], and Wilson's disease (0.67%)[16]. Liver adenoma has been reported to be more frequently associated with glycogen storage disease (GSD) type 1, but rarely in types 3 and 4, and has not been reported in type 6 or 9[15]. Interestingly, the association between non-alcoholic fatty liver disease (NAFLD) with or without fibrosis and HCC has recently been reported in adults, raising concern and indicating the need for surveillance strategies for early lesion detection[17]. Nonetheless, there has only been one case report describing NAFLD and HCC in a child[18]. Moreover, apart from HCC, the prevalence of benign liver tumors such as FNH and NRH in children with chronic liver diseases has been underreported[8].

PATHOGENESIS AND CHARACTERISTICS OF LIVER TUMORS IN CHR-ONIC LIVER DISEASES

In the adult population, the pathogenesis of liver tumors in chronic liver diseases has been generally associated with a liver injury causing hepatocellular proliferation. However, up to 70% of pediatric HCC develops in normal liver tissue[19]. Potential genetic factors predisposing liver tumor in children without chronic liver diseases include familial adenomatous polyposis (FAP), Fanconi anemia, ataxic telangiectasia, Beckwith-Wiedemann syndrome (BWS), trisomy 18, neurofibromatosis, and tuberous sclerosis, which will not be the discussed in this review. With regard to chronic liver diseases, the process of liver injury and inflammation may promote liver cell regeneration[20]. If the injury continues or includes other predisposing factors, it could lead to liver cirrhosis and progression to liver neoplasm. Predisposing factors (Table 1) include the dysregulation of liver proliferation and promotion of telomere shortening [21]. In addition, primary liver injury or liver injury secondary to oxidative stress could induce dysregulation of signaling pathways involving protumorigenic growth factors and cytokines[22] such as insulin-like growth factor, hepatocyte growth factor, the wingless signaling pathway, transforming growth factor-α, epidermal growth factor, and transforming growth factor-β. For example, increasing oxidative stress resulting from an deficiency of antioxidant enzymes caused by the homozygous PiZZ mutation of α-1 antitrypsin could induce liver damage[23] and rarely, HCC in children [24,25]. Furthermore, procarcinogenic genetic factors such as p53 mutations leading to telomere-induced genomic instability are strongly associated with malignant liver neoplasm.

Infections from HBV and hepatitis C virus (HCV) may result in allelic deletions and p53 mutations, and are considered strong inducers of hepatocarcinogenesis leading to HCC[26]. Toxic substances such as the accumulation of toxic metabolites in tyrosinemia type 1 disorder (including maleyl acetoacetate, fumaryl acetoacetate and succinyl acetone) may also lead to the development of liver neoplasm. Hence, the incidence of HCC in children is reportedly 13%–37%, when tyrosinemia is diagnosed beyond 2 years of age[27,28]. Another example of a metabolic disturbance causing liver neoplasm is GSD type 1, and rarely type 4, in patients with poor dietary control. A decrease in tumor suppressor kinase-1 expression might explain the pathogenesis of adenoma. Interestingly, obesity is a well-known major risk factor for cancer involving a process of a low-grade, chronic inflammatory responses. Consequently, lipotoxicity from the ectopic deposition of fat in the liver may contribute to the development of liver neoplasm in the obese population with NAFLD[29]. Moreover, cholestasis and bile salt accumulation, which may cause liver neoplasm due to an increased risk of liver tumors, has been reported in patients with BA and PFIC type 2 and 3[30,31]. Several case reports have described HCC in infants with BA and cirrhosis at the age of 1 year[32] and HB has been reported in three children diagnosed with congenital hepatic fibrosis and polycystic disease at the age of 2 years[7]. Finally, it has been hypothesized that both intrahepatic and extrahepatic shunts are associated with neoplasms of the liver due to local hemodynamic instability[33].

CLINICAL MANIFESTATIONS AND CHARACTERISTICS OF LIVER TUMORS

Although children with liver tumors are commonly asymptomatic, some children present with tumor complications, including abdominal pain, fever, jaundice, cholangitis, anemia, fatigue, and portal hypertension. Specific sequelae can be commonly observed in individual tumors and include tumor bleeding in HA, lung and bone metastasis in HCC[7,34], and fever with thrombosis in HB[35]. Liver tumors in chronic liver diseases are summarized in Table 2.

HB

HB is the most common malignant liver tumor in children aged less than 5 years[4]. The majority of predisposing factors of HB include premature birth with very low birth weight and genetic diseases such as FAP, BWS, trisomy 21, Li-Fraumeni syndrome, congenital portosystemic shunt, GSD type 1 and 3, and tyrosinemia. Nonetheless, HB has a better prognosis compared with other malignant liver tumors, especially with early detection. In terms of risk stratification, the Children's Hepatic Tumors International Collaboration identified younger age (< 1 year), PRETEXT classi-

Table 1 Predisposing factors in developing hepatic tumors in chronic liver diseases

Predisposing factors
Oxidative stress
Dysregulation of protumorigenic growth factors and cytokines
Genetic factors: p53 mutation, telomere shortening, homozygous PiZZ mutation, tumor suppressor kinase-1 expression
Hepatocarcinogenesis: HBV, HCV, HIV
Toxic substances: Tyrosinemia type I (maleyl acetoacetate, fumaryl acetoacetate and succinyl acetone), PFIC type 2 and 3 (bile salt)
Metabolic disturbance: Glycogen storage disease type 1 and 4, obesity and NAFLD
Vascular disruption: Congenital absence of portal vein, noncirrhotic portal hypertension, Budd-Chiari syndrome

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; NAFLD: Non-alcoholic fatty liver disease; PFIC: Progressive familial intrahepatic cholestasis.

Table 2 Liver tumors identified in chronic liver diseases

Liver disease and main pathogenesis	Tumor type
Genetic or metabolic syndromes	
Hereditary tyrosinemia type 1[80-83]	HCC
GSD type 1, 3, 4	HA, HCC, HB
Alagille syndrome	HCC, regenerative nodule
Other familial cholestatic syndromes	HCC
NAFLD	HCC
α-1 antitrypsin deficiency	HCC
Infections	
HBV	HCC
HCV	HCC
Vascular	
Abernethy	FNH, HCC, HA
Noncirrhotic portal hypertension	NRH
Congenital portosystemic shunt	HCC, HB
Cirrhosis and cholestatic conditions	
Biliary atresia	HCC, FNH, pseudotumor
Autoimmune hepatitis	HCC
Wilson disease	HCC
Congenital hepatic fibrosis	HCC
Cryptogenic cirrhosis	HCC

FNH: Focal nodular hyperplasia; GSD: Glycogen storage disease; HA: Hepatic adenoma; HB: Hepatoblastoma; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; NRH: Nodular regenerative hyperplasia.

fication I and II, and well-differentiated or fetal cell subtype could predict good outcomes[36] (Figures 1 and 2).

HCC

Unlike HB, HCC is a rare malignant tumor in children. However, the incidence increases in patients with underlying chronic liver diseases or in the presence of a specific genetic syndrome. HBV and HCV are fatal causes of HCC in endemic areas of South Africa and Asian countries. Genetic and metabolic diseases that closely
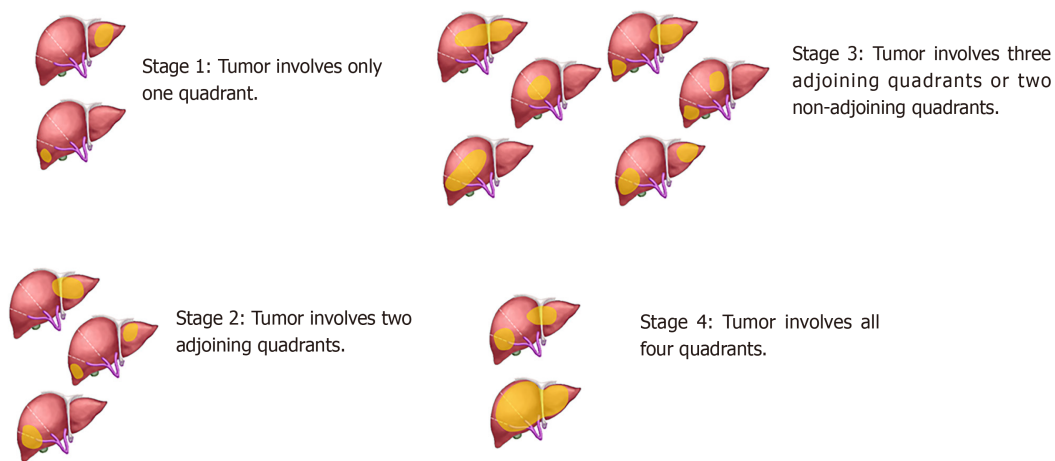
Figure 1 Definition of the pretreatment extent of disease or PRETEXT classification for the malignant liver tumor.
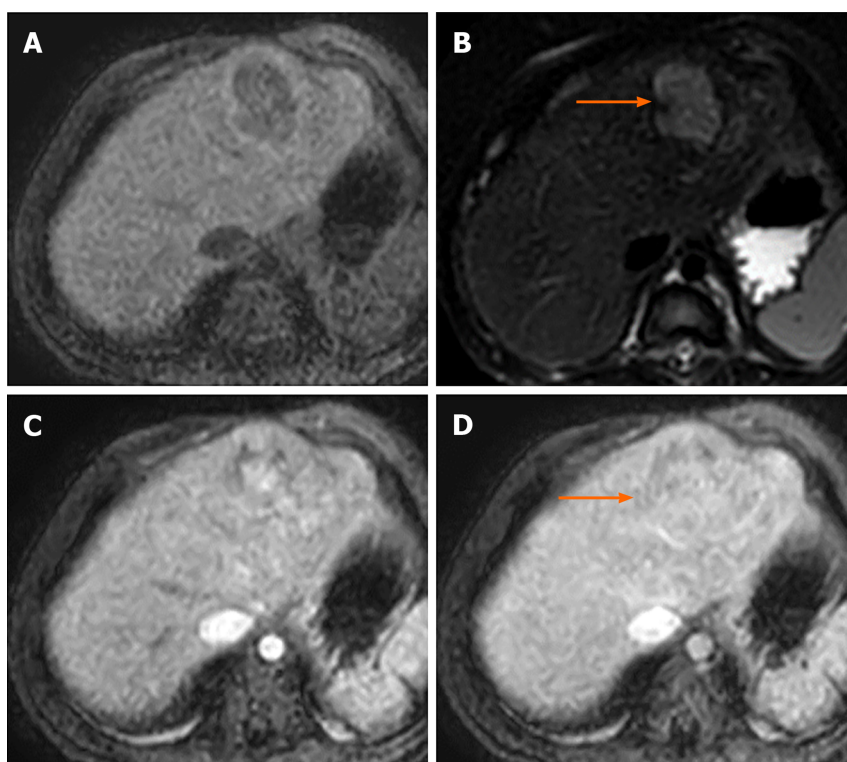
Figure 2 Magnetic resonance images of a 2-year-old boy with underlying abernethy malformation presenting with an incidentally identified liver mass with pathological tissue diagnosed hepatoblastoma. A: Mass showing well-defined hypointense liver parenchyma on T1W and; B: Hyperintense parenchyma on T2W images; C: This mass revealed heterogeneous arterial hyperenhancement; and D: Venous enhancement after the administration of gadolinium-based contrast agent.

associated with HCC are tyrosinemia, and PFIC types 2 and 3. Because of the initial nonspecific symptoms, only 50% of cases present high levels of alpha-fetoprotein (AFP), most children have been diagnosed with more advanced disease with only a 20% possibility of complete removal of the tumor mass. Unfortunately, HCC is largely chemoresistant to therapy. If a tumor is unresectable but there is no evidence of extrahepatic metastasis, liver transplantation must be considered[8].

HA

HA is a spherical or ovoid, well-circumscribed tumor without vascular or bile duct involvement, and usually presents as a solitary mass (70%–80%). Multiple adenomas are commonly observed in GSD type 1[37], and might be associated with a high frequency of β-catenin mutations and a lack of hepatocyte nuclear factor-1 alpha inactivation[38]. Other liver conditions involved include GSD type 3 and 4,

tyrosinemia, galactosemia, and congenital or acquired portosystemic shunts[39,40]. The regression of HA is possible if predisposing factors are eliminated. The tumor is usually complicated by rupture or hemorrhage (10%)[41] especially if > 5 cm in size. Malignant transformation rarely occurs but requires long-term monitoring (Figures 3 and 4).

FNH

FNH comprises normal focal liver parenchymal with bile duct proliferation and vascular anomalies[42,43]. As the hypothesis of FNH pathogenesis involves the response of liver cells to local vascular abnormalities[44], FNH is usually associated with portal vein agenesis or hypoplasia and the Budd-Chiari syndrome[45]. This tumor is typically a single lesion less than 5-cm in size, located near the liver surface [46]. Despite the homogenous normal liver, central scars or fibrous areas surrounding the large vessels are the main characteristics. This tumor is a true benign neoplasm and is usually asymptomatic. Moreover, the tumor may regress if the underlying vascular disturbance is corrected. However, a case of FNH in a child with biliary atresia and cirrhosis has been reported by our center (Figure 5).

NRH

NRH is defined as normal parenchyma with small diffuse regenerative nodules without or with minimal fibrosis. It is a very rare tumor that might be the result of microcirculatory disturbances. Vascular disorders leading to atrophic hepatocytes are followed by compensatory regeneration. Liver conditions related to NRH include chronic Budd-Chiari syndrome, human immunodeficiency virus, antiviral agents, congenital absence of the portal vein[47], and post-liver transplantation[48]. Portal hypertension may occur in up to 50% of patients[49]. Imaging of NRH is very similar to that observed in cirrhosis with nodule sizes usually between 1 and 3 mm. The absence or only presence of 0–1 fibrous septa in histopathology may distinguish NRH from cirrhosis[47,50]. Long-term follow-up is recommended as malignant transformation has been reported[51], with a 5-year cumulative incidence of 4%[11].

Other hepatic lesions

Pseudotumor or giant regenerative nodule is an unusual benign hepatic lesion in the background of chronic liver disease or cirrhosis. The incidence of pseudotumor in children with BA is reportedly 3.8%[52]. Well-formed tumors are rarely bleeding or necrotic. The tumor characterization is similar to FNH but with no central scars. A peripheral tumor capsule could distinguish the pseudotumor from HCC. The typical imaging features of this pseudotumor have not been well described. Computed tomography (CT)-guided biopsy is sometimes needed in inconclusive cases[53,54]; however, needle-track seeding[55] should be considered if the tumor is proven malignant. Pseudotumors should be included in the differential diagnosis of liver masses in children with chronic liver diseases or cirrhosis.

Dysplastic nodules are considered precancerous nodules present in chronically diseased livers. It is believed that these dysplastic nodules are responsible for the malignant transformation of nodules progressing towards HCC. Histological evaluation of the nodule reveals hepatic parenchyma with some degree of cellular atypia. Magnetic resonance imaging (MRI) studies, with special contrast agents such as extracellular contrast, hepatic-specific contrast, and reticuloendothelial contrast are the best techniques for the differential diagnosis of this small nodule from HCC[56].

SCREENING AND INVESTIGATION

Most liver tumors identified in chronic liver diseases are malignant. Although there is no international guidelines available that address the frequency for screening liver tumors in high-risk children, early detection might be necessary for timely management. HCC can develop very early, particularly in chronic liver diseases. A recent case report described the development of HCC in a 4-year-old child with vertical transmission of HBV infection[32]. HCC and HB in infants with cirrhosis due to BA have been described as early as 1 year of age[31,32], and in a 2-year-old child with congenital hepatic fibrosis and autosomal recessive polycystic disease[7]. However, children with tyrosinemia who are under medical management or those who have undergone liver transplantation, remain at low risk for developing HCC[13, 57]. Consequently, early and long-term surveillance is recommended.
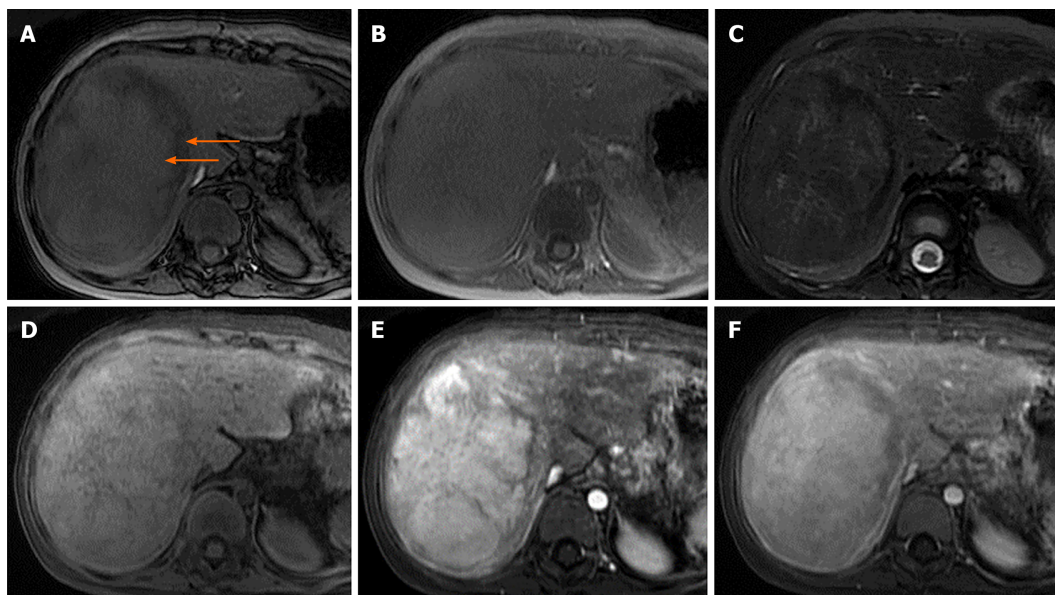
Figure 3 Magnetic resonance images of a 10-year-old girl with extrahepatic hypertension from portal vein thrombosis status post-splenectomy and proximal splenorenal shunt with developing liver mass with pathological tissue diagnosis of hepatic adenoma. A: Axial dual gradient echo opposed-phase images revealed a heterogeneous drop in signal intensity (arrows); B: Axial dual gradient echo in the in-phase image revealing the heterogeneous microscopic fat in the mass; C: Heterogeneously mild hyperintense mass in T2-weighted (T2W) image; D: Iso-to-slightly hyperintense mass in the T1W image; E: Intense arterial hyperenhancement after gadolinium-based contrast administration; F: Heterogeneous venous enhancement after gadolinium-based contrast administration.

AFP

In cases of underlying liver disease, screening with noninvasive modalities is suggested with AFP followed by imaging studies after risk stratification. AFP is the preferred tumor marker to evaluate liver masses with an increase of 90% for HB and 50% for HCC in children. However, young children present high baseline levels of AFP, which decrease over time to adult levels at 8 mo of age[58]; thus, interpretation is challenging for infants at this age. An increase in normal AFP levels in some benign tumors and HB have also been evidenced[59,60]. Hence, AFP alone is not recommended for the initial screening for liver tumors. Imaging as another screening modality is also required in parallel.

Imaging studies

Imaging modalities are the primary diagnostic investigations as they are less invasive and informative[33]. Abdominal ultrasound (US), CT, and MRI are optional and depend on the availability of resources. Abdominal US is frequently used as this technique is non-radiating and rarely requires deep sedation or anesthesia. US with Doppler may provide additional tumor information including size, echogenicity, focality, border, vascular involvement, and presence of thrombi. A limitation is operator dependence. Recently, contrast enhanced US (CEUS) has been proposed to be a promising imaging modality as its performance is comparable to CT and MRI, and has a specificity of 98% for identifying benign liver lesions and a negative predictive value of 100%[61]. This technique uses an US contrast agent such as SonoVue® that was approved for use in both adults and children by the United States Food and Drug Administration in 2016[62]. CEUS should be considered for use as a follow-up measure in children with known hepatic diseases thus minimizing radiation exposure using a cost-effective approach[63,64]. SonoVue® is reportedly safe in children[65] and extensive data from a prospective multicenter study of 23188 adults showed the rate of adverse events was 0.125% and serious adverse occurred in 0.0086%[66]. Although US could define the origin of the liver tumor, CT or MRI are able to more accurately describe tumor characteristics, particularly the tumor border and eventual extensions to adjacent organs or into vessels. MRI is the most sensitive imaging modality for regenerative and dysplastic nodules but is comparable with CT for HCC detection with a lower false positive rate than MRI[67] (Table 3). Annual screening for liver tumors by imaging modalities in high-risk patients is reasonable. Once the tumor is detected and the size is < 3 cm, the American Association of the Study of Liver Disease and the European Association for the Study of the Liver recommend US screening at 3-

Table 3 Typical imaging appearances of liver tumors

Tumors	US with doppler	CT	MRI
HB	Well circumscribed hyperechoic or heterogenous echogenic lesion	Hypoattenuating lesion in non-contrast image with heterogeneous arterial and venous enhancement	T1W; hypointense
			T2W; hyperintense
			Heterogeneous arterial and venous enhancement
HCC	Variable from hypo-, iso-, or hyperechoic from internal fat, necrosis or hemorrhage	Well- or poorly defined, hypoattenuating lesion with arterial hyperenhancement and venous "wash-out" with/without delayed capsular enhancement	T1W; hypointense
			T2W; hyperintense
			Early arterial enhancement and wash-out with relative low signal intensity on venous and delayed phases
		Tumor thrombus enhancement in portal vein	Delayed capsular enhancement
FNH	Homogenous, well-circumscribed	Homogeneous, well-circumscribed iso- to slightly hypoechoic lesion	T1W; iso- to slightly hypointense with hypointense scar
			T2W; iso- to slightly hyperintense with hyperintense scar
		Hypoattenuating scar	Enhancement pattern same as CT
	Internal color flow in the central scar extending to the periphery in a spoke-wheel pattern	Arterial and early portal venous enhancement and becomes isoattenuating to liver in the late portal venous and delayed phases	Normal or increased uptake on delayed hepatobiliary phases of the hepatocyte specific contrast agent
Adenoma	Hyperechoic lesion in the normal liver	Well-circumscribed hypoattenuating lesion with hyperattenuation if hemorrhaging	T1W; hyperintense
			T2W; hyperintense
			Fat component; Signal dropout on opposed-phase or fat suppression images
	Hypoechoic lesion in the background of diffuse fatty infiltration or glycogen storage	Intense arterial enhancement and isoattenuating in venous and delayed phases	Peripheral pseudocapsular enhancement
			Enhancement pattern same as CT
NRH	Multiple tiny and typically isoechoic lesions, difficult to detect.	Slightly hypo- or isoattenuating lesion to liver	T1W; homogenous and slightly hyperintense.
			T2W; variable
		Isoattenuation to liver in both arterial and portal venous phases	Enhancement in portal phase like normal liver parenchyma

CT: Computed tomography; FNH: Focal nodular hyperplasia; HB: Hepatoblastoma; HCC: Hepatocellular carcinoma; MRI: Magnetic resonance imaging; NRH: Nodular regenerative hyperplasia; US: Ultrasonography.

to 6-mo intervals for adult patients[68], as this interval growth is the best indicator for malignant liver tumor transformation. There are no international guidelines for tumor surveillance in children and thus, many centers adopt adult guidelines instead.

Histopathology evaluation of liver tissue

Liver biopsy is considered an invasive procedure. The reported incidence of complications after percutaneous liver biopsy in children is 6.83%, of which 2.4% experience a major complication[69]. However, liver biopsy for histopathology evaluation might be necessary in cases with inconclusive findings from imaging and with the surrogate marker AFP. Identification of the cytologic malignancy and hepatocellular differentiation are important for HCC diagnosis. In cases with inadequate liver samples or with no distinction between two diagnostic features of focal distribution in other areas or well-differentiated HCC in origin, it is extremely difficult to distinguish HCC from HA or dysplastic nodules. Special staining should be helpful if malignancy is not clearly evidenced. Markers favoring HCC over HA include glycipan-3 (GPC3), and loss of reticulin network by reticulin staining. In addition, markers favoring HCC over high-grade dysplastic nodules are GPC3, heat shock protein 70, glutamine synthetase, and cluster of differentiation 34 (diffuse staining) (Figure 6A-C)[70].

With regard to FNH, which is a truly benign lesion and does not require active management, differential diagnosis from HA and HCC is sometimes challenging. Atypical FNH lesions on imaging studies, in which no central scar pattern is present
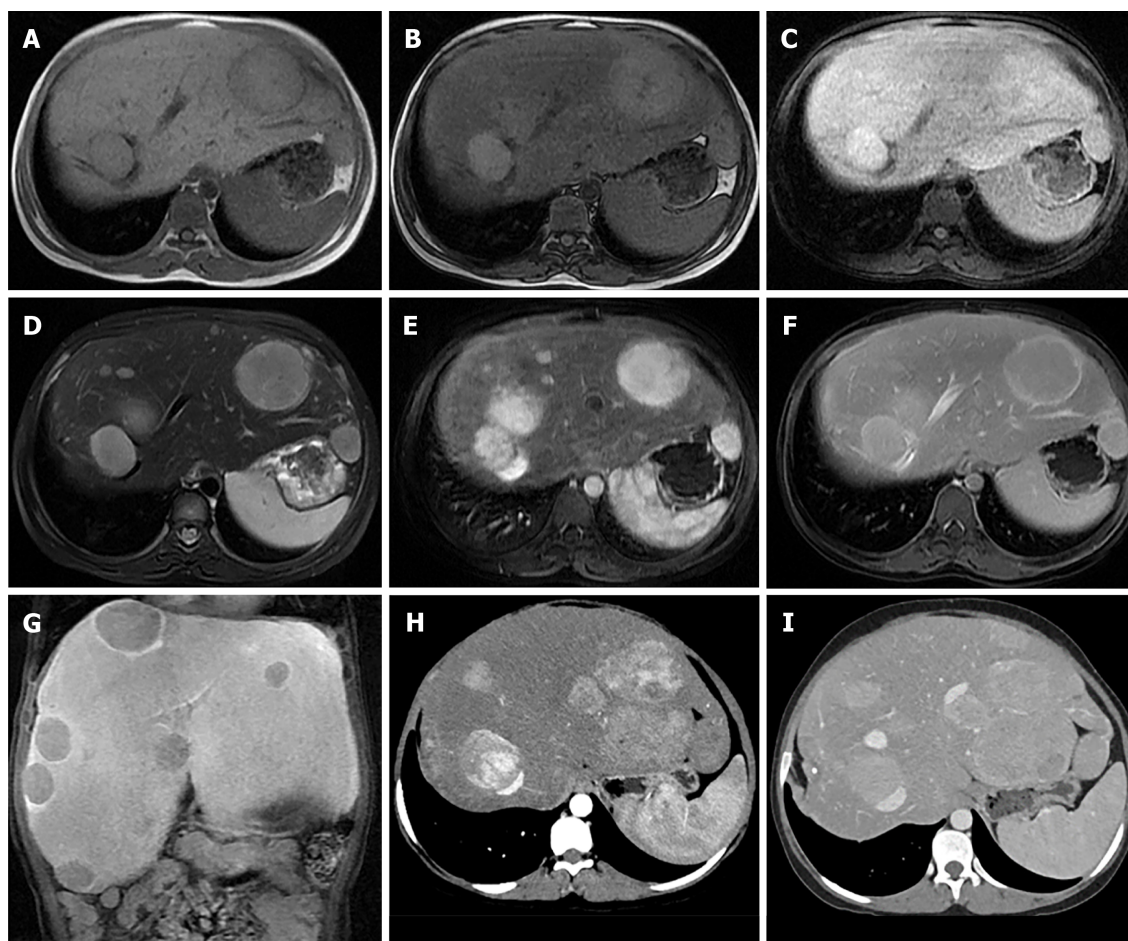
Figure 4 Magnetic resonance images of a 15-year-old girl with underlying GSD type 1 (A-G) and at the 5-yr follow-up, computed tomography was performed (H-I). One of the identified nodules presented histopathological findings compatible with hepatic adenoma. A and B: Axial dual gradient echo images showing several slightly hyperintense nodules in both hepatic lobes on the background of diffuse hepatic steatosis with dropout in signal intensity of liver parenchyma on contrast-phase images (A) as compared to in-phase images (B); C: Hypointense nodules on the T1-weighted (T1W) image; D: Hyperintense nodules on the T2W image; E-G: Intense arterial hyperenhancement of nodules (E), iso-to mild venous enhancement (F) and hypointense nodules on delayed hepatobiliary phases at 20 min (G) after injection with gadolinium-base hepatocyte specific contrast agent; H: Intense arterial hyperenhancement and increased in size of the nodules; I: Slight hyperenhancement in the venous phase.

and there is delay in the wash-out period, may not allow differentiation from HCC. In liver biopsy, typical findings reveal benign hepatocytes separated by fibrous septa that typically contain large dystrophic vessels with eccentrically thickened walls and narrowed, often thrombosed, while lamina and the presence ductular reaction are more helpful to confirm FNH diagnosis[71]. In addition, the diagnosis is aided by immunohistochemical staining for glutamine synthetase[72], which also presents a characteristic pattern in FNH (Figure 7A and B). Unlike the FNH pattern, HA in benign hepatocytes contains thin-walled arteries unaccompanied by bile ducts. The hepatocyte itself usually contains glycogen and fat that are sometimes entirely steatotic. If the hepatocytes present atypia with mitoses or an acinar growth pattern (pseudogranular), it is very challenging to differentiate from well-differentiated HCC. Immunohistochemistry staining indicating β-catenin activation will be useful if positive[71].

TREATMENT

Long-term follow-up and surveillance

Benign tumors such as HA and FNH may resolve if the primary liver disease is suitably managed. Nearly 40% of children with FNH with a confirmed liver biopsy have resolved lesions[73]. Follow-up by US every 6–12 mo is suggested as complications (bleeding, necrosis or rupture) could occur in HA, although they are rarely observed in FNH.
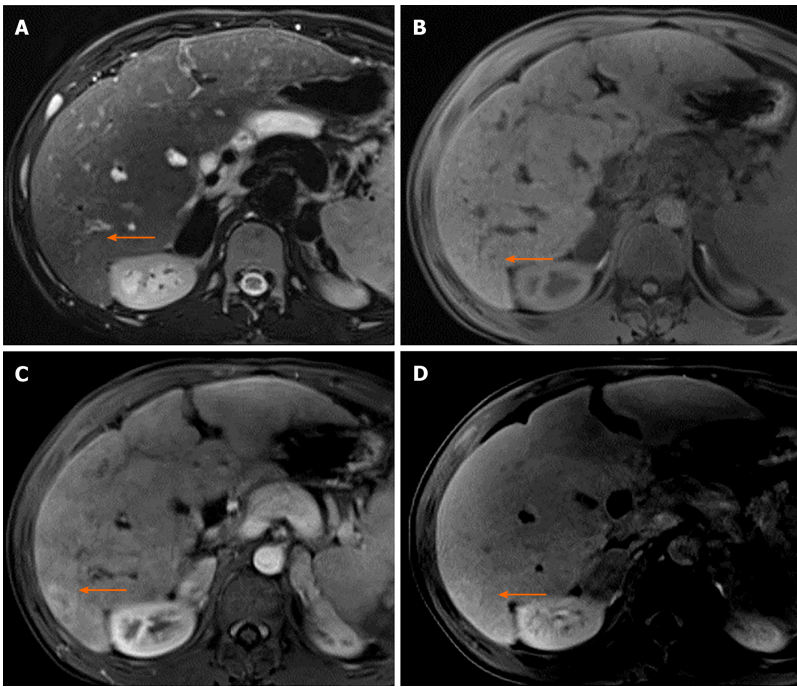
Figure 5 Pre-operative magnetic resonance images of a 13-year-old boy with biliary atresia identified liver nodule (arrows) before liver transplantation. The histopathological findings of the nodule were compatible with FNH. A: Isointense nodule on the T2W image; B: Iso-to-slightly hyperintense nodule on the T1W image; C: Arterial hyperenhancement of the nodule and; D: Persisted delayed enhancement on delayed hepatobiliary phase at 30 min after the administration of gadolinium-based hepatocyte specific contrast agent.
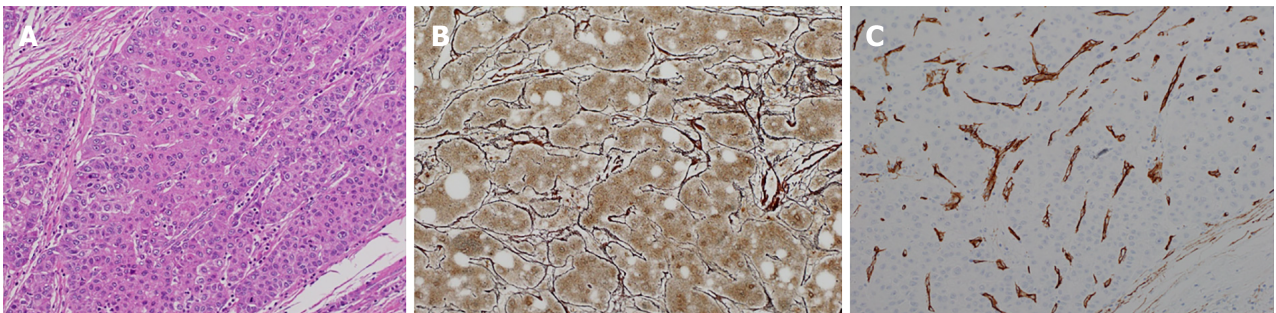
Figure 6 Histopathology from liver tumor demonstrates hepatocellular carcinoma. A: Hematoxylin and eosin staining shows tumor cells in a trabecular pattern. The cell plates are three cells thick or wider in most of this tumor; B: Reticulin staining highlights loss of normal cord architecture; C: Cluster of differentiation 34 immunostaining highlights the increased vascularity of hepatocellular carcinoma.

Surgical removal

Surgical excision is the only curable modality for malignant liver tumors with a favorable outcome if early detection is achieved. Surgical removal is also indicated in symptomatic benign tumors such as bleeding HA. For FNH, which is a true benign tumor, tumor resection has been performed because of symptoms (48%), inability to rule out malignancy (31%), and rapid tumor growth (15%)[44].

Liver transplantation

Liver transplant is the mainstay treatment and is required in cases of unresectable malignant liver disease[74]. Cisplatin-based chemotherapy prior to liver transplantation to achieve suitable margins for HB resection is under debate and requires additional data[75]. For HCC, early transplantation should be considered at the earliest possible opportunity because of its chemo- and radio-resistance. In cases of benign liver tumor, liver transplantation might have a role in the presence of multiple tumors that are difficult to resect, if these tumors are at high risk of transformation, or in patients experiencing symptoms[76,77] (Figure 8).
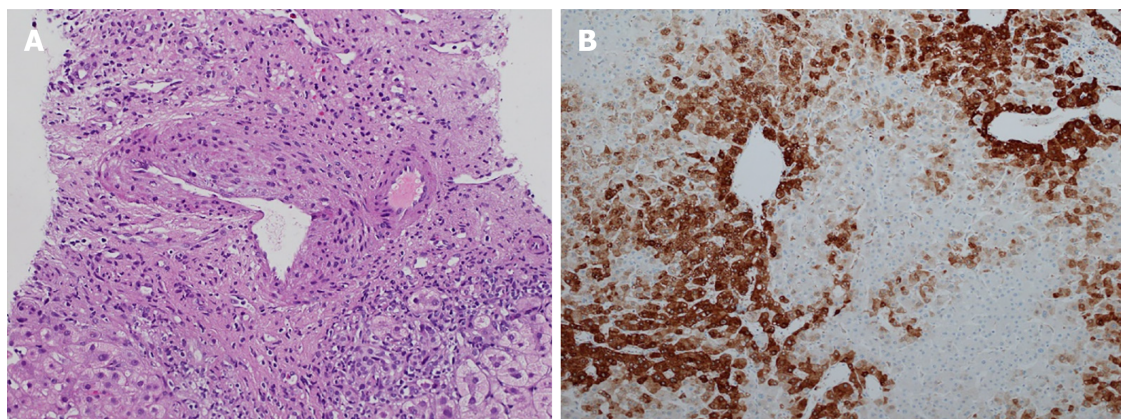
Figure 7 Histopathology of the liver tumor demonstrates focal nodular hyperplasia. A: Hematoxylin and eosin staining reveals a muscular vessel with irregular wall thickness, and ductular reaction; B: Glutamine synthetase immunostaining of focal nodular hyperplasia shows increased overall staining in a map-like pattern.
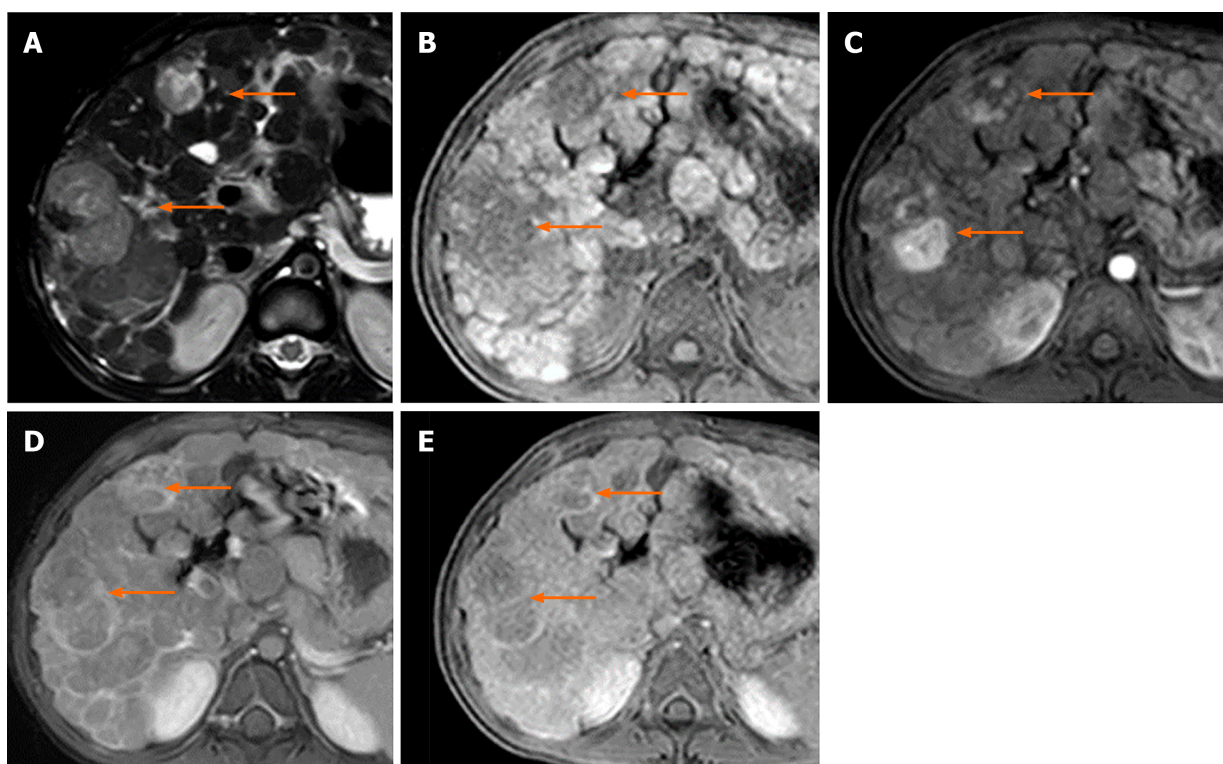
Figure 8 Magnetic resonance images of a 7-yr-old boy with tyrosinemia type I and renal Fanconi syndrome presenting nodules with histopathologic findings indicative of hepatocellular carcinoma. He underwent liver transplantation with favorable outcome and no evidence of tumor recurrence after a 1-yr follow-up. A: Multiple hyperintense nodules in T2-weighted (T2W) image; B: Hypointense nodules on the T1W image on the background of macronodular cirrhotic liver; C: Heterogeneous venous "wash-out" enhancement of the nodules; and D: Delayed capsular enhancement after administration of gadolinium-based contrast agent.

Other treatments

Transarterial chemoembolization and radiofrequency ablation are currently considered trial modalities in children with malignant tumors. This option might be considered in patients who are not eligible for tumor resection or liver transplantation [78,79].

CONCLUSION

Liver tumors in children with chronic liver disease are more common than expected. Early detection with noninvasive and highly specific diagnostic modalities are

necessary as children require routine monitoring. Liver histopathology is required in equivocal cases. Treatment outcome is favorable with timely management even in cases of malignant tumor. Liver transplantation is the preferred treatment option. In cases of benign tumor, long-term follow-up and surveillance are encouraged as tumor transformation has also been evidenced.

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Non-surgical treatment of hilar cholangiocarcinoma

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Abstract

Cancer of the biliary confluence also known as hilar cholangiocarcinoma (HC) or Klatskin tumor, is a rare type of neoplastic disease constituting approximately 40%-60% of intrahepatic malignancies, and 2% of all cancers. The prognosis is extremely poor and the majority of Klatskin tumors are deemed unresectable upon diagnosis. Most patients with unresectable bile duct cancer die within the first year after diagnosis, due to hepatic failure, and/or infectious complications secondary to biliary obstruction. Curative treatments include surgical resection and liver transplantation in highly selected patients. Nevertheless, very few patients are eligible for surgery or transplant at the time of diagnosis. For patients with unresectable HC, radiotherapy, chemotherapy, photodynamic therapy, and liver-directed minimally invasive procedures such as percutaneous image-guided ablation and intra-arterial chemoembolization are recommended treatment options. This review focuses on currently available treatment options for unresectable HC and discusses future perspectives that could optimize outcomes.

Core Tip: Most patients with hilar cholangiocarcinoma (HC) are not candidates for surgery or liver transplant at the time of diagnosis. Recently, several options for the management of unresectable HC have emerged and due to the complexity of this disease, a multi-disciplinary approach with multimodal treatment is recommended, including surgery, medical oncology, radiation oncology, diagnostic radiology, interventional radiology, gastroenterology, and pathology. Recent data suggest an improvement in overall survival, better response rates, and tumor control in patients with unresectable HC can be achieved by combining chemotherapy and minimal invasive ablatives strategies.

INTRODUCTION

Cancer of the biliary confluence also known as hilar cholangiocarcinoma (HC) or Klatskin tumor, is a rare type of neoplastic disease constituting approximately 40%-60% of intrahepatic malignancies, and 2% of all cancers[1]. It mainly affects subjects over 65 years of age and established risk factors are primary sclerosing cholangitis, biliary tract lithiasis, and parasitic liver disease (biliary ascariasis, liver schistoso-miasis, and fluke infestation), while other associated risk factors include, chronic pancreatitis, cirrhosis, inflammatory bowel disease, advanced age and male gender[2]. Typical symptoms are painless jaundice, cachexia, fatigue, and abdominal pain, usually reflecting the advanced stage of the disease at presentation, while concomitant cholangitis is present only in up to 10% of the cases. The prognosis is extremely poor as the majority of Klatskin tumors are deemed unresectable upon diagnosis and most patients with unresectable bile duct cancer die within the first year after diagnosis, due to hepatic failure, and/or infectious complications secondary to biliary obstruction[3].

Recommended imaging modalities for the diagnosis and staging of HC include computed tomography, magnetic resonance imaging, and magnetic resonance cholan-giopancreatography, which provide detailed information regarding the location and extent of HC (Bismuth–Corlette classification), vessel involvement and metastases. Criteria of unresectability include locally advanced (LA) tumor (mainly vessel involvement), lymph node metastases beyond the hepatoduodenal ligament, distant metastases, and patient's performance status[4]. Endoscopic retrograde cholangiopan-creatography (ERCP) and percutaneous transhepatic cholangiography (PTC) are mainly reserved for biopsy and/or palliative procedures to relieve obstruction (endoscopic or percutaneous biliary drainage). The most frequent HC histological type is the mucinous adenocarcinoma followed by the papillary type which is correlated with a more favorable prognosis. The only curative treatment remains surgical margin-negative (R0) resection (extended hemi-hepatectomy in most cases) with extrahepatic bile duct resection, hepatectomy, and en-bloc lymphadenectomy and if surgery is not an option, liver transplantation provides acceptable outcomes in highly selected patients. Nevertheless, survival rates for surgical after resection range between 10% and 40% at 5 years, with reported recurrence rates up to 50%-70%, even after R0 resection[5]. However, the percentage of patients eligible for resection remains low, around 25%[4]. For unresectable disease, radiotherapy, chemotherapy, and photodynamic therapy (PDT) are included in the treatment algorithm, while liver-directed, minimally invasive treatments such as percutaneous image-guided ablation options and intra-arterial chemoembolization have been more recently developed[6-8]. Due to the variety of diagnostic and treatment modalities involved in HC

management, a multi-disciplinary approach is recommended including hepatobiliary and transplant surgeons, medical and radiation oncologists, diagnostic and interventional radiologists, gastroenterologists, and pathologists[3,5]. This review focuses on available treatment options for unresectable HC and discusses future perspectives that aim in the optimization of current outcomes.

ABLATIVE THERAPIES

In the case of LA inoperable tumors in patients who are not suitable for liver transplant, locoregional therapies could be considered as a valid alternative to treat such patients. Different ablative therapies have been studied for the treatment of advanced HC, including irreversible electroporation (IRE), PDT, and endobiliary radiofrequency ablation (ERFA) (Table 1).

IRE

IRE is an image-guided ablation technique based on creating short-pulsed high-voltage current fields which applied for local control and progression of the primary LA tumor.

IRE may be considered not only for LA HC but also for patients with late-onset resection-site recurrence (after 6 mo).

As clinical practice and literature reported not all hepatic lesions are suitable for thermal ablation with radiofrequency ablation (RFA) or microwave ablation due to the possibility of damaging adjacent structures such as central bile ducts and gallbladder; moreover ablation close to large vessels can be ineffective because of heat sink effects or can cause vessel thrombosis[9]; IRE may potentially overcome the limitations of other modalities, such as skin phototoxicity in PDT, possible heat-sink effect in thermal ablation and the need for multiple fractions in stereotactic body radiotherapy (SBRT) [10]. As the effect of IRE is confined to the cell membrane and, in contrast to other ablative techniques, no thermal tissue damage occurs, thus avoiding vessels or duct injury.

Depending on the magnitude of the electric field and its exposure time, pulsed electric fields (PEFs) provoke either temporary (reversible) permeabilization of cell membranes and when the PEFs exceed a certain threshold value (w650 V/cm) delivered in 70–80 microseconds, irreversible injury to the membranes or permanent (irreversible) is induced with membrane disruption resulting in massive cell apoptosis [11].

There are no strict size criteria, IRE seems to be most effective for tumors < 3 cm in diameter.

IRE requires to be carried out under general anesthesia with complete neuromuscular block to thereby reducing muscle contractions caused by the electrical pulses of the stimulation and under cardiac gating either in the operating room or in the interventional radiology suite. During the procedure, the cardiac rhythm is continuously monitored, with a defibrillator present at all times.

The neoplastic mass is surrounded by a defined number of needles ranging from two to six. In order to perform a macroscopic complete ablation with a 5 mm margin, the interelectrode distances should range from 10 to 24 mm, with a maximum angulation between electrodes of 15°.

Given the complex anatomy of the liver hilum and the proximity of the hepatic duct, portal vein, and hepatic arteries, IRE may be associated with severe complications.

Dollinger *et al*[12] analyzed injury to venous structures and bile duct structures within 1 cm of an IRE ablation zone in hepatic tumors[12]. Only 10% of vessels demonstrated lesions, including portal vein thrombosis and vessel narrowing, which resolved in most patients. However, partial portal vein thrombosis is a relative contraindication because of the increased risk of worsening of the thrombus. Severe cardiac arrhythmias or cardiac dysfunction are considered contraindications to IRE procedure[10].

Bile leak and hepatic artery or portal vein thrombosis are possible complications associated with the procedure, necessitating careful monitoring and instruction of patients on discharge.

IRE has advantages of effective local tumor control, safety, fewer complications, and an absence of heat-sink effects. In literature is reported high efficacy in local tumor control with overall survival (OS) of 24.8 ± 6.84 mo and disease progression-free survival (PFS) of 18.5 ± 8.41 mo[13].

Table 1 Main published data from various minimally invasive treatment options

Ref.	Study design	Treatment	No. of patients	Outcomes	Complications/Adverse events
Hsiao *et al* [13]	Single-center, single-arm, retrospective	IRE	9	Median overall survival: 26 mo; progression-free survival: 18 mo	None reported
Martin *et al* [14]	Single-center, single-arm, retrospective	IRE	26	Median survival without biliary drainage: 305 d (range 92–458); disease-free: 11.5%	Complications: 3/26 (11.5%; severe 7.7%)
Li *et al*[18]	Single-center, comparative, retrospective	PDT + stent *vs* stent-only	62 (30 *vs* 32)	Median survival: PDT + stent 14.2 *vs* stent-only 9.8 mo, *P* = 0.003	Adverse events: 24 (38.7%) *vs* 20 (29.0%), *P* = 0.239
Mizandari *et al*[22]	Single-center, single-arm, retrospective	Endobiliary RFA	39	Median survival: 89.5 d (range 14-260)	None reported
Andrašina *et al*[27]	Single-center, prospective, multimodal oncological therapy	TACE or IA chemotherapy with or without SC *vs* IV SC	40 (17 *vs* 23)	Median overall survival: 13.5 mo (range, 11.0-18.8 mo). Median overall survival IA: 25.2 mo (range, 15.2-31.3 mo) *vs* IV SC 11.5 mo (range, 8.5-12.6 mo) in (*P* < 0.05)	None reported

IRE: Irreversible electroporation; PDT: Photodynamic therapy; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; IA: Intraarterial; IV: Intravenous; SC: Systemic chemotherapy.

In properly selected patients with obstructive jaundice, it safely achieves biliary decompression, therefore IRE can be used to increase catheter-free days and optimize the overall quality of life[14].

However, there are no reports available describing the median or long-term survival of patients with HC following IRE procedure.

Overall, based on current literature, IRE represents a promising technique concerning safety and local control for HPB tumors ineligible for resection or thermal ablation due to their proximity to vital structures.

Preclinical studies have demonstrated that IRE creates a well-defined boundary between ablated and non-ablated tissue; thus, the cells are either destroyed or remain intact. Compared with thermal ablation, perivascular tumor ablation with IRE appears to result in less frequent recurrence, indicating that the effectiveness of IRE is not influenced by the heat sink effect[15].

On the other hand, this technique presents some disadvantages compared to other thermal ablation such as RF and Mowat Wilson syndrome, because IRE needs to be performed under general anesthesia, is more complex and is much more expensive [16].

Although more clinical trials and comparative studies are required to validate the efficacy of ire in comparison with others non surgical treatment for HC.

PDT
PDT is a two-step procedure with either percutaneous transhepatic cholangioscopy (PTCS) or ERCP. At the time of ERCP, a bougie catheter choledochoscope is advanced to the level of the malignant stricture and used to deliver the laser fiber. The first step of the procedure involves the intravenous administration of photosensitizing agents that accumulate within cancer cells; subsequently, after an interval required for the drug to accumulate in the cancer, the tumor is exposed to non-thermal laser light of the appropriate photoactivation wavelength. Light activation leads to the formation of singlet oxygen free radicals and the destruction of nearby cells.

There are two major PDT methods for HC, ERCP and PTCS ones. ERCP is the preferred method but requires X-ray fluoroscopy to display the optical fiber marker at the tumor site. On the other side, the major advantage of PTCS is direct viewing of the tumor for more accurate localization and assessment of therapeutic response, while disadvantages include relatively greater trauma due to percutaneous approach.

Common adverse events after PDT include acute cholangitis, pancreatitis, haemobilia, liver abscess, and skin photosensitivity reactions. Severe skin photo-toxicity is reported in up to 30% of patients[17].

Recent studies have shown that PDT for unresectable cholangiocarcinoma can reduce bile duct stenosis, improve quality of life, and prolong survival[18].

Multiple prospective and retrospective series have demonstrated an increase in survival of 2-3 mo with the addition of PDT to biliary stenting in a palliative setting. A phase II pilot study by Wiedmann *et al*[19] evaluating PDT as a neoadjuvant modality

demonstrated a 1-year survival of 83%[19].

ERFA

There are few studies about the clinical applicability of RFA for malignant bile duct obstruction. ERFA catheters (Figure 1) were first introduced less than 10 years ago [20], and these catheters are easily used with a standard-sized duodenoscope, therefore RFA procedure can be performed either through endoscopic or percutaneous access. The rationale of those catheters is to destroy locally the malignant biliary stricture; local coagulative necrosis caused by RFA has the potential to delay tumor growth, prolonging the duration of stent patency[21].

ERCP-directed RFA is a novel procedure that induces local coagulative necrosis by delivering thermal energy *via* a bipolar probe by using high-frequency alternating current over a guidewire to the level of the stricture of interest by using fluoroscopic guidance, during ERCP or through endoscopic ultrasonography[22].

Several studies concerning endoscopic RFA procedures of malignant biliary strictures have been published. However, data are limited, because of small sample sizes, lack of randomization, and study heterogeneity (biliary tumor site). Complications reported after ERFA are sepsis, cholecystitis, and pancreatitis.

Most studies using ERCP-guided RFA in the treatment of HC assessed improvements in stent patency duration and luminal diameter. In the treatment of malignant tumors, RFA can induce high temperatures locally, which leads to coagulation necrosis of tumor cells and controls tumor re-growth[23,24].

Reports comparing the beneficial effects of endoscopic RFA therapy for the survival of patients with biliary cancer are rare.

Endoscopic RFA can significantly alleviate jaundice, reduce the thickness of tumor lesions, prolong HC stent patency, improved the quality of life, without increasing complications' rate.

In his study, Yang *et al*[25] reported that bilirubin levels at 2 wk were significantly reduced in the RFA + stent group compared with the stent-only group, suggesting that RFA could reduce jaundice more rapidly. Moreover, stent patency of the RFA + stent group was significantly longer than that of the stent-only group. RFA combined with stent placement can prolong biliary tract patency and OS without increasing the incidence of adverse events in patients with cholangiocarcinoma[25].

Also, the percutaneous approach of intrabiliary tract RF ablation, firstly described in 2013 by Mizandari *et al*[21] in patients with unresectable malignant hilar biliary obstruction is considered a feasible and safe procedure because it can be performed following biliary decompression with minimal discomfort to the patient.

INTRA-ARTERIAL THERAPIES

In the last few years, very little scientific literature has been produced about locoregional palliative intra-arterial therapies for unresectable HC.

Even in the last expert consensus statement by Mansour *et al*[3], there is no mention of intra-arterial therapies, considering systemic chemoradiation with or without intraluminal brachytherapy (ILBT) as the best choice in tumor control rate[3].

A retrospective cohort study conducted at the Liaoning Cancer Hospital by Zheng *et al*[26] investigates the clinical efficacy of cisplatin-based and gemcitabine transcatheter arterial chemoembolization combined with radiotherapy after biliary drainage or biliary stent implantation in patients with HC, thus obtaining a median survival time of 20 mo, almost doubling that of the control group (10.5 mo). The median patency time of the biliary stent (15.6 mo) was also more than doubled compared with the control group[26].

In 2010, Andrašina *et al*[27] published a prospective study on multimodal oncological therapy for unresectable cholangiocarcinoma, selecting 43 patients who underwent metallic-stent implantation followed by ILBT; 38 of these (88%) had hilar involvement. Patients have been divided into two arms: The intra-arterial arm consisted of patients treated with a locally intra-arterial infusion *via* a Port catheter percutaneously inserted into the hepatic artery of Cisplatin and 5-fluorouracil (5-FU) completed by a non-selective embolization with iodized oil (Lipiodol) and/or systemic chemotherapy, while the intravenous arm was treated only with systemic chemotherapy. The median OS from diagnosis was 25.2 mo in the IA arm and 11.5 mo in the IV arm[27].

This was a not randomized study and patients were selected according to the principle of individually tailored multimodal oncological therapy. Highly vascularized

Figure 1 The Habib™ EndoHPB bipolar radiofrequency catheter (Boston scientific).

tumors, which could be the target of chemoembolizations, have a naturally better prognosis than hypovascular ones.

CHEMOTHERAPY

The role of chemotherapy is sometimes associated with transplantation in the unresectable disease limited-stage; since 2005 some experience is described to investigate the role of liver transplantation after chemoradiation in stage I and II HCs. In this protocol, seventy-one patients were enrolled in the transplant protocol and received neoadjuvant external beam radiotherapy to a target dose of 4500 cGy in 30 fractions. Concomitantly, intravenous fluorouracil (5-FU) was given. Two to three weeks after the completion of external beam radiotherapy, a transluminal boost of radiation was delivered using a transcatheter Iridium-192 brachytherapy wire; authors conclude that liver transplantation with neoadjuvant therapy currently appears to have greater efficacy than resection for selected patients with localized, node-negative HC. Despite differences in the patient groups, transplantation with neoadjuvant therapy achieved better local control and higher patient survival than did conventional resection[28].

Darwish Murad *et al*[29] analyze data from 12 United States participating centers reported 319 patients; Patients with HC who were treated with neoadjuvant therapy followed by liver transplantation had a 65% recurrence-free survival rate after 5 years, demonstrating this therapy to be highly effective in very selected cases[29]. This was not a randomized controlled trial, so further study are needed.

In the unresectable disease, palliative chemotherapy or chemoradiation is the only treatment that must be attempted. Consistent data suggest the use of first-line gemcitabine and cisplatin chemotherapy in patients with advanced disease; the trial randomly assigned 410 patients with ECOG-PS ≤ 2 to systemic chemotherapy with gemcitabine alone or cisplatin–gemcitabine; the study showed an OS benefit in favor of cisplatin–gemcitabine (hazard ratio 0.64)[30]; in some selected and limited stage cases, oncologists use the combination of gemcitabine and cisplatin as neoadjuvant intent; in cases of stable disease or partial response, it can be considered external beam radiotherapy with concomitant capecitabine oral administration. But there are no randomized trials to confirm the use.

Some trials are ongoing to investigate the role of triple-chemotherapy combinations in the first-line setting, such as cisplatin-gemcitabine combined with nab-paclitaxel or with S1 (tegafur, gimeracil, and oteracil), and FOLFIRINOX (5-FU, oxaliplatin, and irinotecan; AMEBICA study, NCT02591030). Acelarin, (NUC-1031) a first-in-class nucleotide analog, with cisplatin will be compared with gemcitabine and cisplatin combination therapy in a phase III study (NCT04163900). At the progression of first-line chemotherapy, the choice of the second-line chemotherapy is unclear. The ABC-06 trial showed a higher although modest median OS in the FOLFOX arm, differences in survival at 6 mo (35.5% *vs* 50.6%) and 12 mo (11.4% *vs* 25.9%) and the treatment is clinically meaningful[31]. FOLFOX can be considered a new standard of care in the second-line setting.

At the moment, the role of chemotherapy is mainly related to the advanced disease with a palliative purpose.

TARGET THERAPIES AND IMMUNOTHERAPY

New target therapies have demonstrated a potential role in the intrahepatic cholangiocarcinoma treatment with isocitrate dehydrogenase (*IDH*) *1-IDH2* mutation and *FGFR2* fusion[32]. So some phase III trials with *IDH1–IDH2* or *FGFR* inhibitors as first- and/or second-line treatment are ongoing[33].

Due to the various inter-tumoral and intra-tumoral heterogeneity of cholagiocarcinoma, the hilar and peri-HC are considered different subtypes with different genetic alterations such as the mutations of AT-rich interactive domain (ARID)1B, E74-like factor (ELF)3, protein polybromo-1 (PBRM1), protein kinase cAMP-activated catalytic subunit alpha (PRKACA), and sub unit beta (PRKACB)[32]. At the moment there are not studies to investigate the role of specific drugs in this setting. Other and several functional studies with the *PKCACA* and *PKCACB* fusion genes will be mandatory for understanding pathogenesis in perihilar and distal cholangiocarcinoma[34].

The role of immunotherapy in that kind of disease is uncertain and under investigation. The programmed cell death protein 1 (PD-1), the programmed death-ligand 1 (PD-L1) and T lymphocyte-associated antigen 4 are the most known immune check point inhibitors drug targets. Studies are ongoing with monoclonal antibodies such as ipilimumab or tremelimumab (anti- CTL4) or antibodies targeting PD-L1, such as durvalumab, or its receptor PD-1, such as nivolumab or pembrolizumab. Preliminary data suggest a higher response rate in intrahepatic cholangiocarcinoma treatment with the genetic signature of microsatellite instability that can predict the response to the immune check point inhibition[35].

So the immune-modulating therapies could be promising options for the subgroup of patients with cholangiocarcinoma harboring high mutational loads[36].

The future direction of the medical treatment of HC it might be a combination of therapies involving immunotherapy plus chemotherapy, immunotherapy and radiotherapy[37].

For example, it is well established that the sensitivity of the immune system to the tumors is increased during the radiotherapy with a synergistic effect due to the changing of micro environment and apposition of new neo antigens. Some cases are reported of refractory advanced intrahepatic or HC that were treated in a satisfied way with anti-PD-1 antibody following or concurrent with SBRT[38].

Further studies are necessary to validate their efficacy and safety and to become the basis and direction for future researches for the treatment of HC patients.

RADIOTHERAPY

In patients with inoperable or metastatic disease combined radio-chemotherapy or exclusive chemotherapy may be proposed.

Radiation therapy (external beam RT ± brachytherapy) with or without concomitant chemotherapy (5-FU or gemcitabine) is a potential choice in the treatment of patients with LA disease in good performance status. Since local progression of unresectable cholangiocarcinoma can lead to pain, biliary obstruction with severe hepatic insufficiency, this modality can control tumor-related symptoms and prolong survival.

However, the rarity of cancer associated with the lack of literature in this field of study with few clinical trials available (retrospective and non-randomized) means that the role of RT in this setting of patients is not yet well defined[39].

Some studies have shown improvements in symptoms of HC patients treated with radiotherapy with a median survival rate between 9 and 14 mo[40,41]. Classically the dose used is about 45-50 Gy delivered at 1.8-2 Gy/fraction with or without ILBT boost [42].

In a phase 2 study, 128 patients with intrahepatic malignancies, including 46 patients with cholangiocarcinoma, patients received a median dose of 60.75 Gy delivered in 1.5 Gy/fraction twice daily with conformational 3D technique. An improvement in survival compared to historical controls was observed, with 12 patients (of 33 evaluable patients with cholangiocarcinoma) achieving a complete or partial response to disease[43].

A retrospective analysis of 48 patients with gallbladder carcinoma and cholangiocarcinoma treated between 1998 and 2018 and a median radiotherapy dose of 50.4 Gy, achieved a median OS of 12.0 mo with OS at 2, 3, and 5 years of 33%, 20%, and 7%, respectively. In the univariate analysis, biologically effective dose (BED) > 59.5 Gy 10 was associated with improved PFS and OS and primary tumor size was associated with worsening PFS[44].

In the last decades, modern technological advances such as intensity-modulated RT, the ability to perform SBRT treatments (Figure 2), respiratory management methods, and imaging guidance during therapy, have enabled potentially ablative doses to be delivered for the treatment of cholangiocarcinoma[45,46].

However, the results of dose escalation studies for the treatment of HC were not clearly as favorable as those for intrahepatic cholangiocacinoma[47]. A multi-center retrospective study of patients with HC reported improved median survival in patients receiving > 40.0 Gy compared with those receiving less. A retrospective analysis of 52 patients with unresectable HC, suggested a possible association between increased radiation dose and improved LC[48].

In a recent study, 80 patients treated with RT for unresectable HC were retrospectively analyzed[49] in which RT was administered at doses of 30-75 Gy for a median BED of 59.5 Gy. The cohort was divided into a conventional dose group (BED ≤ 59.5) and a high dose RT (HDRT) group (> 50.4 Gy in 28 fractions, BED > 59.5) and. The HDRT group did not demonstrate better freedom from local progression or OS. Furthermore, HDRT was associated with the onset of grade 3 or higher lymphopenia [50]. These results suggest that higher doses do not provide elevated LC and OS benefits in HC. The proximity of HC tumors to the duodenum and/or small intestine is the factor limiting the ability to completely cover the tumor with high doses of radiation (tolerance doses < 50 Gy)[46].

Historically, the use of ILBT has shown an advantage in treating HC as a boost after EBRT or as a definitive treatment, given the possibility of limiting high doses to the liver or intestine[50] and studies are supporting its association with improved stent preservation and survival[51].

An Italian pooled analysis collected retrospective data from 3 radiotherapy Centers analyzing, from 1992 to 2017, 73 patients treated with EBRT + ILBT or EBRT alone in combination with chemotherapy or exclusive ILBT (with Ir 192 both HDR and LDR). The results demonstrated excellent local control, especially in patients treated with EBRT + LIRT + CHT or exclusive LIRT, in the absence of a clear impact on OS. Surely, careful selection of patients could allow us to evaluate who could benefit most from treatment with ILBT obtaining greater benefits[52].

SBRT has also been extensively explored as a potentially curative treatment strategy for patients with LA cholangiocarcinoma or in patients with local relapse. The total doses used ranged from 45 to 60 Gy in 3-5 fractions resulting in median survival of 11-29 mo[53]. The SBRT not only has the advantage of limiting doses to surrounding organs but also of limiting treatment times by increasing compliance with therapies and facilitating integration with systemic treatment.

Sandler *et al*[54] analyzed 31 patients with intrahepatic cholangiocarcinoma (19%) or extrahepatic cholangiocarcinoma (81%) who received SBRT at a median dose of 40 Gy in 5 fractions[54]. The median OS was 15.7 mo, the 2-year OS was 33%, and the 2-year LC was 47%. Serious adverse events occurred in 16% of patients (9% with grade 3-4 duodenal ulceration or bleeding).

A recent systematic review analyzed 10 studies (none of which were randomized) with at least 10 patients enrolled *per* study, in which SBRT was used for the treatment of intra- and extrahepatic cholangiocarcinoma[55]. Dose prescribing methods and total dose/fraction were highly variable with a median prescribed SBRT dose between 30 and 60 Gy in 3-5 fractions and median BED between 57.6 and 180.0 Gy. The survival results were almost comparable to those of standard chemoradiotherapy and CHT with a median OS of 15.0 mo. Results in terms of LC and toxicity would also demonstrate that SBRT treatment is reasonably effective with acceptable treatment-related toxicities. Overall, treatment-related acute and late toxicities were found to be acceptable and at rates almost comparable to those reported after chemoradiotherapy ± ILRT boost.

However, all the studies conducted so far show that the minimum available evidence in the setting of SBRT for cholangiocarcinoma highlights the need for high-quality studies in this area. In terms of OS, the preliminary results do not appear much different from those of standard chemoradiotherapy. Therefore, SBRT can be considered a therapeutic option in selected patients with cholangiocarcinoma, in association with adjuvant CHT.
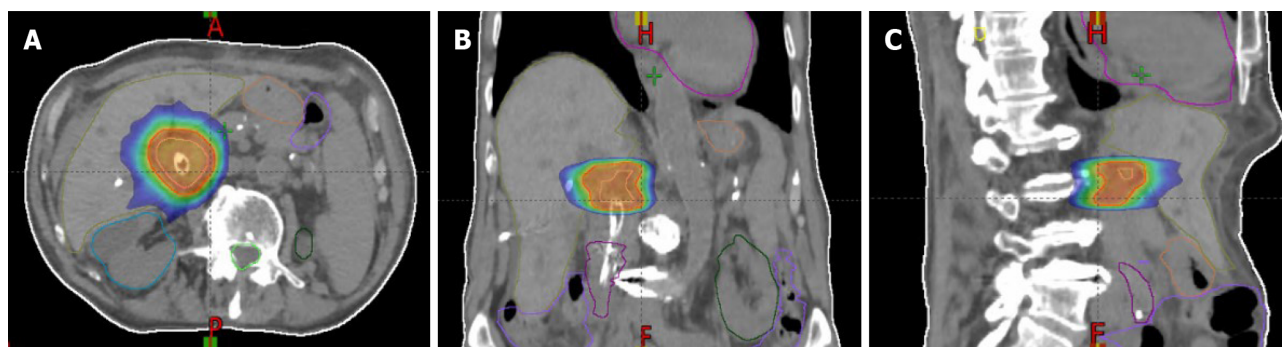
Figure 2 Radiation treatment plan for a patient treated with stereotactic body radiotherapy for hilar cholangiocarcinoma. The plans show isodose levels in the axial plane, coronal plane, and sagittal plane. A: Axial plane; B: Coronal plane; C: Sagittal plane.

A new field of study in this setting of patients is certainly carbon ion radiotherapy (CIRT) which offers a higher relative biological efficacy (RBE) compared to photons and the Bragg peak and limited lateral scattering of the beam offer higher dose delivery than photons, allowing higher dose delivery to the tumor, reducing the dose to healthy tissue[56]. However, very few CIRT studies exist for cholangiocarcinoma, based on a small cohort of patients and a single randomized but retrospective multicenter study[57]. In the latter, 56 patients with cholangiocarcinoma treated with CIRT were analyzed; more than 80% were inoperable. The most commonly prescribed CIRT dose was 76 Gy (RBE) in 20 fractions [effective biological dose (BED) of 105 with $\alpha/\beta = 10$]. This study revealed a median MST survival time of 14.8 mo for all 56 patients, 23.8 mo for 27 patients with intrahepatic cholangiocarcinoma, and 12.6 mo for 29 patients with HC after CIRT. Among the serious toxicity events noted, liver failure or sepsis following bile duct stenosis or cholangitis may occur during the natural course of HC, which may have adversely affected tolerance to treatment; moreover, biliary tract stenosis and pre-CIRT cholangitis have been observed in patients with HC and persisting even after CIRT could directly influence the prognosis. The study's OS and MST rates were comparable to those of previous proton or SBRT treatments, however, given the numerous limitations (retrospective study, different fractionations used, numerous cases lost to follow-up, and short median follow-up) and the safety of CIRT for cholangiocarcinoma remains poorly understood, although CIRT may be considered a promising therapy for patients with cholangiocarcinoma non fit surgery.

In conclusion, the role of radiotherapy in its different approaches for the treatment of LA HC is not yet clear in terms of modalities, timing, and doses for which clinical trials would be necessary. Furthermore, intensifying treatment for cholangiocarcinoma with novel systemic agents, in combination with radiation, could broaden therapeutic prospects.

FUTURE DIRECTIONS

Ongoing research is focused on the concept of personalized therapy and precision medicine, based on the heterogeneity of the molecular profile of HC[58]. Whole exome and transcriptome sequencing has detected that intrahepatic HC demonstrates IDH1/2 and *BAP1* mutations and *FGFR2* gene fusions and research findings indicate that immune checkpoint inhibitors could be used to patients with a poor prognosis subtype of high mutational load and increased immune activity[32]. The goal of molecular research is to develop a tailored therapy protocol based on molecular profiling, in order to minimize toxicity and optimize efficiency. Only recently, Wang *et al*[59] published a retrospective study investigating the molecular profile of intrahepatic cholangiocarcinoma in the Chinese population, using next-generation sequencing. The identified genomic alterations were used for personalized therapy and targeted or immunotherapy agents demonstrated superior survival and tumor response outcomes compared to standard chemotherapy[59]. Moreover, genome sequencing and animal model studies suggest that gain-of-function mutations in the *IDH* gene, could be involved in a subset of cancers with inflammatory signature and trials with IDH inhibitors are ongoing[60].

Nevertheless, prospective randomized control trials investigating precision medicine protocols are still awaited and several issues remain to be resolved as the

complexity of HC requires in depth analysis of the biological mechanisms of the disease.

Recent advances in percutaneous minimally invasive treatment options include endoluminal RFA following tumor in growth in the hilum and the use or drug-eluting stents which is been investigated in both experimental animal models and extremely limited human trials[61-64].

Multimodality treatment protocols combining percutaneous minimally invasive therapies with systemic chemotherapy, modern RT, and PDT have been previously described and seem promising, however, large-scale studies are missing[65].

According to current evidence, future research could be focused on the comparison of the efficacy of IRE and other therapeutic modalities, RFA plus stent placement compared to RFA alone or stents alone, and the combination of various percutaneous therapies with individualized drug-therapy based on molecular profiling, in order to provide more solid evidence supporting the efficacy of multidisciplinary approaches.

CONCLUSION

Over the past two decades, several options for the management of unresectable HC have emerged. Due to the complexity of this disease, a multi-disciplinary approach with multimodal treatment is recommended, including surgery, medical oncology, radiation oncology, diagnostic radiology, interventional radiology, gastroenterology, and pathology. Recent studies suggest an improvement in OS, better response rates, and tumor control in patients with unresectable HC can be achieved by combining chemotherapy and ablatives strategies.

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Basic Study

Genome-wide CRISPR-Cas9 screening identifies that hypoxia-inducible factor-1a-induced *CBX8* transcription promotes pancreatic cancer progression *via* IRS1/AKT axis

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Abstract

BACKGROUND
Pancreatic cancer (PC) is one of the most lethal malignancies worldwide. It is known that the proliferation of PC cells is a critical process in the disease. Previous studies have failed to identify the key genes associated with PC cell proliferation, using bioinformatic analysis, genome-wide association studies, and candidate gene testing.

AIM
To investigate the function of the chromobox 8 (CBX8)/receptor substrate 1 (IRS1)/AKT axis in PC.

METHODS
A genome-wide CRISPR-Cas9 screening was performed to select genes that could facilitate PC cell proliferation. Quantitative reverse transcription-polymerase chain reaction was used to detect the expression of *CBX8* in PC tissues and cells. The regulatory roles of CBX8 in cell proliferation, migration, and invasion were verified by *in vivo* and *in vitro* functional assays.

RESULTS
CBX8 was upregulated in PC tissues and shown to drive PC cell proliferation.

Higher expression of CBX8 was correlated with worse outcomes of PC patients from two independent cohorts comprising a total of 116 cases. CBX8 was also proved to serve as a promising therapeutic target for a PC xenograft model. We demonstrated that hypoxia-inducible factor (HIF)-1a induced CBX8 transcription by binding to the promoter of *CBX8*. CBX8 efficiently activated the PI3K/AKT signaling by upregulating insulin IRS1.

CONCLUSION

CBX8 is a key gene regulated by HIF-1α, and activates the IRS1/AKT pathway, which suggests that targeting CBX8 may be a promising therapeutic strategy for PC.

Key Words: CRISPR-Cas9 screening; Pancreatic cancer; Chromobox 8; Hypoxia; pI3K/AKT signaling

Core Tip: The authors demonstrated that hypoxia-inducible factor-1a induced chromobox (CBX)8 transcription by binding to the promoter of *CBX8*. CBX8 efficiently activated the PI3K/AKT signaling by upregulating insulin receptor substrate 1. The newly identified signaling axis may support the development of new therapeutic strategies for pancreatic cancer.

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal cancers worldwide. It has become the second most fatal cancer in the United States. The 5-year survival rate of pancreatic ductal adenocarcinoma (PDAC) is < 10% due to late diagnosis and resistance to systemic therapies[1]. Many studies have shown that the prognosis of patients with PC remains poor after complete surgical resection. At the same time, due to the difficulty of diagnosis, many patients with PC have no chance of surgery after diagnosis. Therefore, it is important to study the occurrence and development of PC itself and the corresponding targeted therapy.

Hypoxia is one of the important characteristics of PC[2]. Activation of hypoxia-inducible factors (HIFs; particularly HIF-1a and HIF-2a) is an important mechanism for tumor cells to adapt to a hypoxic microenvironment. Our previous studies indicated that HIFs not only regulate the growth and metastasis of PC cells, but also mediate the immunosuppression[3] and angiogenesis[4] in PC.

Chromobox 8 (CBX8) (also known as human polyclonal 3), together with CBX2, CBX4, CBX6, and CBX7, are members of the CBX protein family. CBX8 plays an oncogenic role in different types of cancer. Zhang *et al*[5] reported that CBX8 upregulates Leucine Rich Repeat Containing G Protein-Coupled Receptor 5 (LGR5), leading to increased stemness and decreased chemosensitivity of colon cancer cells. CBX8 promotes tumor growth and metastasis in hepatocellular carcinoma (HCC)[6] and breast cancer[7]. However, whether CBX8 is involved in the proliferation of PC cells remains unknown.

Genome-scale CRISPR-Cas9 knockout (GeCKO) library is a powerful tool for the assessment of gene function[8] and the screening for genes involved in cancer cell proliferation and metastasis. In this study, Bxpc-3 and PANC1 cells were transduced with library lentiviruses. The cells were then injected subcutaneously into nude mice and removed 21 d later to sequence the single guide RNA (sgRNA) in the tumor tissue. Among all candidate genes, *CBX8* was selected for further analysis because of

its upregulation in cells. Furthermore, higher *CBX8* expression was correlated with worse clinical outcomes of PDAC patients from two independent cohorts. We also showed that *CBX8* was a key gene that was regulated by HIF-1α, and could activate the IRS1/AKT pathway. The above findings suggest that targeting CBX8 may be a promising therapeutic strategy for PC.

MATERIALS AND METHODS

Cell culture and reagents
PC cell lines (PANC1 and Bxpc-3) and 293T cells were purchased from American Type Culture Collection (Manassas, VA, United States) and maintained in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin in a 95% air/5% CO_2 environment at 37 °C. Cells at passages 3–15 were used in this study. For hypoxic culture, cells were cultured under 1% O_2.

Lentiviral packaging and infection
The Human CRISPR Knockout Pooled Library was obtained from Addgene (http://www.addgene.org/crispr/Libraries/geckov2/, United States). GeCKO library plasmids, pVSVg (AddGene) and psPAX2 (AddGene), were added to 100 μL Opti-MEM at a ratio of 1:0.5:1.5. After 15-min incubation with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, United States), the mixture was added to 293T cells. Forty-eight hours later, cell supernatants containing lentiviruses were collected. PANC1 and BXPC-3 cells were transduced at a low multiplicity of infection (0.3) to ensure that most cells received only one viral construct. The cells were selected with puromycin (1 μg/mL) for 14 d. Only cells transduced with a LentiCRISPR construct could survive. After transfection with GeCKO library, cells were transplanted subcutaneously into the right flank of 4-wk-old male nude mice. Twenty-one days later, the mice were killed and primary tumors were removed for sgRNA sequencing.

Cytotoxicity assay
Following infection, cells were treated with gemcitabine for 48 h. Cell viability was assessed by CCK-8 assay (Beyotime, China) and the IC_{50} values were calculated using GraphPad Prism 7 (GraphPad, La Jolla, CA, United States). All measurements were performed in triplicate. The abundance of sgRNA was determined by deep sequencing.

Colony formation assay
Cells were treated with DMSO or chemotherapeutic agents for 24 h and then seeded in six-well plates (200/well). The cultures were maintained in a 5% CO_2 incubator at 37 °C for 2 wk. The colonies were fixed with 4% paraformaldehyde, followed by staining with 0.5% crystal violet. The number of colonies in each group was counted under a microscope. Independent experiments were performed in triplicate.

Luciferase activity assay
The promoter regions of *CBX8* and *IRS1* were amplified by polymerase chain reaction (PCR) and cloned into RB reporter plasmids (Ribo, China). *CBX8* knockdown plasmids were cotransfected into 293T cells. Twenty-four hours later, cells were harvested and Renilla and firefly luciferase activities were detected using a dual-luciferase reporter assay kit. The Renilla luciferase activity was used for normalization.

Chromatin immunoprecipitation and chromatin immunoprecipitation sequencing
A chromatin immunoprecipitation (ChIP) assay was carried out using an EZ-ChIP Chromatin Immunoprecipitation Kit (Millipore, Bedford, MA, United States). Formaldehyde (1%) was used to crosslink proteins and DNA for 10 min. Cell lysates were sonicated to obtain DNA fragments, which were subjected to IP with primary antibodies or negative control IgG. Purified DNA was analyzed by quantitative reverse transcription PCR (qRT-PCR) with SYBR Green Master Mix (Promega, Beijing, China). The relative enrichment values were calculated through normalization of the results to the input values and are expressed relative to the values obtained with normal IgG.

In vivo experiment
All animal experimental procedures were approved by the Ethics Committee for

Animal Research of Shanghai Jiaotong University School of Medicine (Shanghai, China). Four-week-old male nude mice were subcutaneously transplanted in the right flank with CBX8-silenced or control PC cells (PANC1, Bxpc-3, 2×10^6 cells per mouse) in 100 µL PBS mixed 1:1 with BD Matrigel Basement Membrane Matrix (Corning, Corning, NY, United States) as previously reported. Tumor size was measured every 3 d with a digital caliper. When tumor volume reached approximately 100 mm^3 (day 6 postinoculation), mice were randomly assigned to each group. After 21 d, the tumor weight was detected and mice were killed.

Patients' samples

This study was approved by the Human Research Ethics Committee of Shanghai General Hospital (Shanghai, China) and performed following the United States Common Rule. Each patient provided written informed consent. A total of 116 archived PDAC specimens were collected. Patients were followed over time.

Statistical analysis

All data are shown as the mean ± SD. Statistical analyses were performed using Graphpad Prism 7. Student's *t* test or analysis of variance was used to compare continuous variables.

RESULTS

Genome-wide pooled sgRNA library screening identifies the genes affecting the growth of PC cells in vivo

To identify the genes responsible for growth of PC cells, we transduced PANC1 and Bxpc-3 cells with GeCKO library lentiviruses. After 14 d of puromycin selection, cells were transplanted subcutaneously into the right flank of 4-wk-old male nude mice. Twenty-one days later, the mice were killed and primary tumors were removed for sgRNA sequencing (Figure 1A). About 1.6×10^6 sgRNA sequences were obtained from each tumor sample. Genomic DNA was extracted from tumor tissues for PCR and next-generation sequencing (NGS) analysis. The expression of genes in each sample was measured. The genes were ranked according to the numbers of sgRNA and NGS reads. Compared to the control group, 872 cell growth related candidate genes were identified in PANC1 cells, while 819 were found in Bxpc-3 cells. There were 244 genes identified in both groups with differentially enriched sgRNA. We performed qPCR to confirm the candidate top 10 differently expressed sgRNAs from NGS analysis (Figure 1B), which indicated that *CBX8* had the lowest expression level of sgRNA (Figure 1C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene set enrichment analysis (GSEA) showed that these universal differentially expressed sgRNAs were enriched in the PI3K/AKT pathway (Figure 1D and E).

Higher CBX8 expression induces worse clinical outcome in PC patients

To determine whether CBX8 is involved in PC progression, we examined the mRNA level of *CBX8* in 116 paired PC and adjacent nontumor tissue samples and found that *CBX8* expression was significantly increased in PC tissues compared with corresponding noncancer tissues (Figure 2A). Kaplan–Meier survival analysis indicated that patients with higher *CBX8* expression had a shorter overall survival than those with low *CBX8* expression (Figure 2B). The protein content of CBX8 in these tissue samples was obtained by Western blot analysis (Figure 2C). Compared to normal neighboring tissues, the CBX8 protein content in PC tissues was increased.

We analyzed the expression of CBX8 in The Cancer Genome Atlas (TCGA) database to confirm its potential regulatory pathways and cellular functions. KEGG pathway analysis found that genes positively correlated to CBX8 (Figure 2D) were enriched in the PI3K/AKT and pancreatic adenocarcinoma pathway (Figure 2E).

CBX8 is required for efficient proliferation of PC cells in vitro and in vivo

We measured the protein level of CBX8 in PC cell lines and found that CBX8 was upregulated in PANC1 and BXPC-3 cells compared with normal pancreatic epithelial cells (Figure 3A). CCK-8 assay was used to assess the effect of CBX8 on PC cell proliferation, which indicated that CBX8 knockdown (Figure 3B) reduced the proliferation of PC cells (Figure 3C and D). Colony formation assay revealed that CBX8 silencing impeded the proliferation of PC cells (Figure 3E).
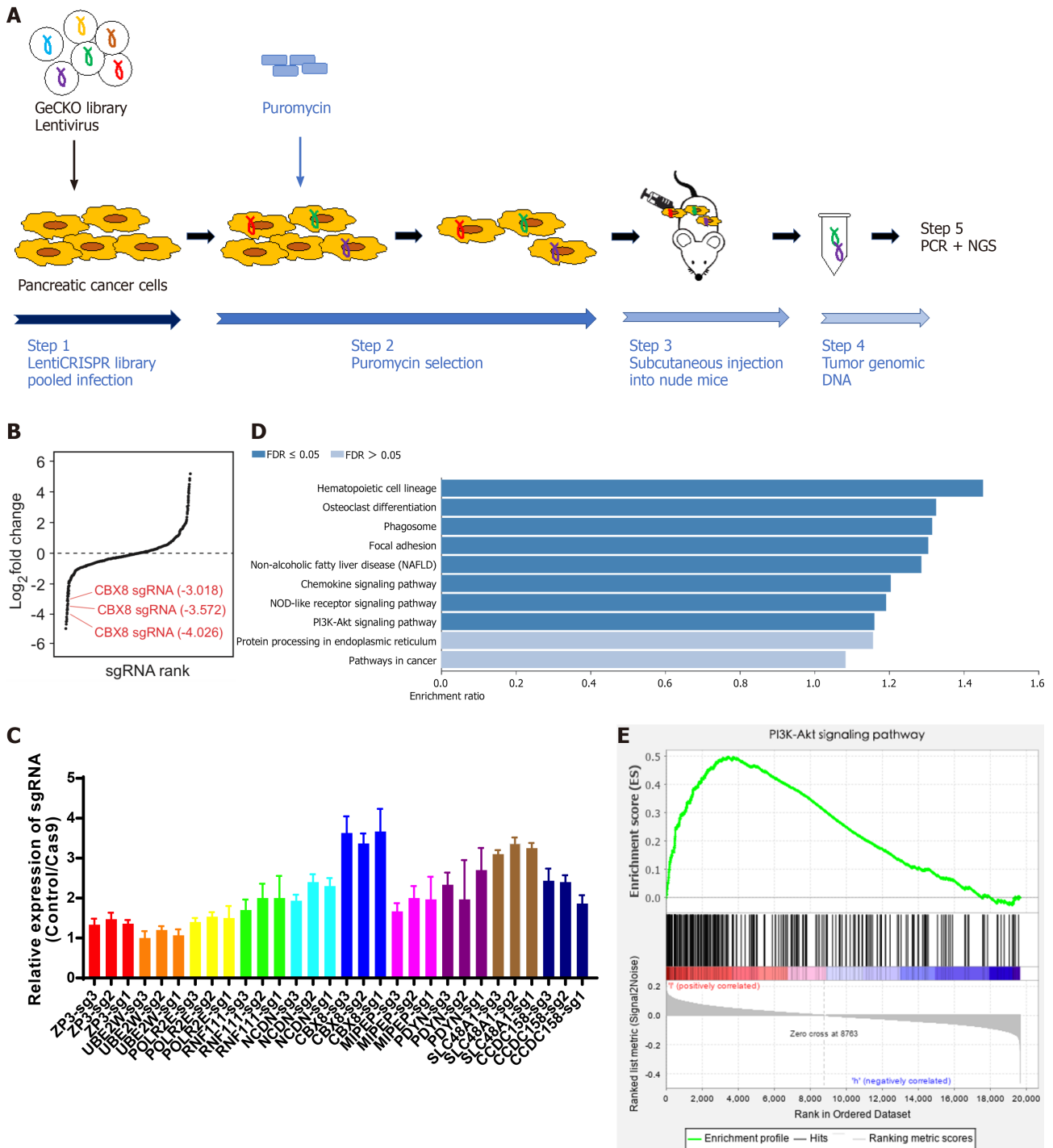
Figure 1 Genome-wide pooled sgRNA library screening identifies genes affecting growth of pancreatic cancer cells *in vivo*. A: Schematic overview of experimental timeline and procedures of CRISPR/Cas9-based screening; B: Respective sgRNAs scored in negative selection CRISPR/Cas9 screening; C: Relative mRNA levels of top six sgRNAs detected by qPCR; D: KEGG pathway analysis of sgRNAs in negative selection CRISPR/Cas9 screening; E: GSEA of sgRNAs in the PI3K/AKT pathway.

To further explore the role of CBX8 in PC tumorigenesis, BXPC-3 cells were transfected with CBX8 knockdown lentiviral vectors (sh-CBX8) or empty vectors (sh-NC). A PC mouse model was established by subcutaneous injection of two groups of cells into the right flank of nude mice. The tumor size of the sh-CBX8 group was significantly smaller than that in the control group (Figure 3F). The kinetics of tumor growth of each group are shown in Figure 3H. A significant difference was found in the tumor weight between the two groups (Figure 3I).

For further investigation, immunohistochemistry (IHC) for Ki67 was performed and indicated that CBX8 knockdown could restrain the proliferative ability of PC cells (Figure 3J).

E
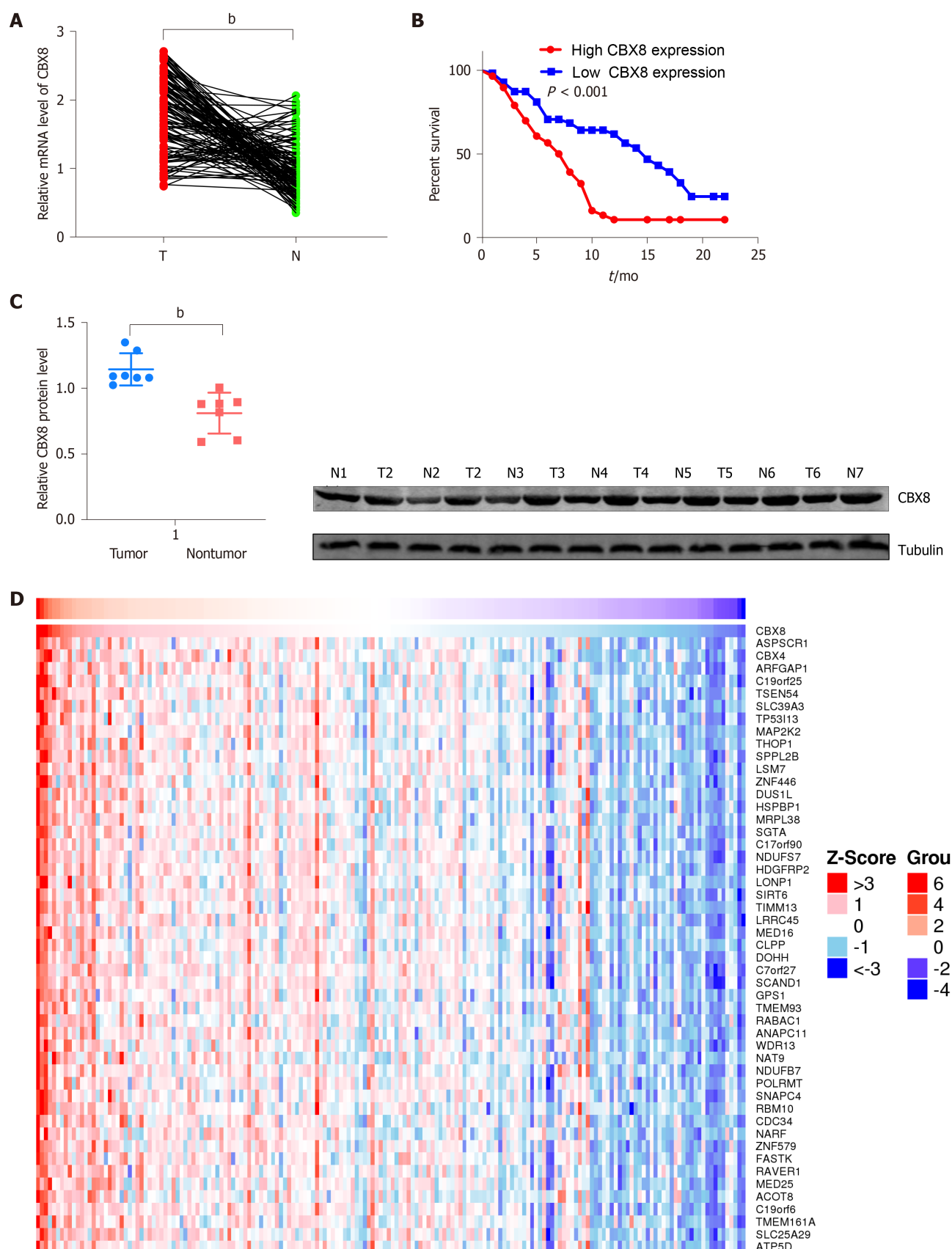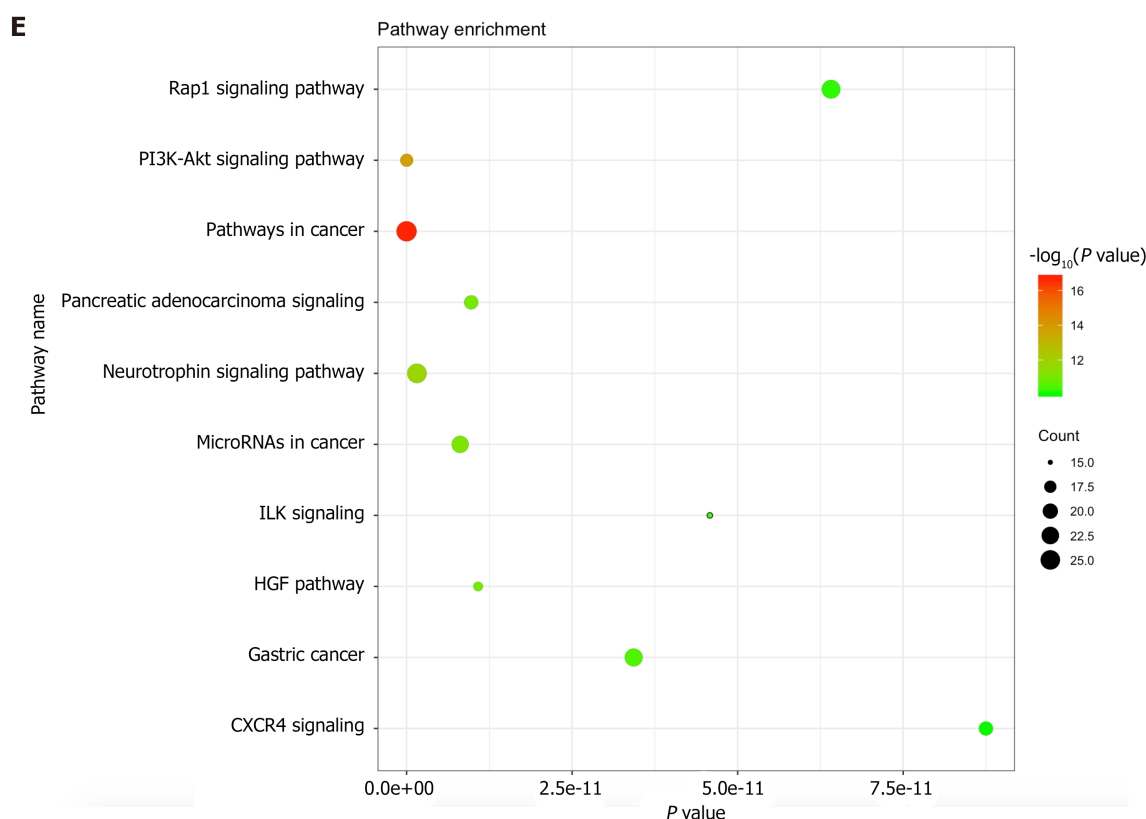
Figure 2 Higher CBX8 expression induces worse clinical outcome in pancreatic cancer tissue. A: The mRNA expression of *CBX8* in 116 paired pancreatic cancer (PC) and normal tissues measured by quantitative reverse transcription-PCR; B: Kaplan–Meier survival of patients with high and low *CBX8* mRNA level; C: Protein expression of CBX8 in 20 paired PC and normal tissues measured by Western blot; D: Heatmap indicating genes positively related with CBX8; E: KEGG pathway analysis of genes in positive relation with CBX8. [b]$P < 0.01$.

HIF-1α modulates CBX8 expression transcriptionally in PC cells

Hypoxia is one of the important characteristics of PC. Among these complex mechanisms, HIF-1α is an important molecule for cells to adapt to hypoxia. We sought to investigate the regulatory mechanism of HIF and CBX8.

By using active chromatin markers in the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/), we identified the proximal *CBX8* promoter and found the potential hypoxia-responsive elements (HREs) between -1100 and -875 bp before the transcriptional start site (TSS) (Figure 4A). Although HRE sequences are widely distributed in all genes, < 1% show hypoxia-dependent binding of HIFs; therefore, we evaluated the function of these HREs. We transfected the wild-type and mutant *CBX8* promoter into 293T cells and exposed them to normoxia or hypoxia. In mutant *CBX8* promoter, we deleted the -875 bp site. This demonstrated that the HRE of *CBX8* was responsive to hypoxia (Figure 4B). We knocked down HIF-1α (Figure 4C) and detected *CBX8* mRNA level under normoxia or hypoxia, which indirectly proved the transcriptional regulation of CBX8 by HIF-1α (Figure 4D). Consistently, ChIP and qPCR demonstrated that HIF-1α bound to *CBX8* promoter under hypoxia (Figure 4E). These data revealed that *CBX8* was a transcriptional target of HIF-1a under hypoxia. Knockdown of HIF-1α with two different siRNAs prevented accumulation of HIF-1α protein under hypoxia (Figure 4F), and the protein expression of CBX8 under hypoxia was not significantly different from that under normoxia (Figure 4G).

CBX8 promotes PC cell proliferation by targeting IRS1/PI3K pathway

To explore the specific mechanism of the regulation of CBX8 on PC cell proliferation, we analyzed the transcriptome of wild-type and CBX8 knockdown PC cells. The analysis revealed 312 differently expressed genes (DEGs) between the two groups and the heatmap showed the top 30 genes (Figure 5A and C). KEGG analysis showed that the DEGs were significantly enriched in the PI3K/AKT, Rap1, and neurotrophin signaling pathways (Figure 5B and C). To investigate the target genes regulated by CBX8, ChIP-seq was performed and we found 62 135 peaks compared to input signals. The pie chart indicates the CBX8-binding distribution (Figure 5D). Overlapping genes
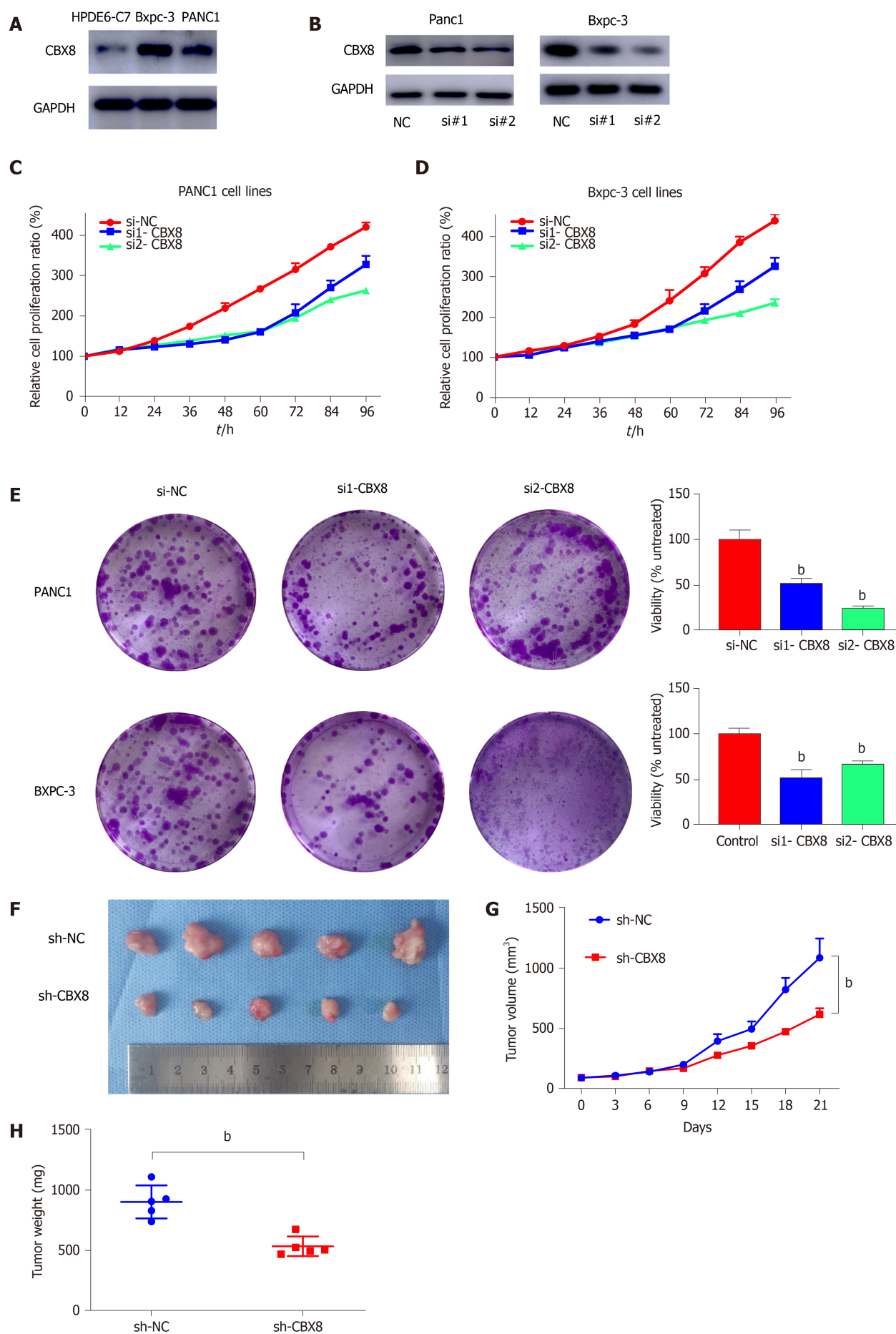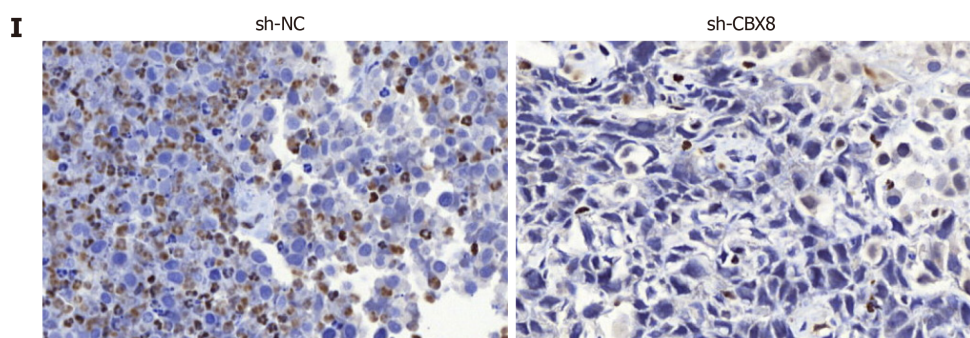
Figure 3 CBX8 is required for efficient proliferation of pancreatic cancer cells *in vitro* and *in vivo*. A: Protein expression of CBX8 in pancreatic cancer (PC) cells and normal pancreatic cells; B: Western blot analysis of CBX8 knockdown efficiency in PC cells; C: Viability of PANC1 cells after transfection with CBX8 knockdown and control plasmids; D: Viability of BXPC-3 cells after transfection with CBX8 knockdown and control plasmids; E: Colony formation assay for evaluation of the effect of CBX8 on PC cell proliferation; F: Images of tumors harvested from each group; G: Tumor growth curve drawn based on the tumor size measured each week; H: Weight of tumors in each group; I: Representative images of immunohistochemistry staining for Ki67 in xenograft tumors. [b]*P* < 0.01.

between CBX8 knockdown and the ChIP-seq data were investigated and there were 35 downregulated genes among the CBX8 target genes. IRS1, which regulates PI3K/AKT pathway activation, was in the set of target genes. We performed ATAC-seq and integrated the multiple tracks in the IGV diagram, where CBX8 and H3K27ac were found to co-occupy the *IRS1* enhancer region (Figure 5E). Upregulation of CBX8 increased the expression of IRS1 (Figure 5F), whereas CBX8 silencing led to a significant downregulation of IRS1 in PC cells (Figure 5G).

We measured CBX8 and IRS1 expression levels in PC tissues, which showed that the endogenous IRS1 level was positively correlated with CBX8 in PC tissues (Figure 5H). We conducted a luciferase reporter assay in 293T cells. The luciferase activity of *IRS1* promoter was enhanced by overexpression of CBX8 but reduced by CBX8 silencing (Figure 5I). Combined ChIP and qPCR analysis revealed that CBX8 bound to regions 2–3 in the *IRS1* promoter (Figure 5J), which were included in the -721/235 fragment. The expression of IRS1 in BXPC-3 cells was downregulated after CBX8 knockdown and upregulated after CBX8 overexpression. The phosphorylation levels of AKT and mammalian target of rapamycin (mTOR) in CBX8-overexpressing BXPC-3 cells were increased, while CBX8 knockdown suppressed the phosphorylation of AKT and mTOR (Figure 5K).

Cbx8 promotes the growth and proliferation of pancreatic cancer cells through IRS1 in vivo

After identifying IRS1 as the possible target of CBX8, we explored whether CBX8 promoted the proliferation of PC cells through IRS1. CCK-8 and colony formation assays were performed to assess the effects of CBX8 and IRS1 on PC cell proliferation, which indicated that CBX8 knockdown reduced the proliferation of PC cells, while IRS1 overexpression inhibited the facilitating effect of CBX8 on PC cell proliferation (Figure 6A and B). IRS1 overexpression and CBX8 knockdown plasmids were cotransfected into PC cells. IRS1 overexpression partly rescued the anti-tumorigenicity of CBX8 knockdown in PC cells (Figure 6C-E). IHC for Ki67 indicated that CBX8 knockdown decreased the proliferation of PC cells, but this was reversed partly by IRS1 overexpression (Figure 6F). In conclusion, our results showed that the CBX8/IRS1 axis regulated PC cell proliferation and HIF-1α promoted the expression of CBX8 in PC cells under hypoxia (Figure 6G).

DISCUSSION

In recent years, CRISPR screening has made great progress in cancer research. Many studies have used CRISPR systems to screen key genes that mediate tumor drug resistance or immune escape[8-10]. In our study, we used the CRISPR screening system to screen genes related to tumor growth. We screened many growth-related genes, among which *CBX8* was associated with a poor clinical prognosis of PC, and *CBX8* deletion can slow down the proliferation of PC cells. Our data showed that knockdown of CBX8 decreased PC cell proliferation, as demonstrated by weakened colony formation *in vitro* and in mouse xenografts. The role of CBX8 in PC has not
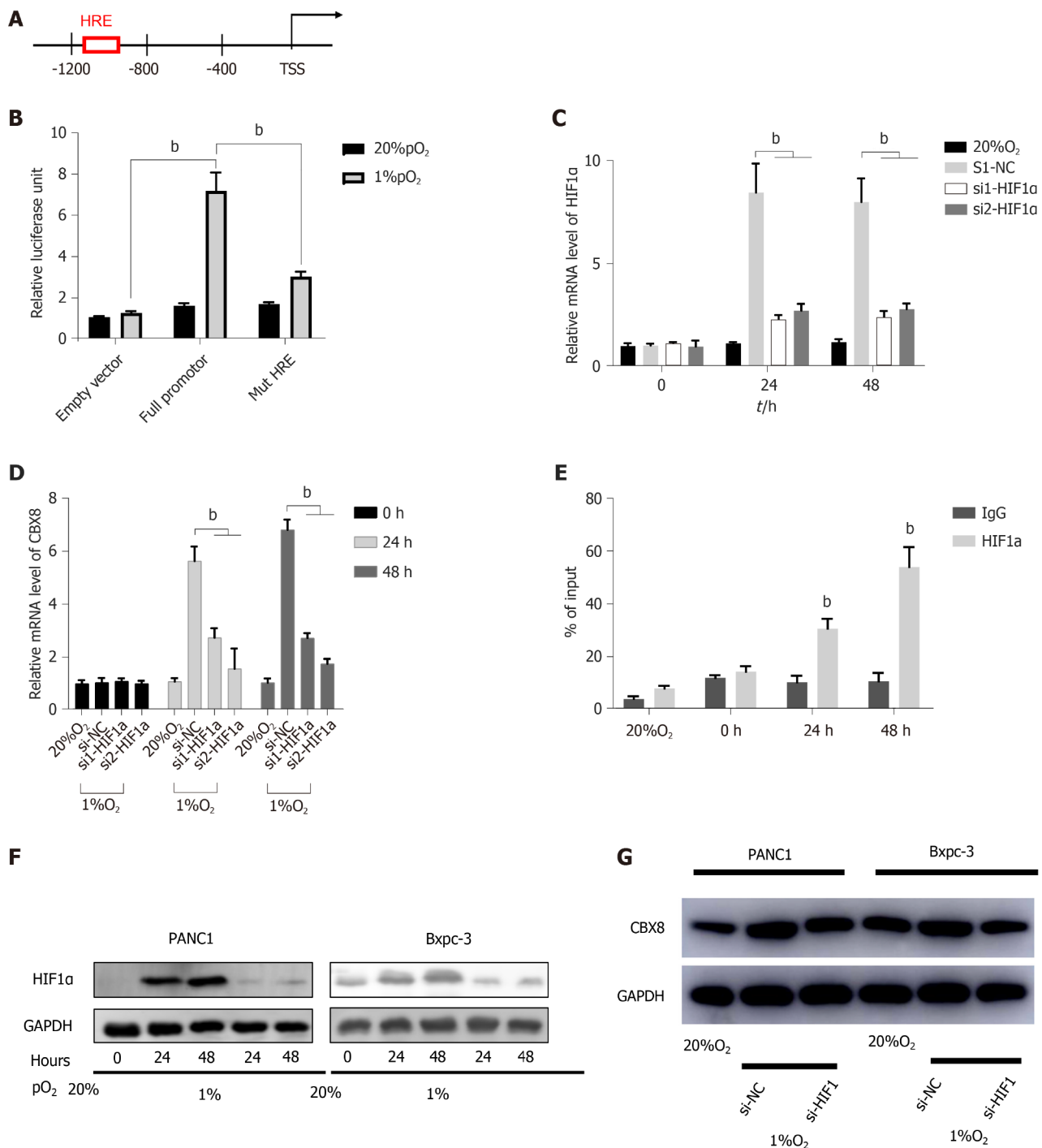
Figure 4 Hypoxia-inducible factor-1α modulates CBX8 expression transcriptionally in pancreatic cancer cells. A: Human *CBX8* promoter shown with HRE; B: 293T cells transfected with full HRE promoter and mutant HRE were cultured under normoxia or hypoxia for 48 h, and firefly luciferase activity determined relative to control Renilla luciferase; C: Quantitative reverse transcription-polymerase chain reaction for detecting hypoxia-inducible factor-1α (HIF-1α) knockdown efficiency under hypoxia; D: Expression level of *CBX8* mRNA was evaluated and normalized to control group; E: Chromatin immunoprecipitation-qPCR analysis was used to determine the binding affinity of HIF-1α to *CBX8* promoter regions; F and G: Expression levels of HIF-1α and CBX8 protein were evaluated and normalized to control group. $^bP < 0.01$.

been studied before, and it may become a new therapeutic target.

CBX8 is associated with the stemness and chemosensitivity of many types of cancer. It has been reported that CBX8 upregulates LGR5 expression in a noncanonical manner by interacting with KMT2b and Pol II, leading to increased cancer stemness and decreased chemosensitivity in CC[5]. CBX8 also promotes the proliferation of HCC cells through YBX1-mediated cell cycle progression, and high CBX8 expression is related to a poor prognosis of HCC patients[11]. A recent study showed that CBX8 recruited KMT2b to the *LGR5* promoter and modulated H3K27me3 in the promoter of bone morphogenetic protein (BMP)4, resulting in increased *BMP4* transcription and
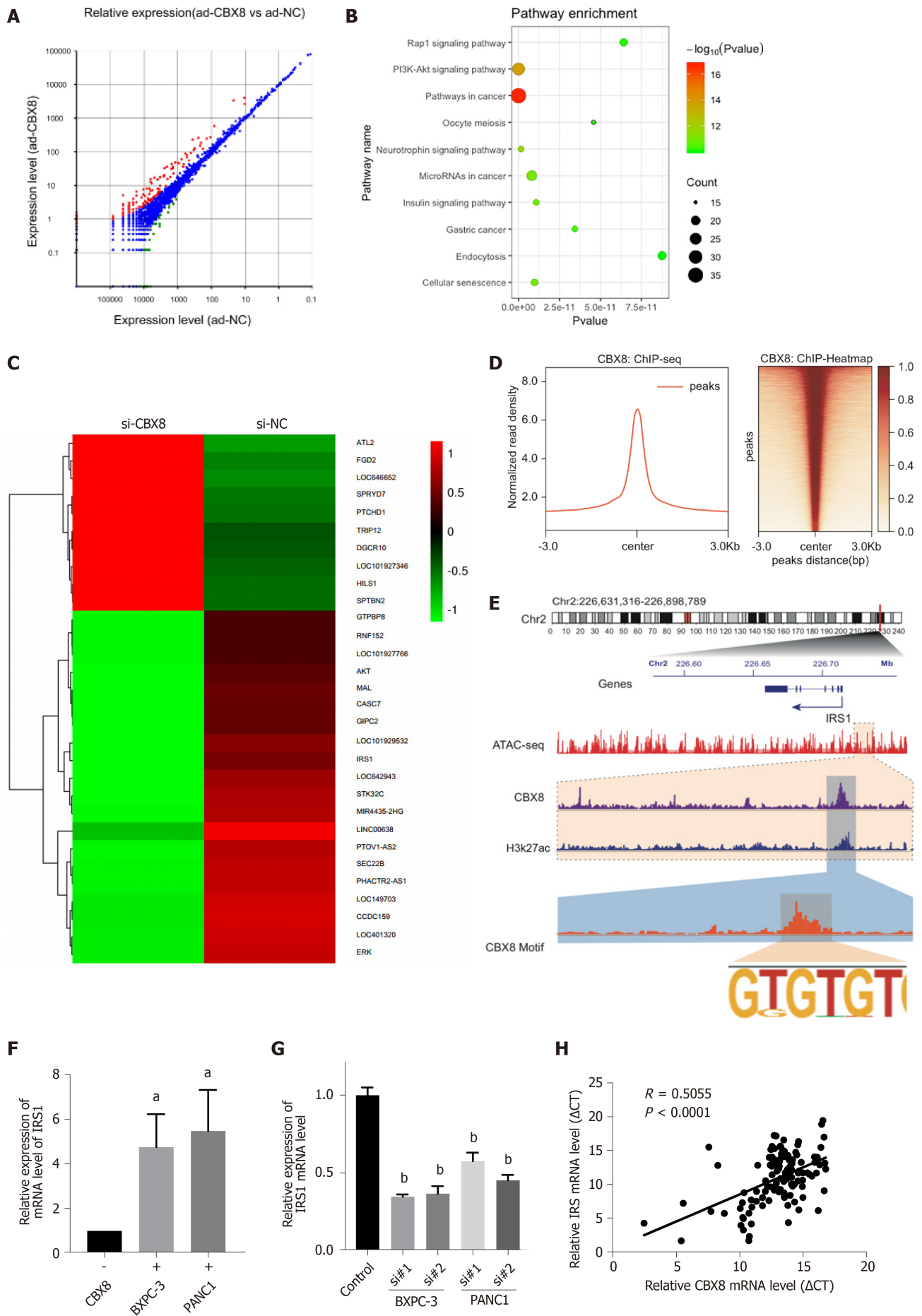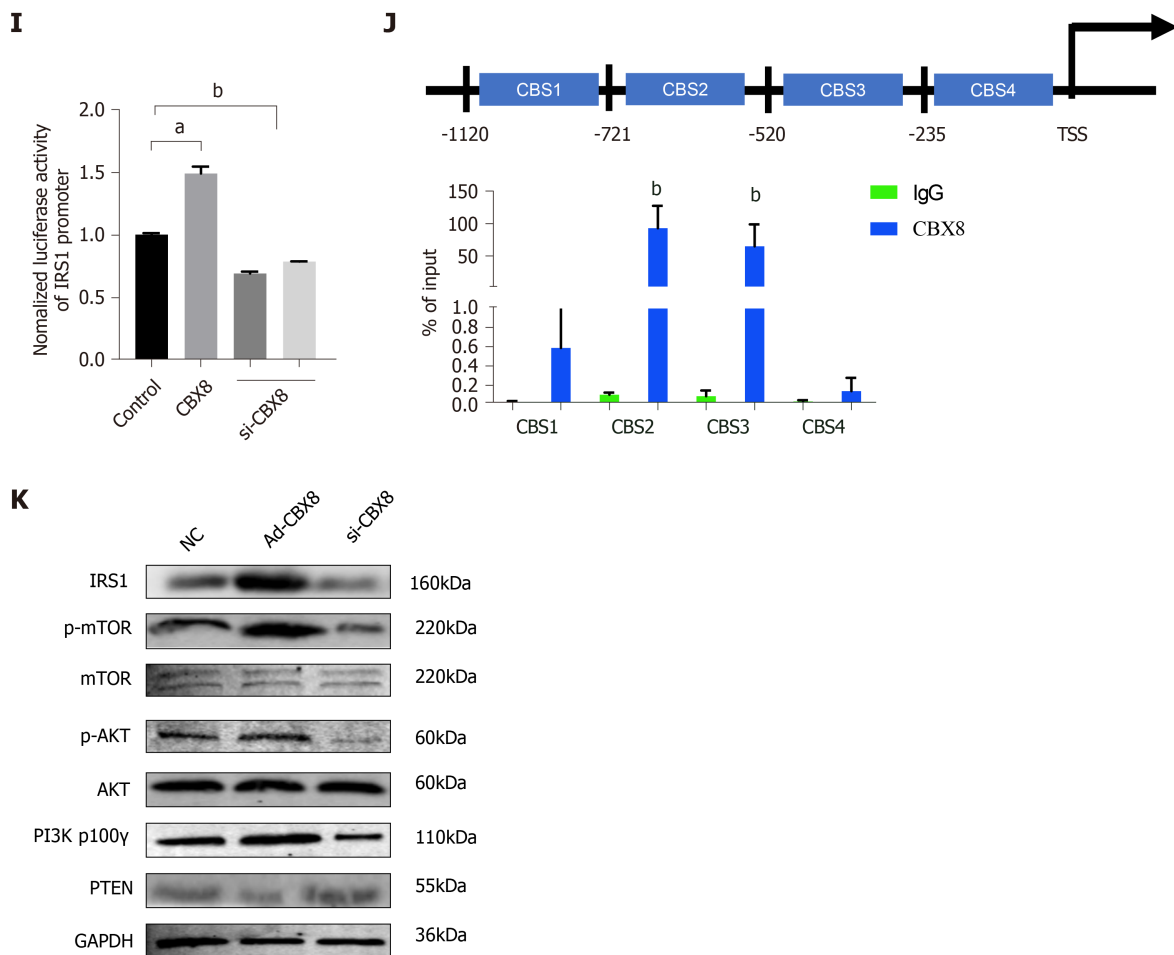
Figure 5 CBX8 promotes pancreatic cancer cell proliferation by targeting IRS1/PI3K pathway. A: Differently expressed genes (DEGs) after CBX8 knockdown; B: KEGG pathway analysis of DEGs; C: Heatmap indicating the top 30 DEGs; D: Chromatin immunoprecipitation (ChIP)-Seq summary plot of CBX8-binding intensities across CBX8 peaks in BXPC-3 cells; E: Multiple tracks in the IGV diagram exhibited the co-occupancy of CBX8 and H3K27ac in the IRS1 enhancer region; F: Expression level of *IRS1* mRNA was evaluated and normalized to the control group; G: Expression level of *IRS1* mRNA was evaluated and normalized to untransduced controls; H: Expression of CBX8 and IRS1 exhibited a positive correlation in PC tissues; I: Luciferase reporter assay was performed to confirm the binding of CBX8 to the promoter region of *IRS1* in 293T cells; J: ChIP-qPCR analysis was used to determine the binding affinity of CBX8 to *IRS1* promoter regions; K: Expression of PTEN and PI3Kγ and the phosphorylation levels of IRS1, AKT, and mTOR in PC cells were assessed by Western blot. $^aP < 0.05$, $^bP < 0.01$.

subsequent activation of Smads and mitogen-activated protein kinases. In our study, the level of CBX8 in PC tissues was higher than that in adjacent normal tissues. The CRISPR/Cas9 screening also indicated that CBX8 knockout might decrease the proliferation of PC cells.

Hypoxia is a significant feature of PC. Due to the rapid growth of tumor, the blood vessels in PC often show immature microvascular lumina. This results in hypoxic features in PC tissues. In our previous studies, we found that hypoxia mediated the invasion, metastasis, and angiogenesis of PC by promoting the secretion of exosomes [4,12]. In the present study, we found that hypoxia-upregulated HIF-1α bound to *CBX8* promoter region and regulated *CBX8* expression at the transcriptional level.

The IRS family is composed of four proteins (IRS1–IRS4), which were initially considered as typical cytolytic adaptor proteins. They are involved in IR and insulin-like growth factor I receptor signal transduction[13,14]. Post-translational modification of IRS1 can activate the mTORC1 signaling pathway through chronic elevation of multiple serine phosphorylation sites[15].

In our study, CBX8 modulated H3K27me3 in the promoter of *IRS1*, resulting in increased *IRS1* transcription and subsequent activation of PI3K and AKT. At the same time, *in vitro* colony formation assay and mouse xenograft model confirmed that CBX8 promoted the growth of PC cells through IRS1.

Through CRISPR screening, we identified a group of genes related to the growth of PC. Combined analysis of clinical samples of patients with PC demonstrated that CBX8 was higher in PC tumor tissue and high expression of CBX8 predicted a poor clinical outcome. HIF-1α regulated expression of CBX8 transcriptionally under
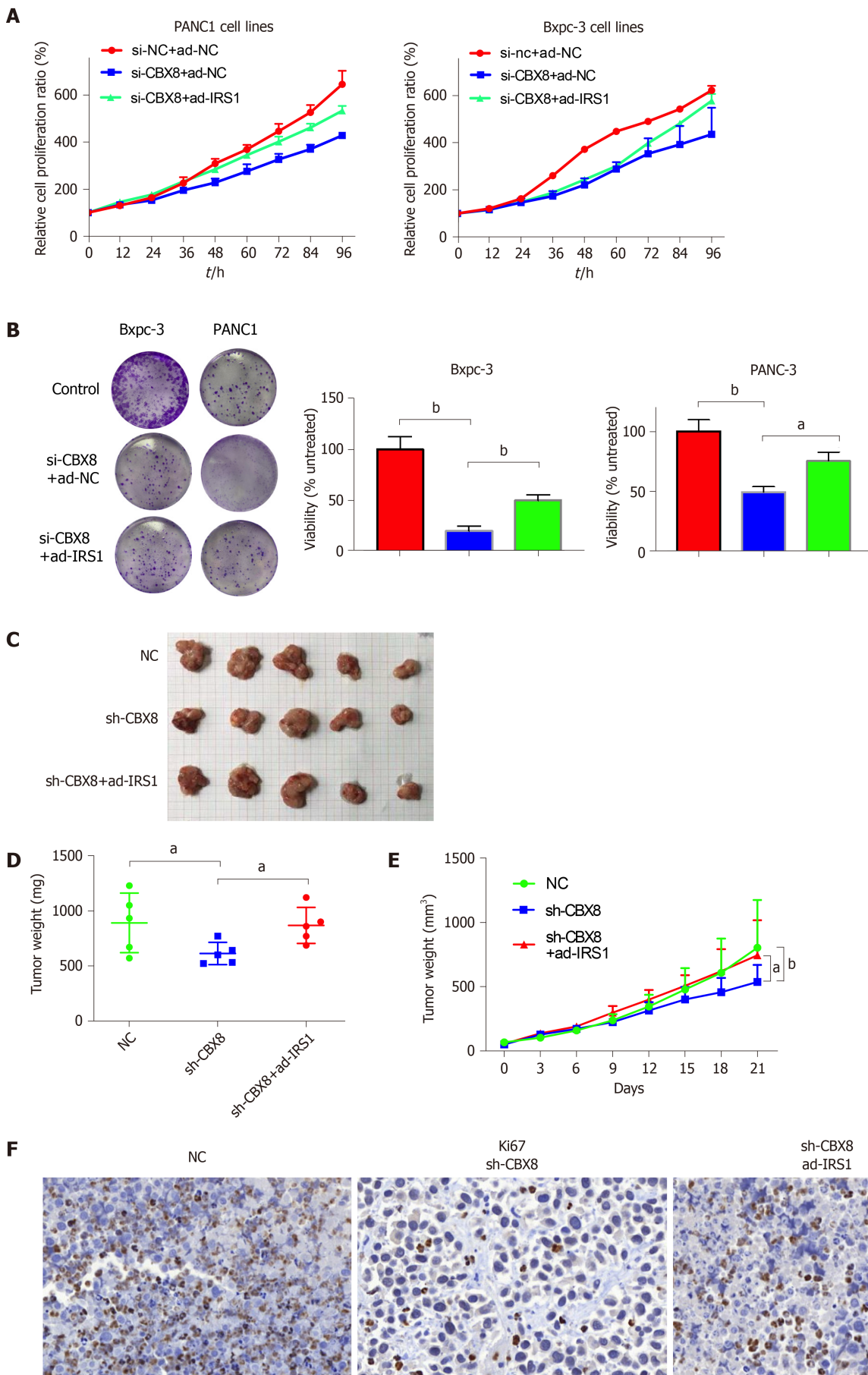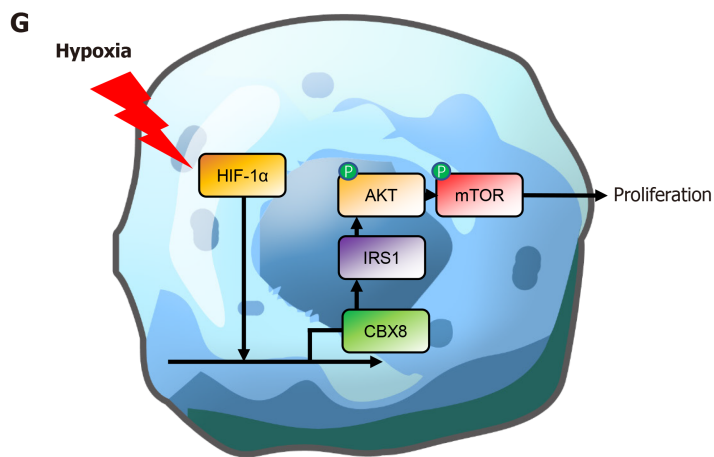
Figure 6 CBX8 promotes the growth and proliferation of pancreatic cancer cells through IRS1 *in vivo*. A: Viability of BXPC-3 and PANC1 cells after transfection with CBX8 knockdown and/or IRS1 plasmid; B: Colony formation assay was performed to evaluate the effect of CBX8 on PC cell proliferation; C: Images of tumors harvested from each group; D: Weight of tumors in each group; E: Tumor growth curve drawn based on the tumor size measured each week; F: Representative images of immunohistochemistry staining for Ki67 in xenograft tumors; G: Schematic model illustrating that HIF-1a-meditated CBX8 transcription promotes pancreatic cancer progression *via* IRS1/AKT axis. $^{a}P < 0.05$, $^{b}P < 0.01$.

hypoxia and CBX8 induced PC cell proliferation by targeting IRS1, which activated the PI3K/AKT pathway. These studies revealed the mechanism of the promotion effect of CBX8 on the development of PC, and provided potential therapeutic targets.

CONCLUSION

Our results suggest that CBX8 could function as an oncogenic factor in PC progression. High CBX8 expression is correlated with poor clinical outcomes of PDAC patients from two independent cohorts. We also showed that CBX8 is a key gene that is regulated by HIF-1α, and can activate the IRS1/AKT pathway. The above findings suggest that targeting CBX8 may be a promising therapeutic strategy for PC.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer (PC) is one of the most lethal cancers worldwide. It has become the second most fatal cancer in the United States. Chromobox (CBX)8 promotes tumor growth and metastasis in other cancers. However, whether CBX8 is involved in the proliferation of PC cells remains unknown.

Research motivation

Many studies have shown that the prognosis of patients with PC remains poor after complete surgical resection. Therefore, it is important to study the occurrence and development of PC and the corresponding targeted therapy. We hope to provide a novel therapeutic target for patients with PC.

Research objectives

The present study aimed to investigate the function of the CBX8/IRS1/AKT axis in PC.

Research methods

Genome-wide CRISPR-Cas9 screening was performed to select genes that could facilitate PC cell proliferation. A total of 244 candidate genes were identified as being responsible for proliferation of PC cells using deep single guide RNA sequencing. Quantitative reverse transcription-polymerase chain reaction was used to detect the expression of CBX8 in PC tissues and cells. The regulatory roles of CBX8 in cell prolif-eration, migration, and invasion were verified by CCK-8 and Transwell assays.

Research results

CBX8 was upregulated in pancreatic tumor tissues and shown to drive PC cell proliferation. Higher expression of CBX8 was correlated with worse outcomes of PC patients from two independent cohorts with a total of 116 cases. CBX8 also served as a promising therapeutic target for a PC xenograft model. We demonstrated that HIF-1a induced CBX8 transcription by binding to the promoter of CBX8. CBX8 efficiently activated the PI3K/AKT signaling pathway by upregulating insulin receptor substrate (IRS)1.

Research conclusions

CBX8 promotes PC cell progression by activating the IRS1/AKT pathway.

Research perspectives

CBX8 could promote PC progression, which might provide a potential treatment strategy for this malignancy.

ACKNOWLEDGEMENTS

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ORIGINAL ARTICLE

Basic Study

Shuyu pills inhibit immune escape and enhance chemosensitization in hepatocellular carcinoma

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Abstract

BACKGROUND

Hepatocellular carcinoma (HCC) is characterized by dysregulation of the immune microenvironment and the development of chemoresistance. Specifically, expression of the programmed cell death protein 1 (PD-1)/programmed cell death

1 ligand 1 (PD-L1) axis, an immune checkpoint, may lead to tumour immune escape, resulting in disease progression. The latest research shows that tumour immune escape may be caused by the upregulation of PD-L1 mediated by hypoxia-inducible factor-1 alpha (HIF-1α), and simultaneous inhibition of HIF-1α and PD-L1 has the potential to enhance the host's antitumour immunity. Moreover, inhibition of the PD-1/PD-L1 axis may mitigate tumour chemoresistance. Shuyu pills (SYPs) contain immunity-enhancing and antitumour components, making them a potential HCC treatment.

AIM
To investigate the efficacy of SYPs for HCC treatment *via* simultaneous HIF-1α and PD-L1 inhibition and the mechanism involved.

METHODS
A subcutaneous xenograft tumour model was first established in BALB/c nude mice by the subcutaneous injection of 1×10^7 SMMC-7721 cells. Male mice (male, 5 weeks old; $n = 24$) were then randomly divided into the following four groups ($n = 6$): Control (0.9% normal saline), SYP (200 mg/kg), SYP + cisplatin (DDP) (200 mg/kg + 5 mg/kg DDP weekly *via* intraperitoneal injection), and DDP (5 mg/kg cisplatin weekly *via* intraperitoneal injection). The dose of saline or SYPs for the indicated mouse groups was 0.2 mL/d *via* intragastric administration. The tumour volumes and body weights of the mice were measured every 2 d. The mice were euthanized by cervical dislocation after 14 d of continuous treatment, and the xenograft tissues were excised and weighed. Western blot assays were used to measure the protein expression of HIF-1α, PD1, PD-L1, CD4+ T cells, and CD8+ T cells in HCC tumours from mice. Quantitative reverse transcription polymerase chain reaction was used for real-time quantitative detection of PD-1, PD-L1, and HIF-1α mRNA expression. An immunofluorescence assay was conducted to examine the expression of CD4+ T cells and CD8+ T cells.

RESULTS
Compared to mice in the control group, those in the SYP and SYP + DDP groups exhibited reduced tumour volumes and tumour weights. Moreover, the protein and mRNA expression levels of the oncogene HIF1α and that of the negative immunomodulatory factors PD-1 and PD-L1 were decreased in both the SYP and SYP + DDP groups, with the decrease effects being more prominent in the SYP + DDP group than in the SYP group (HIF-1α protein: Control *vs* SYP, $P = 0.0129$; control *vs* SYP + DDP, $P = 0.0004$; control *vs* DDP, $P = 0.0152$, SYP + DDP *vs* DDP, $P = 0.0448$; HIF-1α mRNA: control *vs* SYP, $P = 0.0009$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P = 0.0003$, SYP *vs* SYP + DDP, $P = 0.0192$. PD-1 protein: Control *vs* SYP, $P = 0.0099$; control *vs* SYP + DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P = 0.0009$; SYP + DDP *vs* DDP, $P < 0.0001$; PD-1 mRNA: control *vs* SYP, $P = 0.0002$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P = 0.0003$, SPY *vs* SYP + DDP, $P = 0.0003$; SYP + DDP *vs* DDP, $P = 0.0002$. PD-L1 protein: control *vs* SYP, $P < 0.0001$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P = 0.0040$; SYP + DDP *vs* DDP, $P = 0.0010$; PD-L1 mRNA: Control *vs* SYP, $P < 0.0001$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P < 0.0001$; SYP + DDP *vs* DDP, $P = 0.0014$). Additionally, the quantitative and protein expression levels of CD4+ T cells and CD8+ T cells were simultaneously upregulated in the SYP + DDP group, whereas only the expression of CD4+ T cells was upregulated in the SYP group. (CD4+ T cell quantitative: Control *vs* SYP + DDP, $P < 0.0001$, SYP *vs* SYP + DDP, $P = 0.0005$; SYP + DDP *vs* DDP, $P = 0.0002$. CD4+ T cell protein: Control *vs* SYP, $P = 0.0033$; Control *vs* SYP + DDP, $P < 0.0001$; Control *vs* DDP, $P = 0.0021$, SYP *vs* SYP + DDP, $P = 0.0004$; SYP + DDP *vs* DDP, $P = 0.0006$. Quantitative CD8+ T cells: Control *vs* SYP + DDP, $P = 0.0013$; SYP *vs* SYP + DDP, $P = 0.0347$; SYP + DDP *vs* DDP, $P = 0.0043$. CD8+ T cell protein: Control *vs* SYP + DDP, $P < 0.0001$; SYP *vs* SYP + DDP, $P < 0.0001$; SYP + DDP *vs* DDP, $P < 0.0001$). Finally, expression of HIF-1α was positively correlated with that of PD-1/PD-L1 and negatively correlated with the expression of CD4+ T cells and CD8+ T cells.

CONCLUSION
SYPs inhibit immune escape and enhance chemosensitization in HCC *via*

simultaneous inhibition of HIF-1α and PD-L1, thus inhibiting the growth of subcutaneous xenograft HCC tumours.

Key Words: Shuyu pills; Hepatocellular carcinoma; Tumour microenvironment; Immune escape; Chemoresistance

Core Tip: Hepatocellular carcinoma is characterized by both dysregulation of the immune microenvironment and chemoresistance. This study demonstrated that the components of Shuyu pills (SYPs) inhibit the growth of subcutaneous xenograft tumours in nude mice and act synergistically when used in combination with cisplatin (DDP). SYPs exert their effects by simultaneously inhibiting the expression of hypoxia-inducible factor-1 alpha and programmed cell death 1 ligand 1 (PD-L1) and promoting that of CD4+ T and CD8+ T cells, thereby inhibiting immune escape of the tumour cells. Additionally, the programmed cell death protein 1/PD-L1 axis was inhibited, which mitigated the resistance of the tumours to DDP, thereby sensitizing them to chemotherapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy that ranks 6th in cancer incidence and 4th in cancer-related mortality worldwide[1]. Elucidation of the molecular changes underlying HCC development should unveil novel molecular targets for the development of therapies aimed at controlling tumour progression and improving patient survival. Changes in the tumour microenvironment play a crucial role in tumour development and progression. One of the most important changes is the development of immunosuppressive mechanisms by tumour cells, which allow them to escape the host's immune system, thereby enhancing their survival and proliferative, migratory, and invasive capabilities[2]. An important mechanism that mediates the immunosuppressive microenvironment is the overactivation of immune checkpoints, a major one of which is formed by programmed cell death protein 1 (PD-1) and its ligand programmed cell death 1 ligand 1 (PD-L1). PD-L1 is expressed on the surface of many types of tumour cells, and PD-1 is expressed on the surface of tumour-infiltrating lymphocytes (TILs). The binding of PD-L1 to PD-1 molecules can inhibit the function of T cells, limiting their ability to destroy tumour cells. This promotes tumour immune escape and leads to disease progression[3]. Additionally, PD-L1 overexpression is associated with a poor prognosis in many cancer types and development of tumour cell resistance to anticancer therapies[4]. Therefore, inhibition of the PD-1/PD-L1 signalling pathway, which would restore the normal tumour cell surveillance and destruction activities of T cells, may represent an effective therapeutic strategy against HCC[5,6].

Hypoxia, which is associated with an imbalance in rapid tumour growth and an insufficient blood supply, is another common change that occurs in the microenvironment of solid tumours[7]. Extensive research has demonstrated that the expression of hypoxia-inducible factor-1 alpha (HIF-1α), which plays a crucial role in tumour angiogenesis, invasion, and metastasis, is elevated in hypoxic tumours[8,9]. Additionally, hypoxia has been shown to induce tumour chemoresistance[10,11]. HIF-1, the major transcription factor mediating the adaptive response to hypoxia, is a heterodimeric complex consisting of the HIF-1α and HIF-1β subunits, with HIF-1α being the major functional protein[12]. Recent studies have identified a significant positive correlation between HIF-1α and PD-L1 in a variety of tumour cell lines[13],

where the upregulation of PD-L1 mediated by HIF-1α constitutes a tumour immune escape mechanism[14-17]. In one study, knockdown of HIF-1α using small interfering RNA prevented the accumulation of HIF-1α protein, which inhibited the hypoxia-mediated increase in PDL1 mRNA and consequently its protein expression on the cell surface[18]. The molecular mechanism underlying this correlation was explored in a previous study, wherein hypoxia was shown to cause rapid, significant, and selective upregulation of PD-L1 in bone marrow mesenchymal stem cells, macrophages, dendritic cells, and tumour cells[19]. This upregulation of PD-L1 was dependent on HIF-1α, an upstream regulator of PD-L1 mRNA and protein expression. HIF-1α binds directly to the transcriptional active site—the hypoxia response element—of the PD-L1 proximal promoter, leading to rapid PD-L1 accumulation and subsequent tumour immune escape[19]. Therefore, simultaneous inhibition of PD-L1 and HIF-1α expression represents a promising novel strategy in cancer immunotherapy.

Traditional Chinese medicine is an important treatment modality for HCC. Shuyu pills (SYPs) are composed of a compound formulation that has long been used as a traditional Chinese medicine for improving energy metabolism and immune function. The pills exert myriad health-benefiting effects, such as boosting one's immunity, and can be used as an adjunct cancer treatment. Yam polysaccharides, a component of SYPs, can enhance the antioxidative capacity and free radical-scavenging activities of the body and thereby reduce cellular oxidative damage[20]. They can also improve the immunomodulatory activities of splenic lymphocytes and enhance immune function [21]. Other components of SYPs, such as ginsenosides, can regulate signal transduction pathways associated with inflammation, oxidative stress, angiogenesis, and tumour cell metastasis[22]. Ginsenosides also regulate the cell cycle and inhibit the multidrug resistance of cancer cells and are involved in cancer immunomodulation[23]. Trichos-anthin, another SYP component, can inhibit the growth of tumour cells and induce their apoptosis[24] and displays potent immunosuppressive activity[25]. Thus, it is evident that the components of SYPs exert a multitude of effects that can contribute to antitumour activity. As a solid tumour characterized by hypoxia and immune dysfunction, HCC is also prone to developing resistance to chemotherapeutic drugs during clinical treatment. Therefore, in this study, we investigated the efficacy of the combination of SYPs and cisplatin (DDP) for the treatment of HCC and the mechanism involved.

The therapeutic efficacy and mechanism of action of SYPs against HCC were explored from the perspectives of immune escape in the tumour microenvironment and chemoresistance. We hypothesized that SYPs exert antitumour effects by simultaneously inhibiting HCC cellular expression of HIF-1α and PD-L1, which would improve the immunosuppressive state of the tumour microenvironment. We also hypothesized that the SYP and DDP combination would mitigate chemoresistance by inhibiting the PD-1/PD-L1 axis.

MATERIALS AND METHODS

The preparation of SYPs and DDP
Each SYP contained the following components: *Rhizoma Dioscoreae*, *Radix Angelicae Sinensis*, *Ramulus Cinnamomi*, *Medicinal Fermented Mass*, *Radix Rehmanniae*, *Ginseng*, *Radix Glycyrrhizae*, *Rhizoma Chuanxiong*, *Radix Paeoniae Alba*, *Rhizoma Atractylodis Macrocephalae*, *Radix Ophiopogonis*, *Semen Armeniacae Amarae*, *Radix Bupleuri*, *Radix Platycodi*, *Poria*, *Colla Corii Asini*, *Rhizoma Zingiberis*, *Radix Saposhnikoviae*, *Radix Ampelopsis*, and *Fructus Jujubae* in an 8:2:2:4:2:4:4:2:2:2:2:2:1:2:2:1:2:2:8 radio. Granules of the traditional Chinese medicinal formula were used, and all medicinal substances were purchased from Guangdong Yifang Pellet Pharmaceutical Co., Ltd. (Guangdong, China; Lot No. 17043278). All study parameters fulfilled standard quality require-ments. All medicinal substances were dissolved in warm water, and the mixtures were then vortexed into a suspension. Based on the pharmacological dose requirements of SYPs, the daily dose of SYPs for human adults was 156 g. Therefore, the equivalent dose for each nude mouse was 200 mg/kg according to the Table of Equivalent Dose Ratio Conversion between Human and Animal by Body Surface Area. DDP injections (Cat No. 9E0214B02) were purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, China). The DDP dose was 5 mg/kg, administered intraperitoneally once a week.

The study of SYPs using high resolution mass spectrometry
SYPs were extracted using 100% methanol, and 5.000 g SYPs were extracted using 20.00 mL anhydrous methanol by ultrasonic extraction for 45 min. Then, the super-

natant was centrifuged at 8000 RPM for 5 min. After centrifugation, the supernatant was filtered through a 0.22 μm microporous filter membrane, and the filtrate was analysed using UPLC-Q-TOF-MS (1290 UPLC-6540, Agilent Technologies Inc., United States). We used an Agilent ZORBAX Eclipse Plus C18 (3.0 mm × 100 mm, 1.8 μm) column, and the mobile phase system consisted of acetonitrile (A) and water (containing 0.1% formic acid). The gradient elution procedure was used under the following conditions: 0–10 min, 5%–15% A; 10–15 min, 15%–20% A; 15–25 min, 25%–45% A; and 25–40 min, 45%–80%. The flow velocity of the 1 μL sample volume was 0.4 mL/min. Mass spectrometry testing conditions were set to ionization mode and electrospray ionization, and accurate mass data correction were performed using electrospray ionization-L Low Concentration Tuning Mix (G1969–85000). Then, positive and negative ion analysis modes and MRE scan modes were adopted to analyse the samples. The sheath gas temperature was 350 °C, and the range of full-mass scanning was 100–1700 m/z. In addition, the capillary voltage was set to 4.0 KV. Nitrogen was chosen as the desolventizer gas, and the temperature was set at 325 °C with a flow rate of 6.8 L/min. Furthermore, to obtain an accurate analysis after primary scanning, secondary mass spectrometry was performed by dependent scanning, and the first three strengths were also selected for collision-induced dissociation. The range of secondary fragment scanning was 50–1000 m/z, and the fragment voltages were set to 10, 20, and 30 kV.

Cells and animal grouping

A subcutaneous xenograft tumour model was established in BALB/c athymic nude mice (n = 24, 5 weeks old, 18 ± 3 g) *via* the subcutaneous injection of 1 × 10^7 SMMC-7721 cells into the right side of the back of each mouse. Once the tumours were palpable in the mice (tumour volume, approximately 100 mm^3), the animals were randomly divided into the following four groups (n = 6): (1) Control (oral dose of 0.2 mL of 0.9% normal saline, daily); (2) SYP (oral dose of 200 mg/kg, daily); (3) DDP (intraperitoneal dose 5 mg/kg, once weekly); and (4) SYP (oral dose of 200 mg/kg, daily) + DDP (intraperitoneal dose 5 mg/kg, once weekly). The tumour volume [calculated as the maximum tumour length × width (2 × 0.5)] and body weight of each mouse were measured every 2 d. Fourteen days after treatment, the mice were euthanized by cervical dislocation according to Animal Research: Reporting *In Vivo* Experiments. This study was approved by the Ethics Review Committee of Experimental Animal Welfare of Central South University and performed in accordance with the European Community Guidelines on the Use and Care of Laboratory Animals, with all laboratory animals being carefully attended to.

Western blot analysis

Protein extraction was performed for western blot analysis. In brief, mouse tumour tissue specimens were first lysed in ice-cold lysis buffer [150 mmol/L NaCl, 20 mmol/L HEPES, 1% Triton X-100, 2 mmol/L EGTA, 20 mmol/L glycerophosphate, 1 mmol/L EDTA, and 10% glycerol plus protease inhibitor (ApplyGene, Inc.)]. Then, the protein concentrations of the tissue lysates were measured using the bicinchoninic acid assay. Next, 50 μg of the denatured protein was loaded onto a 4% sodium dodecyl sulphate-polyacrylamide gel for electrophoresis, after which the separated protein bands were electrotransferred to a PVDF membrane (EMD Millipore) for 2 h. After transfer, the membrane was soaked in a blocking solution (5% milk in 1 × TBST) at ambient temperature for 2 h and then at 4 °C overnight. The next day, the membrane was incubated with the primary antibodies at 37 °C for 60 min and then with the secondary antibodies at 4 °C overnight. The primary antibodies were as follows: Anti-PD-1 (PD-1; Cat No. ab214421; dilution, 1:1000; monoclonal antibody; Abcam); anti-PD-L1 (PD-L1; Cat No. ab238697; dilution, 1 μg/mL; monoclonal antibody; Abcam); anti-CD4 (CD4; Cat No. MA1-146; dilution, 1:1000; monoclonal antibody; Invitrogen Antibodies); anti-CD8 (CD8; Cat No. ab209775; dilution, 1:1000; monoclonal antibody; Abcam); anti-HIF-1α (HIF-1α; Cat No. ab1; dilution, 5 μg/mL; monoclonal antibody; Abcam); and anti-p53 (p53; Cat No. ab26; dilution, 2 μg/mL; monoclonal antibody; Abcam). An anti-actin antibody (Cat No. 60008–1-Ig; dilution, 1:5000; monoclonal antibody; Proteintech Group, Inc.) was used as the protein-loading control. A horseradish peroxidase-conjugated polyclonal secondary antibody (dilution, 1:5000; Proteintech Group, Inc.) was used for detection. SuperECL Plus detection reagent (Thermo Fisher Scientific, Inc.) was used as an enhanced chemiluminescent substrate to enable visualization of the protein bands. The protein signals could be visualized for 3 min and were exposed to Kodak Biomax XAR film (Kodak). ImageJ version 1.80 software (National Institutes of Health) was used to scan and quantify the intensity of each protein band.

Quantitative reverse-transcription polymerase chain reaction

Total RNA was extracted from SMMC-7721 cells using TRIzol reagent (Takara) according to the manufacturer's instructions. Reverse transcription was then performed using cDNA reverse transcriptase. The polymerase chain reaction (PCR) a conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The following primer sequences were used for PCR: Actin, AC ATCCGTAAAGACCTCTATGCC (forward) and TACTCCTGCTTGCTGATCCAC (reverse); p53, CCCCTGTCATCTTTTGTCCCT (forward) and AGCTGGCAGAATA GCTTATTGAG (reverse); HIF-1α, TCCAGCAGACCCAGTTACAGA (forward) and GCCACTGTATGCTGATGCCTT (reverse); PD-1, GCACCCCAAGGCAAAAATCG (forward) and CAATACAGGGATACCCACTAGGG (reverse); and PD-L1, AAAGAC-GAGCATAGCCGAAC (forward) and GCCACACCAATCCAACACC (reverse).

Immunofluorescence assay

The tumour tissues were embedded into paraffin blocks using standard techniques, and the blocks were then sectioned into 4-µm slices. After the sections had been deparaffinized in water, they were subjected to heat-induced antigen retrieval in 0.01 M citrate buffer (pH 6.0). Then, once the sections had cooled to ambient temperature, they were washed 3 times with 0.01 M poly(butylene succinate) (PBS) (pH 7.2–7.6) for 3 min each time. The sections were then placed in sodium borohydride solution for 30 min at ambient temperature and thereafter rinsed with water for 5 min. Next, the sections were soaked in Sudan Black B staining solution at ambient temperature for 5 min, followed by rinsing with water for 3 min. Next, the sections were blocked in 10% normal serum/5% BSA for 60 min and then incubated overnight with appropriately diluted primary antibodies (CD4, CD8) at 4 °C. On the next day, the sections were rinsed 3 times with PBS for 5 min each time and then incubated with the secondary antibody (50–100 µL of anti-rat, rat-IgG-labelled fluorescent antibody) at 37 °C for 90 min. The sections were then rinsed 3 times with PBS for 5 min each. A working solution of 4',6-diamidino-2-phenylindole was used to stain the cell nuclei at 37 °C for 10 min, and the sections were then rinsed 3 times with PBS for 5 min each. Finally, the sections were mounted in buffered glycerol and stored in the dark until subsequent observation under a confocal fluorescence microscope.

Statistical analysis

All experiments were performed at least 3 times, and the results are expressed as the mean ± standard error of the mean. Data were analyzed for statistical significance using Student's *t*-test, with $P < 0.05$ considered statistically significant. SPSS 17.0 software (SPSS, Chicago, IL, United States) was used for all statistical analyses.

RESULTS

Finger-print of SYPs

To elucidate the mechanism of action of SYPs against HCC, the primary components of SYPs were analysed by high-resolution mass spectrometry. The compounds were identified by extracting ion flow diagrams and comparing their molecular formulae with information in the literature and databases. A total of 20 compounds were analysed as follows: Amygdalin, albiflorin, paeoniflorin, prime-O-glucosylcimifugin, liquiritin, cimitin, 5-O-methylvisammil glycoside, apigenin liquiritin, ginsenoside Rg1, ononin, isoliquiritin, platycodon D3,6-shogaol, glyyunnanprosapogenin D, glycyrr-hizic acid, formononetin, licoricesaponine H2, ethylcinamate, glycyrrhisoflavanone, and ligustilide (Table 1). As shown in Figure 1, the 20 pharmaceutical ingredients matched in the fingerprints of SYPs were labelled, and the structure was analysed by mass spectrometry according to the chromatographic retention time.

Effect of SYPs on the growth of subcutaneous xenografts of human HCC in nude mice

A subcutaneous xenograft tumour model was established in male BALB/c nude mice to validate whether SYPs could inhibit tumour growth *in vivo*. Tumour volume and body weight were measured every 2 d for 14 d. The mice were euthanized after 14 d of treatment, and the final tumour volumes and body weights were recorded. As observed from the *in vivo* tumour growth curves, the SYPs and DDP inhibited the growth of the tumours (Figure 2A), with the tumour volumes and tumour weights observed in the SYP, SYP + DDP, and DDP groups being lower than those measured

Table 1 Analysis information of 20 compounds in Shuyu pills

N	Ion mode	RT (min)	Mass	Molecular formula	Name
1	+/-	8.828	457.1584	C20H27NO11	Amygdalin
2	+/-	11.363	480.1632	C23H28O11	Albiflorin
3	+/-	12.406	480.1632	C23H28O11	Paeoniflorin
4	+/-	12.870	468.1632	C22H28O11	Prim-O-glucosylcimifugin
5	+/-	14.593	418.1624	C21H22O9	Liquiritin
6	+/-	15.190	306.1103	C16H18O6	Cimitin
7	+/-	15.786	452.1682	C22H28O10	5-O-methylvisammil glycoside
8	+/-	17.625	550.1686	C26H30O13	Apigenin liquiritin
9	+/-	17.697	800.4922	C42H72O14	Ginsenoside Rg1
10	+/-	17.940	430.1264	C22H22O9	Ononin
11	+/-	18.155	418.1264	C21H22O9	Isoliquiritin
12	+/-	21.717	1386.6303	C63H102O33	Platycodon D3
13	+/-	22.909	276.1725	C17H24O3	6-Shogaol
14	+/-	23.009	838.3987	C42H62O17	GlyyunnanprosapogeninD
15	+/-	24.218	822.4038	C42H62O16	Glycyrrhizic acid
16	+/-	24.665	268.0736	C16H12O4	Formononetin
17	+/-	25.129	822.4038	C42H62O16	Licoricesaponine H2
18	+/-	27.101	176.0837	C11H12O2	Ethylcinnamate
19	+/-	28.227	368.1260	C21H20O6	Glycyrrhisoflavanone
20	+/-	32.319	190.0994	C12H14O2	Ligustilide

in the control group. The inhibitory effect on tumour volume was more prominent in the SYP + DDP group than in either the SYP or DDP individual treatment groups (Figure 2B and C). The overall body weights of the mice did not decrease and were not significantly different among the four groups (Figure 2D). These findings indicate that SYPs inhibited the growth of human HCC tumours in nude mice, and their combined use with DDP exhibited a synergistic effect.

Expression of HIF-1α and PD-1/PD-L1

Next, we sought to investigate the antitumour mechanism of the SYPs. Western blot assays were used to measure the protein expression of HIF-1α, PD-1, and PD-L1 in tumours excised from the mice, whereas quantitative reverse-transcription polymerase chain reaction was used to measure mRNA expression of the three genes (Figure 3). Compared to levels in the control group, the protein and mRNA expression levels of the oncogene HIF-1α were significantly lower in the SYP, DDP, and SYP + DDP groups. Moreover, the expression of HIF-1α protein was significantly lower in the SYP + DDP group than in the DDP group, whereas the expression of HIF-1α mRNA was significantly lower in the SYP + DDP group than in the SYP group (HIF-1α protein: Control *vs* SYP, $P = 0.0129$; control *vs* SYP + DDP, $P = 0.0004$; control *vs* DDP, $P = 0.0152$, SYP + DDP *vs* DDP, $P = 0.0448$; HIF-1α mRNA: Control *vs* SYP, $P = 0.0009$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P = 0.0003$, SYP *vs* SYP + DDP, $P = 0.0192$). Analysis of the immune checkpoint PD1/PD-L1 revealed that compared to its expression in the control group, protein expression of PD-1 was significantly reduced in the SYP and SYP + DDP groups, and the mRNA expression of PD-1 was significantly reduced in the SYP, SYP + DDP, and DDP groups. Moreover, the PD-1 protein and mRNA expression levels in the SYP + DDP group were significantly lower than its levels in the SYP and DDP groups (PD-1 protein: Control *vs* SYP, $P = 0.0099$; control *vs* SYP + DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P = 0.0009$; SYP + DDP *vs* DDP, $P < 0.0001$; PD-1 mRNA: Control *vs* SYP, $P = 0.0002$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P = 0.0003$, SPY *vs* SYP + DDP, $P = 0.0003$; SYP + DDP *vs* DDP, $P = 0.0002$). Similarly, the protein and mRNA expression levels of the oncogene PD-L1
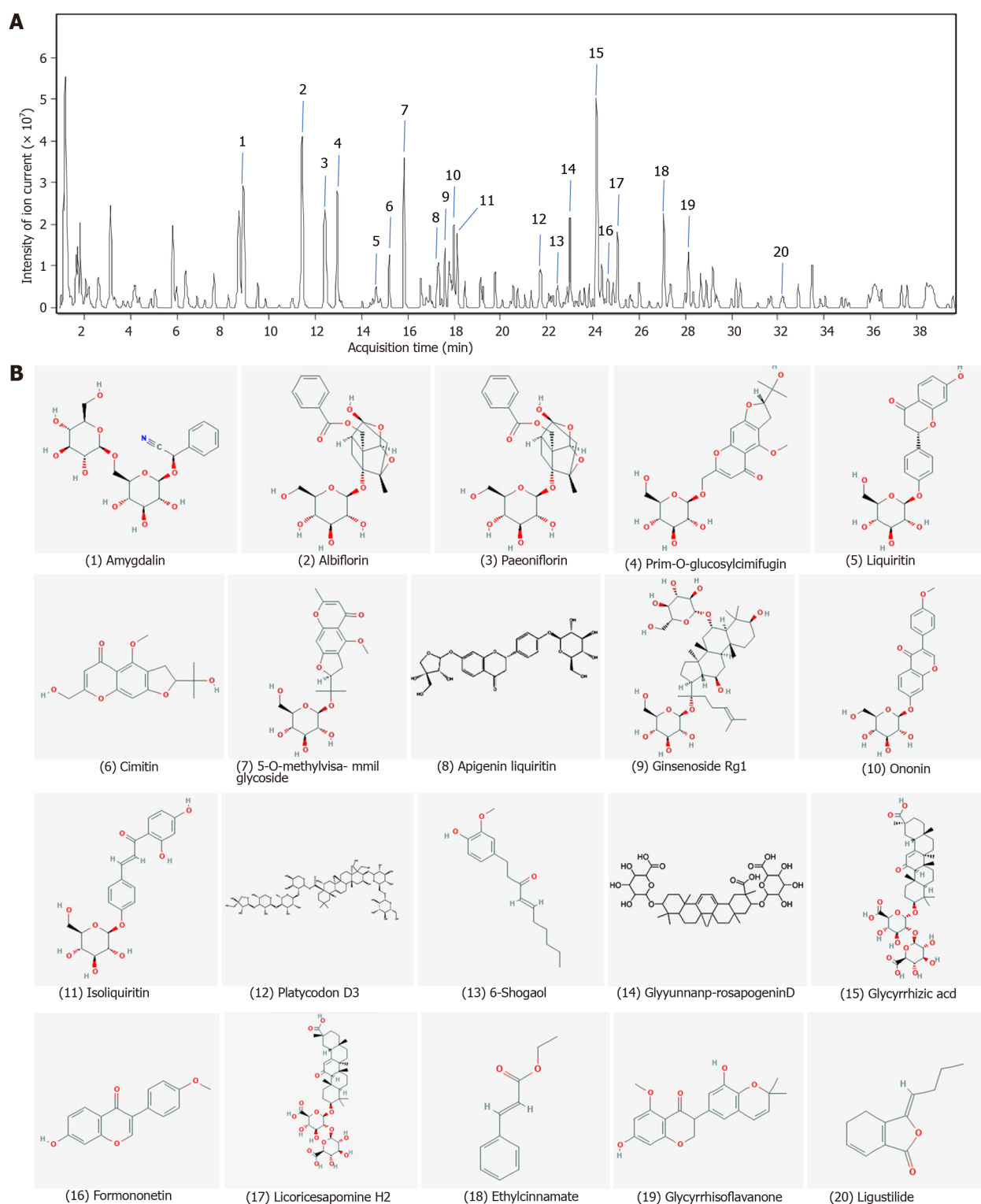
Figure 1 Finger-print of Shuyu pills. The finger-print of Shuyu pills was determined by high resolution mass spectrometry. A: The 20 pharmaceutical ingredients were labeled according to the chromatographic retention time and their structures were analyzed by mass spectrometry; B: Chemical structure formulae of 20 compounds. Chemical structures and formulae of 20 compounds from PubChem (https://pubchem.ncbi.nlm.nih.gov/), numbered according to the compounds information in Table 1.

were significantly lower in the SYP, SYP + DDP, and DDP groups than in the control group. Moreover, the PD-L1 protein and mRNA expression levels were significantly lower in the SYP + DDP group than in the SYP and DDP groups (PD-L1 protein: Control *vs* SYP, $P < 0.0001$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P = 0.0040$; SYP + DDP *vs* DDP, $P = 0.0010$; PD-L1 mRNA: Control *vs* SYP, $P < 0.0001$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P < 0.0001$, SPY *vs* SYP + DDP, $P < 0.0001$; SYP + DDP *vs* DDP, $P < 0.0014$). Notably, the
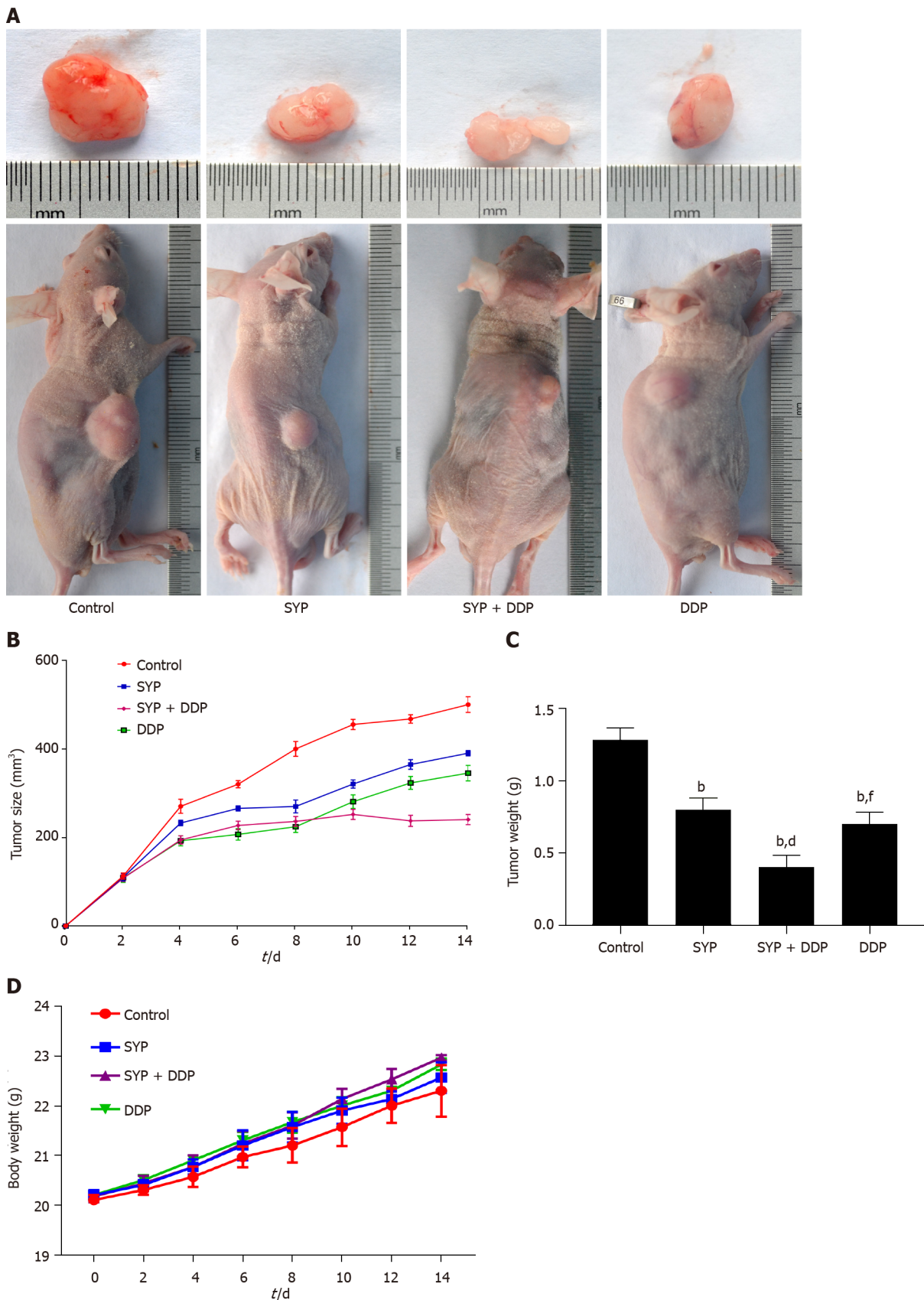
Figure 2 Shuyu pills inhibited the growth of hepatocellular carcinoma *in vivo*. The xenograft mouse model was established in BALB/c nude mice that were then randomly divided into four groups (*n* = 6): Control group (0.9% normal saline, daily), Shuyu pills (SYP) (200 mg/kg, daily), Cisplatin (DDP) (5 mg/kg, once a week), and SYP (200 mg/kg, daily) + DDP (5 mg/kg, once a week). The body weight of each mouse and the tumor volume were measured every 2 d, with the latter calculated as follows: Maximum tumor length × width (2 × 0.5). A: Representative images of the tumors at the end of treatment; B: Average tumor volumes, measured every 2 d; C: Tumor weights at the end of treatment; D: Average body weights of the mice, measured every 2 d. [b]$P < 0.01$ *vs* Control group; [d]$P < 0.01$ *vs* SYP group; [f]$P < 0.01$ *vs* SYP + DDP group. SYP: Shuyu pills; DDP: Cisplatin.
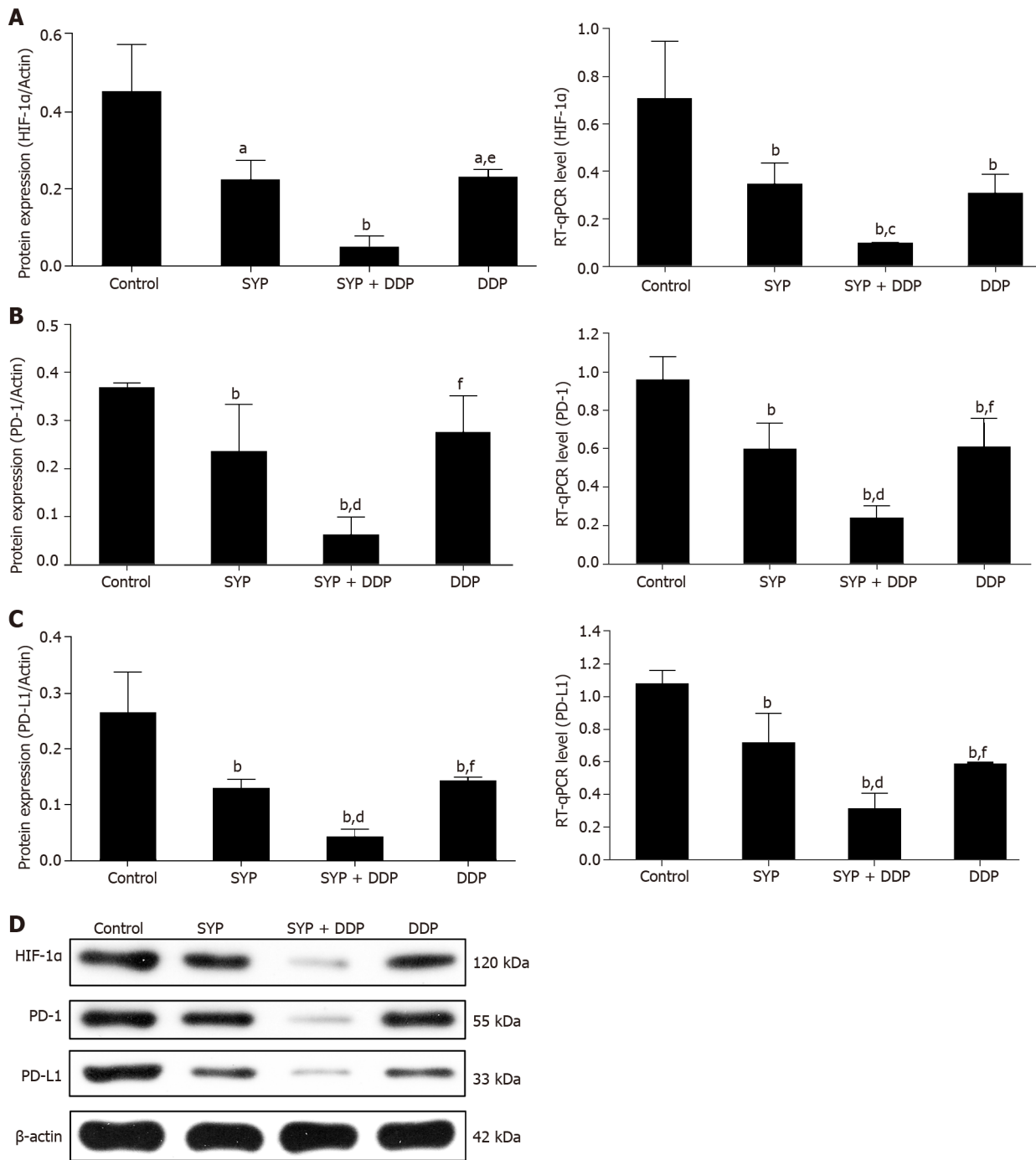
Figure 3 Shuyu pills inhibited the expression of hypoxia-inducible factor-1 alpha, programmed cell death 1, and programmed cell death 1 ligand 1. Nude mice injected subcutaneously with human hepatocellular carcinoma cells were treated with Shuyu pills (SYP), Cisplatin (DDP), or a combination of the two for 14 d, following which the tumor tissues were harvested as indicated. A–C: Western blot and quantitative reverse transcription polymerase chain reaction assays were used to respectively detect the protein and mRNA expression levels of hypoxia-inducible factor-1 alpha (HIF-1α), programmed cell death protein 1 (PD-1), and programmed cell death 1 ligand 1 (PD-L1) in the tumor tissue; D: Representative protein expression patterns of HIF-1α, PD-1, and PD-L1 as measured by western blot assay. Data are presented as the mean ± standard error of the mean, and comparisons between two groups were performed using the least significant difference test or Dunnett's T3 method. [a]$P < 0.05$ and [b]$P < 0.01$ *vs* Control group; [c]$P < 0.05$ and [d]$P < 0.01$ *vs* SYP group; [e]$P < 0.05$ and [f]$P < 0.01$ *vs* SYP + DDP group. SYP: Shuyu pills; DDP: Cisplatin; HIF-1α: Hypoxia-inducible factor-1 alpha; PD-1: Programmed cell death 1; PD-L1: Programmed cell death 1 ligand 1.

protein and mRNA expression trends of HIF-1α, PD-1, and PD-L1 were similar. These findings indicate that SYPs and DDP simultaneously inhibit the expression of HIF-1α, PD-1, and PD-L1 in subcutaneous xenograft tumours in nude mice, with the combination of the two types of drugs displaying a synergistic effect.

Expression of CD4+ T cells and CD8+ T cells
To determine the effect of SYPs on immune function, immunofluorescence assays were conducted to determine the levels of CD4-expressing (CD4+) T cells and CD8-

expressing (CD8+) T cells in subcutaneous xenograft tumours. Images were acquired under a fluorescence microscope for quantitative analysis of the fluorescence signals, and protein expression was determined using the western blot assay (Figure 4). Figure 4A shows immunofluorescence images of CD4+ T cells and CD8+ T cells, where quantitative analysis revealed that expression of CD4+ T cells was higher in the SYP + DDP groups than in the control group, and expression levels were significantly higher in the SYP + DDP group than in the SPY group or DDP group (SYP + DDP *vs* control, $P < 0.0001$, SYP *vs* SYP + DDP, $P = 0.0005$; SYP + DDP *vs* DDP, $P = 0.0002$). Moreover, quantitative expression of CD8+ T cells exhibited similar results (control *vs* SYP + DDP, $P = 0.0013$; SYP *vs* SYP + DDP, $P = 0.0347$; SYP + DDP *vs* DDP, $P = 0.0043$) (Figure 3B). The western blot results showed that expression of CD4+ T cell protein was significantly higher in the SYP, SYP + DDP, and DDP groups than in the control group (control *vs* SYP, $P = 0.0033$; control *vs* SYP + DDP, $P < 0.0001$; control *vs* DDP, $P = 0.0021$). Moreover, expression levels were significantly higher in the SYP + DDP group than in the SYP or DDP group (SYP *vs* SYP + DDP, $P = 0.0004$; SYP + DDP *vs* DDP, $P = 0.0006$). In contrast, protein expression of CD8+ T cells was significantly higher in the SYP + DDP group than in the control, SYP, or DDP group (control *vs* SYP + DDP, $P < 0.0001$; SYP *vs* SYP + DDP, $P < 0.0001$; SYP + DDP *vs* DDP, $P < 0.0001$). These findings indicate that SYPs and DDP upregulated the expression of CD4+ T cells and CD8+ T cells in HCC xenografts in nude mice, whereas the use of SYPs alone only upregulated the expression of CD4+ T cells.

DISCUSSION

HCC is one of the most commonly diagnosed malignant diseases worldwide. Despite the significant progress made in its diagnosis and the advanced developments in cancer treatment modalities (*e.g.*, surgery, chemoradiotherapy, and targeted therapy), the prognosis for patients with HCC remains poor owing to the high recurrence and metastatic potential of the cancer cells[26]. The ability of tumour cells to avoid immune destruction (immune escape) and their development of resistance to chemotherapeutic drugs[27] are key hurdles to the effective control of tumour progression[28,29]. Tumour immune escape is an important mechanism that involves interactions between PD-1 molecules on cytotoxic T lymphocytes (CTLs) and PD-L1 molecules on tumour cells or other immune cells in the body. The PD-1/PD-L1 axis, which is one of several immune checkpoint modulators, primarily acts by inhibiting the adaptive T-cell response. Physiologically, it is implicated in self-tolerance and limitations in the immune response duration and magnitude[30]. Tumour cells exploit the PD-1/PD-L1 immunomodulatory mechanism by activating the PD-1/PD-L1 axis, which deactivates CTLs and leads to their exhaustion, apoptosis, and reduced cytokine production, thereby suppressing the adaptive antitumour response[31]. TILs play a crucial role in the antitumour immune response of the tumour host by specifically binding to and killing tumour cells or inducing their apoptosis[32]. PD-L1 is highly expressed on tumour cells, whereas PD-1 is highly expressed on TILs. The binding of PD-L1 to PD-1 inhibits the activation of TILs and induces their apoptosis[33], which in turn inhibits the host's antitumour immune response, resulting in tumour immune escape[34,35]. CD8, a glycoprotein on the T-cell surface that contributes to antigen recognition by T-cell receptors (TCRs), is also involved in signal transduction during T-cell activation and is known as the coreceptor of TCRs. Upon activation, CD8+ T cells differentiate into CTLs, which can specifically recognize tumour-associated antigens presented on MHC class I molecules on the tumour cell surface. CTLs can also destroy tumour cells directly[36,37]. A higher level of CD8+ T-cell infiltration in a tumour generally indicates a stronger immune response against the tumour and thus a more favourable prognosis[32,38]. CD4+ T cells are equally important immune cells in the human body, where they secrete cytokines that help to initiate and maintain the CD8+ T-cell-mediated antitumour response[39,40]. However, because activation of the PD1/PD-L1 signalling pathway can specifically induce CTL apoptosis, this reduces their ability to kill tumour cells and promotes tumour immune escape. Therefore, the inhibition of PD-1/PD-L1 expression enhances the abilities of CD4+ T cells and CD8+ T cells to kill tumour cells or to induce tumour cell apoptosis, thereby preventing immune escape.

Recently, ample evidence has emerged showing that hypoxia, a common feature of many solid cancers, including HCC, contributes to tumour immune escape through multiple mechanisms[41]. Notably, hypoxia significantly increases the expression of PD-L1 on myeloid-derived suppressor cells, macrophages, dendritic cells, and tumour cells, conferring them with additional resistance to CTL-mediated lysis. This is
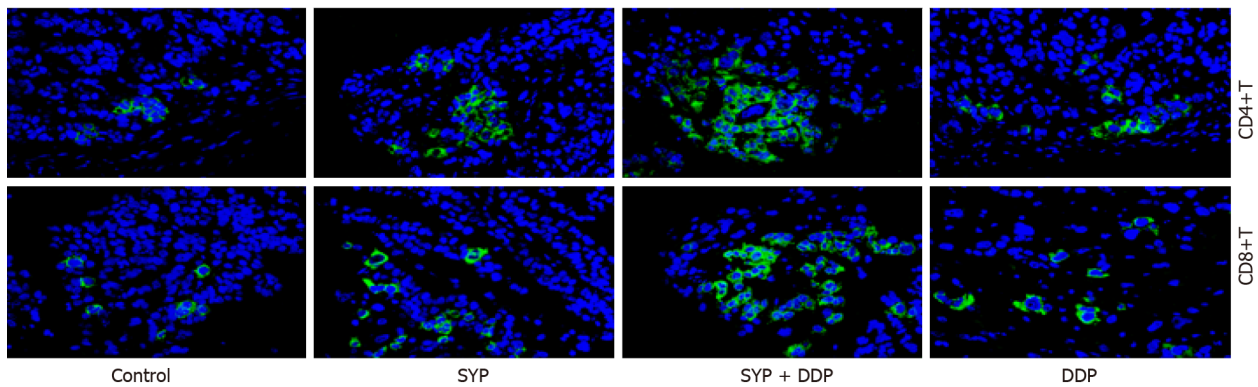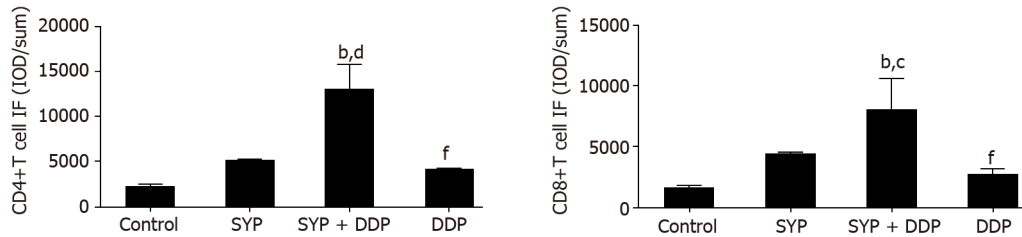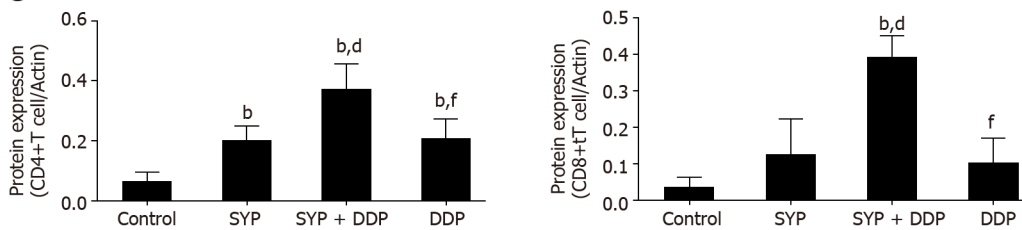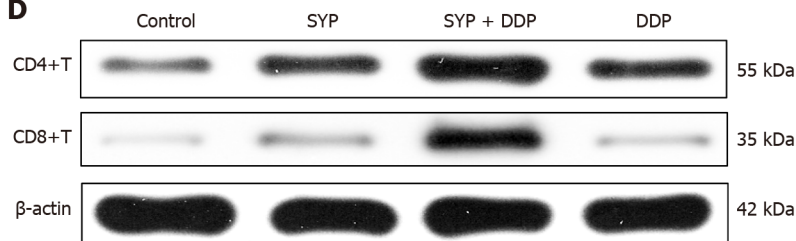
Figure 4 Effects of Shuyu pills on the expression of CD4+ T cells and CD8+ T cells in subcutaneous hepatocellular carcinoma xenografts.
Nude mice injected subcutaneously with human hepatocellular carcinoma cells were treated with Shuyu pills (SYP), Cisplatin (DDP), or SYP + DDP for 14 d. The tumor tissues were then collected and their expression of CD4+ T cells and CD8+ T cells was measured using the immunofluorescence assay. A: Immunofluorescence images of CD4+ T cells and CD8+ T cells; B: Quantitative analysis of the CD4+ T-cell and CD8+ T-cell immunofluorescence intensities; C and D: Protein expression levels of CD4+ T cells and CD8+ T cells as measured by western blot assay. Data are presented as the mean ± standard error of the mean, and comparisons between two groups were performed using the least significant difference test or Dunnett's T3 method. [b]$P < 0.01$ *vs* Control group; [c]$P < 0.05$ and [d]$P < 0.01$ *vs* SYP group; [f]$P < 0.01$ *vs* SYP + DDP group. SYP: Shuyu pills; DDP: Cisplatin.

primarily due to the accumulation of HIF-1α[42] the factor that participates in the hypoxic response of tumour cells. HIF-1α activates myriad genes essential for HCC angiogenesis, proliferation, glucose metabolism, invasion, metastasis, and resistance to radiotherapy and chemotherapy[43-45]. Hypoxia confers cells with resistance to CTL-mediated lysis by upregulating HIF-1α and PD-L1, making these two proteins promising molecular targets for cancer treatment. A positive correlation between PD-L1 and HIF-1α has also been identified in HCC tissues, and patients with HCC tumour tissues overexpressing both of these proteins exhibited a significantly increased risk of recurrence, metastasis, or death[42]. The combination of HIF-1α inhibitors with PD-L1 blockade to target tumour hypoxia may represent a novel immunotherapy for overcoming weakened antitumour cytotoxicity and strengthening the immune system of patients with cancer. However, it remains unclear whether the simultaneous inhibition of HIF-1α and PD-L1 can suppress tumour immune escape in HCC.

Our study revealed that SYPs inhibited the growth of HCC subcutaneous xenograft tumours in nude mice, and the combined use of SYPs with DDP displayed a synergistic effect. With regard to the mechanism, SYPs simultaneously inhibited the expression of HIF-1α and the PD-1/PD-L1 axis and increased the expression of CD4+ T cells, and these effects were synergistic in the presence of DDP. Furthermore, expression of HIF-1α was positively correlated with expression of PD-1 and PD-L1 and negatively with that of CD4+ T cells and CD8+ T cells. These findings suggest that SYPs may inhibit the activation of HIF-1α, which in turn inhibits the expression of the PD-1/PD-L1 axis, thereby promoting the tumour-killing effects of CD4+ and CD8+ T cells and preventing immune escape in the tumour microenvironment. This novel immunotherapeutic approach of simultaneously inhibiting HIF-1α and PD-L1 expression exerts an antitumour effect in HCC.

It is worth noting again that the combination of SYPs and DDP displayed a synergistic effect in the present study. Research indicates that the interaction between PD-1 and PD-L1 contributes to the resistance of tumour cells to conventional chemotherapeutic drugs. Studies using *in vivo* tumour models have shown that anti-PD-L1 therapies that inhibit the PD-1/PD-L1 axis enhance the efficacy of conventional chemotherapy in preventing metastasis. Therefore, blockade of the PD-1/PD-L1 axis is an effective strategy for targeting immune checkpoints. Additionally, the combination of chemotherapy and immune checkpoint blockade is a novel treatment approach that can reduce tumour drug resistance and improve the effectiveness of chemotherapies [4]. Based on these findings, we speculate that the synergistic effect of the SYP and DDP combination may be due to blockade of the PD-1/PD-L1 immune checkpoint, which mitigates the resistance of HCC cells to DDP and sensitizes the cells to this chemotherapeutic drug. Moreover, local hypoxia in the tumour microenvironment induces adaptations of tumour cells and enhances their chemoresistance[46]. Solid tumours are dynamic and heterogeneous structures in which the oxygen tension is significantly lower than that in adjacent normal tissues. Since the vascular system cannot provide sufficient oxygen for the growing tumour, the long diffusion distance between the hypoxic regions and the tumour blood vessels limits the distribution of drugs. Many chemotherapeutic drugs, including platinum, require oxygen as an electron acceptor to induce cell death[47]. Therefore, the regulation of HIF-1α expression can reduce the chemoresistance caused by hypoxia in the tumour microenvironment[48]. Furthermore, we speculate that the synergistic effect of the SYP and DDP combination may also be due to the SYP-mediated improvement of the hypoxic state in the tumour microenvironment, which inhibits the hypoxia-induced chemoresistance of HCC cells and enhances their sensitivity to chemotherapy. Taken together, our data provide valuable insights for future research on HCC chemosensitization.

CONCLUSION

SYPs inhibit immune escape and enhance chemosensitization in HCC *via* simultaneous inhibition of HIF-1α and PD-L1, thus inhibiting the growth of subcutaneous xenograft tumours with HCC.

ARTICLE HIGHLIGHTS

Research background
Hepatocellular carcinoma (HCC) is characterized by dysregulation of the immune microenvironment and the development of chemoresistance. The latest research shows that the simultaneous inhibition of hypoxia-inducible factor-1 alpha (HIF-1α) and programmed cell death protein 1 (PD-1) has the potential to enhance the hosts antitumour immunity. Moreover, inhibition of the PD-1/programmed cell death 1 ligand 1 (PD-L1) axis may mitigate tumour chemoresistance.

Research motivation
Shuyu pills (SYPs) contain immunity-enhancing and antitumour components, making them a potential HCC treatment. The motivation of this research was to study the effect and mechanism of SYPs on HCC.

Research objectives

To investigate the efficacy of SYPs for HCC treatment *via* simultaneous HIF-1α and PD-L1 inhibition and the mechanism involved.

Research methods

The subcutaneous xenograft tumours model was established in BALB/c nude mice. The male mice (male, 5 weeks old; *n* = 24) were then randomly divided into the four groups (*n* = 6): Control group (0.9% normal saline), SYP group (200 mg/kg), SYP + cisplatin (DDP) group (200 mg/kg + 5 mg/kg weekly *via* intraperitoneal injection), and DDP group (5 mg/kg weekly *via* intraperitoneal injection). The tumour volumes and body weights of the mice were measured every 2 d. The mice were euthanized by cervical dislocation after 14 d of continuous treatment, and the xenograft tissues were excised and weighed. The western blot assay was used to measure the protein expression of HIF-1α, PD-1, PD-L1, CD4+ T cells, and CD8+ T cells in the HCC tumours from the mice. Quantitative reverse transcription polymerase chain reaction a was used for the real-time quantitative detection of PD-1, PD-L1, and HIF-1α mRNA expression. The immunofluorescence assay was conducted to examine the expression of CD4+ T cells and CD8+ T cells.

Research results

Compared with the mice in the control group, those in the SYP and SYP + DDP groups had lower tumour volumes and tumour weights. Moreover, the protein and mRNA expressions of the oncogene HIF-1α and that of the negative immunomodulatory factors PD-1 and PD-L1 were decreased in both the SYP and SYP + DDP groups, with the decrease effects being more prominent in the SYP + DDP group than in the SYP group. Additionally, the quantitative and protein expressions of CD4+ T cells and CD8+ T cells were simultaneously upregulated in the SYP + DDP group, whereas only the expressions of CD4+ T cells were upregulated in the SYP group. Finally, the expression of HIF-1α was found to be positively correlated with that of PD-1/PD-L1 and negatively correlated with the expression of the CD4+ T cells and CD8+ T cells.

Research conclusions

SYPs inhibit immune escape and enhance chemosensitization in HCC *via* simultaneous inhibition of HIF-1α and PD-L1, thus inhibiting the growth of subcutaneous xenograft tumours with HCC.

Research perspectives

SYPs inhibit immune escape and enhance chemosensitization in HCC. It is a potential adjuvant drug for the treatment of HCC.

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ORIGINAL ARTICLE

Basic Study

Preventive and inhibitive effects of Yiwei Xiaoyu granules on the development and progression of spasmolytic polypeptide-expressing metaplasia lesions

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Abstract

BACKGROUND
Spasmolytic polypeptide-expressing metaplasia (SPEM) is a potential preneoplastic lesion.

AIM
To elucidate the microRNA (miR)-7-mediated preventive and inhibitive effects of Yiwei Xiaoyu granules (YWXY) in SPEM lesions.

METHODS
Gastric mucosa biopsies were collected from chronic atrophic gastritis patients and healthy people with signed informed consent. YWXY was administered to the mice with induced SPEM by tamoxifen, and the gastric mucosa was harvested on the tenth day of the experiment. Then immunohistochemistry and immunofluorescence were performed to validate the SPEM, lesions and the potential mechanism was investigated. RNA transcripts were detected with reverse transcription-quantitative polymerase chain reaction.

RESULTS
The expression of miR-7 was downregulated in the SPEM lesions, and expression of trefoil factor 2 (TFF2) and clusterin was high in the human gastric mucosa. *In vivo* experiments showed that YWXY could inhibit the cell proliferation in the tamoxifen-induced SPEM lesions by regulating Ki67. Simultaneously, YWXY could restore the expression of miR-7 by regulating TFF2 by detection with immunofluorescence but not with reverse transcription-quantitative polymerase chain reaction, indicating its potential mechanism of targeting miR-7 by mediating

TFF2. The expression of vascular endothelial growth factor-β and gastric intrinsic factor was restored within 3 d of YWXY administration for the SPEM lesions, speculating that the possible mechanism of YWXY is to inhibit the development and progression of SPEM by regulating vascular endothelial growth factor-β and gastric intrinsic factor.

CONCLUSION

miR-7 downregulation is an early event in SPEM through regulation of TFF2 in human gastric mucosa. YWXY is able to inhibit the cell proliferation and restore the expression of miR-7 by mediating TFF2 in the SPEM mouse model.

Key Words: Spasmolytic polypeptide-expressing metaplasia; Yiwei Xiaoyu Granules; MicroRNA-7; Chronic atrophic gastritis; Trefoil factor 2; Gastric precancerous lesions

Core Tip: We showed evidence that microRNA-7 downregulation is an early event in the cascade from metaplasia to gastric cancer and that it contributes to the establishment of an intestinal expression profile through regulation of trefoil factor 2 in both human gastric mucosa and *in vivo* experiments. To the best of our knowledge, we used the spasmolytic polypeptide-expressing metaplasia mouse model for the first time to reveal the effectiveness and the potential mechanism of Chinese medicine Yiwei Xiaoyu granules for the precursor of gastric adenocarcinoma.

INTRODUCTION

Gastric cancer is one of the most frequent and deadly cancers worldwide[1]. The Correa pathway from gastritis to gastric cancer including the oxyntic atrophy (loss of acid-secreting parietal cells) and the development of spasmolytic polypeptide-expressing metaplasia (SPEM) has been well described. SPEM and intestinal metaplasia (IM) have been considered as preneoplastic lesions, whereas SPEM is the first metaplastic lesion to evolve and probably progresses to IM[2-4]. Therefore, it is important to clarify the cause of parietal cell atrophy and the regulatory mechanisms for the chief cell transdifferentiation to explore novel treatments for the gastric precancerous lesions and eventually prevent the occurrence of gastric cancer.

The international consensus has recommended follow-up or endoscopic resection of the precancerous lesions of the stomach; however, it has been reported that gastric IM still persisted even after successful eradication of low-grade dysplasia with radiofrequency ablation[5,6]. In the previous work, our group proved that Yiwei Xiaoyu granules (YWXY) could improve the mucosa atrophy, IM and dysplasia of chronic gastric gastritis (CAG) in the clinic trial[7]. Then we optimized water reflux extraction technology and established the quality standard of YWXY[8,9]. In addition, we explored the mechanism of how YWXY inhibits atrophy and IM of the stomach using a rat model[10,11]. However, the specific mechanism of YWXY still remains largely unknown.

In our previous work, we found that targeting some microRNAs (miRNAs) could prevent or retard the occurrence and development of gastric cancer and its precancerous lesions, such as miR-7 and let-7[12-14]. miR-7a-5p and miR-7a-3p strand consist of a short duplex mature miRNA, and miR-7a-5p has been the focus of the majority of studies and is commonly referred to as "miR-7"[15]. miR-7 has been proved to be a novel prognostic biomarker and potential therapeutic target by mediating p65 and activating NF-κB[16]. Based on our prior investigation, the expression of miR-7 in gastric cancer was decreased compared with the matched normal tissues and adjacent

tissues[14]. Therefore, we hypothesized that miR-7 might serve as a suppressor gene in the process of SPEM progression into gastric cancer. Furthermore, in the light of these facts that YWXY could relieve or even reverse the atrophy and IM of rat mucosa by inhibiting NF-κB[10], we assumed that YWXY could inhibit the progression of gastric mucosa metaplasia even in the earlier phase such as SPEM.

Here, we detected the expression of miR-7 in SPEM with CAG biopsy samples and demonstrated the therapeutic effect of YWXY for the SPEM model induced by tamoxifen, in order to illustrate the possible mechanism.

MATERIALS AND METHODS

Ethics approval and sample collection
Animal experiments and human sample management were performed in accordance with protocols approved by the Ethics Committee of Chongqing Hospital of Traditional Chinese Medicine. All participants signed informed consent forms. From October 2019 to June 2020, 35 pairs of gastric endoscopic biopsy samples, including 30 CAG, and 5 healthy volunteers, were collected from the Department of Gastroenterology, Chongqing Hospital of Traditional Chinese Medicine. The diagnostic standards of CAG followed the Consensus on Chronic Gastritis[17], while the diagnostic standards of normal gastric mucosa with healthy volunteers followed the suggestion from Watanabe *et al*[18].

Animals and drug injections
A total of 24 male wild-type mice were purchased from the Institute of Chinese Medicine in Chongqing. They were divided into four groups randomly, including normal group, model group, low-dose group of YWXY and high-dose group of YWXY, and 6 mice were in each group. From the first to the tenth day of the experiment, the mice in low-and high-dose group of YWXY were gavaged with YWXY (15 g/kg daily and 20 g/kg daily, respectively). To establish the model of SPEM, tamoxifen was intraperitoneally injected (3 mg/20 g mouse body weight) from the eighth day of the experiment for 3 consecutive days[19]. Mice in the normal group were gavaged with saline for 10 consecutive days. On the eleventh day of the experiment, after euthanasia, stomachs were immediately excised, and the gastric body was cut into three parts and fixed with 4% paraformaldehyde. One part was stored at -20 ℃, and the other two parts were stored with paraffin embedding.

Preparation of the medicine
YWXY components were obtained from the pharmacy department of Chongqing Hospital of Traditional Chinese Medicine and identified by two pharmacological experts, and were prepared as described previously[10,11]. Standard extraction technology for YWXY was used[8,9].

Immunohistochemistry
After deparaffinization and hydration, slides underwent antigen retrieval *via* cooking in saline sodium citrate (pH 6.0). Slides were blocked with 3% H_2O_2 at room temperature for 15 min and flushed with distilled water three times (5 min/time). Primary antibodies, including anti-intrinsic factor antibody (1:20, ab171418, Abcam, Cambridge, United Kingdom), anti-trefoil factor 2 (TFF2) antibody (1:200, ab203237, Abcam), vascular endothelial growth factor-β (VEGF-B) antibody (1:200, AF7019, Affinity Biosciences, Cincinnati, OH, United States) and Ki67 antibody (1:200, AF1738, Beyotime, Beijing, China) were incubated overnight at 4 ℃, washed three times (5 min/time) with PBS , and then incubated with DAKO REAL™EnVision™/HRP, Rabbit/Mouse (EVN) (K5007, Glostrup, Denmark) at 25-27 ℃ for 30 min, flushed with PBS three times (5 min/time) according to the manufactures' instructions, developed, dehydrated, cleared, mounted and examined.

Immunofluorescence
The deparaffinized mouse stomach tissue sections underwent antigen retrieval with 10 mmol/L sodium citrate (pH 6.0), washed three times with PBS for 5 min each time, then with blocked with 10% normal goat serum at room temperature for 1 h, followed by overnight incubation with primary antibodies, such as anti-intrinsic factor antibody (1:10, ab171418, Abcam), anti-TFF2 antibody (1:100, ab203237, Abcam) and VEGF-B antibody (1:50, sc-101582, Santa Cruz Biotechnology, Santa Cruz, CA, United States) at

4 °C , then washed three times with PBS for 5 min each time. Goat anti-rabbit IgG (1:100) was added to incubate at room temperature for 1 h and washed three times with PBS for 5 min each time. SABC-DyLight 488 (1:200) was added to incubate at room temperature for 1 h, and PBS washed for 5 min. The diluted DAPI (1:1000) was added to make nuclear condensation at room temperature for 3 min, PBS washed four times, 5 min each time, stained with fluorescence decay resistant medium, mounted and photographed.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted according to the manufacturer's instructions (LS1040, Promega Corporation, Shanghai, China). The PCR primers were as follows: TFF2 forward: 5'-CCTTGGTGTTTCCACCCACT-3' and reverse, 5'-CCCACAATTCTTGC-GAGCTG-3'; GAPDH forward: 5'-ATGGTGAAGGTCGGTGTGAAC-3' and reverse 5'-AATCTCCACTTTGCCACTGC-3'. Reverse transcription was performed using the reverse transcription (K1622, Thermo Fisher Scientific, Waltham, MA, United States) and the Maxima™SYBRGreen/ROXqPCRMasterMi (2X) (K0221, Thermo Fisher Scientific) kits. The thermocycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min on an ABI Step One QPCR System (Applied Biosystems, Waltham, MA, United States). GAPDH was used as an endogenous control, and the ΔΔCt method was used for TFF2 quantification.

Fluorescence in situ hybridization for miR-7a-5p

Fluorescence *in situ* hybridization (FISH) process was performed according to the manufacturer's protocol (MK1030; Boster Biological Technology, Beijing, China). Ten-micrometer paraffin-embedded sections were deparaffinized and rehydrated, then incubated with proteinase and 1 mL 3% citric acid for 30 min at 37 °C, washed with PBS three times (5 min/time) and flushed with distilled water once for 5 min. Then, 20 µL prehybridization solution was added on each slide. To retain moisture, 20% glycerin was put into the dry hybridization chamber and incubated for 2 h at 37 °C . After the prehybridization, the excess liquid was absorbed. The locked nucleic acid probe (mmu-miR-7a-5p FISH probe, 5'FAM-ACAACAAAATCACTAGTCTTCCA-FAM3') was dissoluted with 47.5 µL nuclease-free water and diluted (1:100), added with 20 µL hybridization solution with oligonucleotide probe, incubated at 42 °C overnight, washed, nuclear stained with DAPI (1:1000), mounted and observed under a fluorescence microscope (MF31, MSHOT, Guangzhou, China).

RESULTS

miR-7 was involved in TFF2-induced downregulation in SPEM lesions

To determine if miR-7 is inhibited in the specimens of CAG, we first examined the potential SPEM tissue with hematoxylin and eosin staining[3]. Ten slides were selected and examined with immunohistochemistry (IHC). As it is reported, the clusterin-positive intestinal metaplasia does not express TFF2 in the gastric cancer tissue, whereas SPEM is a metaplasia mucous cell lineage with strong expression of TFF2 and clusterin[4,20]. As a result, the expression of TFF2 and clusterin in SPEM are upregulated compared to the normal stomach tissue with IHC and immunofluorescence (Figures 1 and 2). Also, the expression of Ki67 protein in the SPEM was significantly higher than that in the normal stomach tissue ($P < 0.001$) (Figure 3). It is consistent with the results in the previous animal experiments[23]. It implies that SPEM is actually the precancerous lesion with high proliferative activity.

In our previous study, the expression of miR-7 was significantly downregulated in gastric cancer tissue compared with the normal and adjacent tissue samples, which demonstrated that it was a tumor suppressor of gastric cancer[17]. Here, we sought to identify if miR-7 was dysregulated in the SPEM tissue. By FISH, the results showed that the expression of miR-7 in SPEM was lower than that in normal tissue (Figure 4). Furthermore, to confirm the relationship of miR-7 and TFF2, FISH was performed. The data showed that with the decreased expression of miR-7, the expression of TFF2 was upregulated in the tissue of SPEM (Figure 5). Our results may have major implications for understanding the occurrence and development of SPEM.

YWXY administration could restore the expression of miR-7 by regulating TFF2

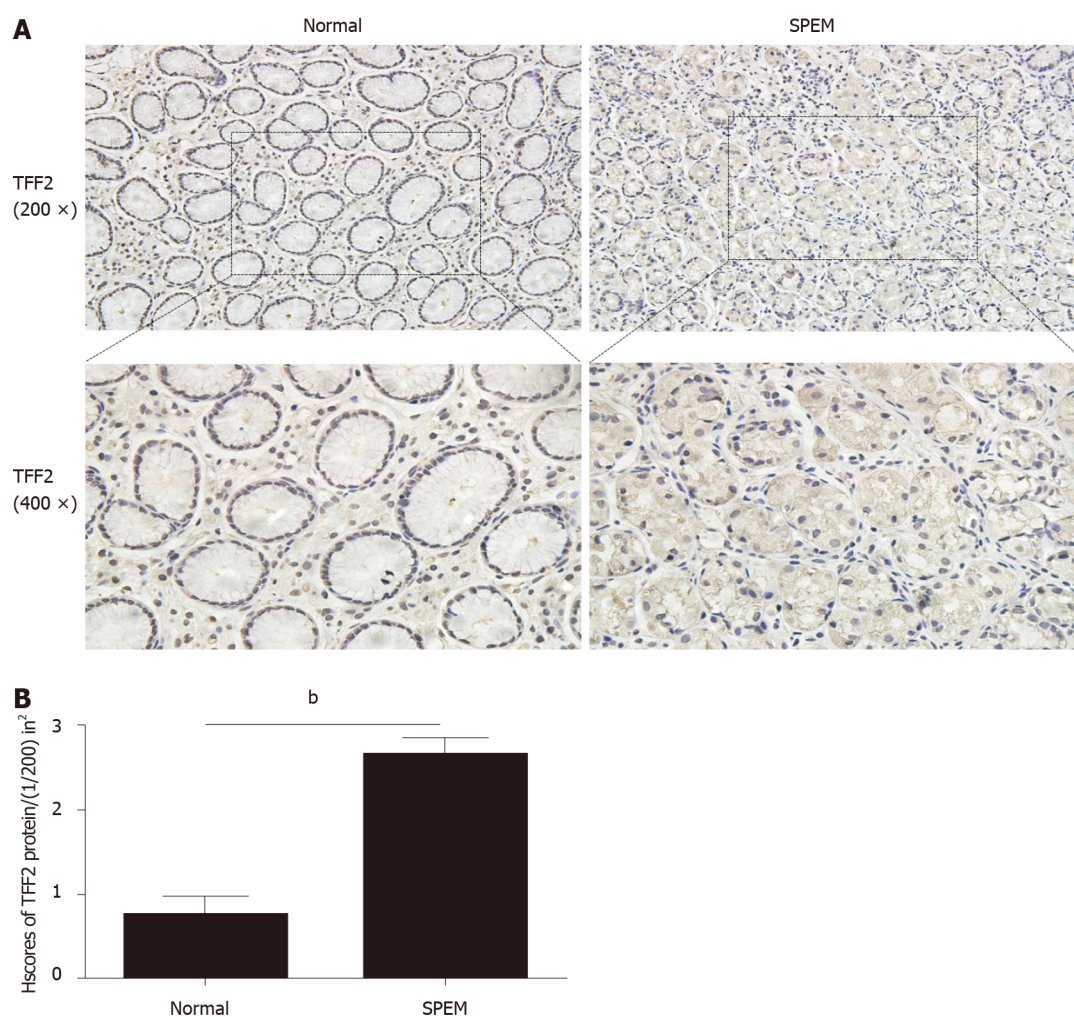We tested, for the first time, the hypothesis that YWXY acts on miR-7 regulating TFF2

A

B

Figure 1 Immunohistochemical analysis of the expression of trefoil factor 2. A: Representative immunohistochemistry images of trefoil factor 2 in normal controls and spasmolytic polypeptide-expressing metaplasia (× 200, × 400). B: The expression of trefoil factor 2 in spasmolytic polypeptide-expressing metaplasia is much higher than that in normal controls ([b]$P < 0.01$). TFF2: Trefoil factor 2; SPEM: Spasmolytic polypeptide-expressing metaplasia.
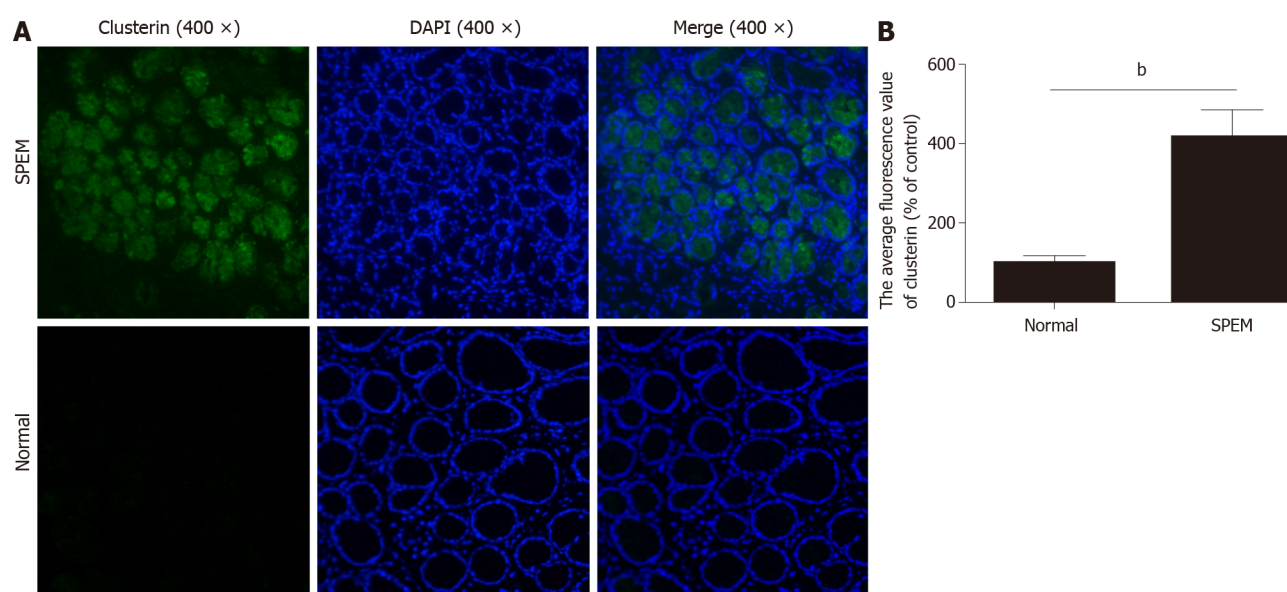
A

B

Figure 2 Representative immunofluorescence images of clusterin. A: The expression of clusterin in normal controls and spasmolytic polypeptide-expressing metaplasia by immunofluorescence (green: clusterin). B: The expression of clusterin in spasmolytic polypeptide-expressing metaplasia is much higher than that in normal controls ([b]$P < 0.01$). SPEM: Spasmolytic polypeptide-expressing metaplasia.
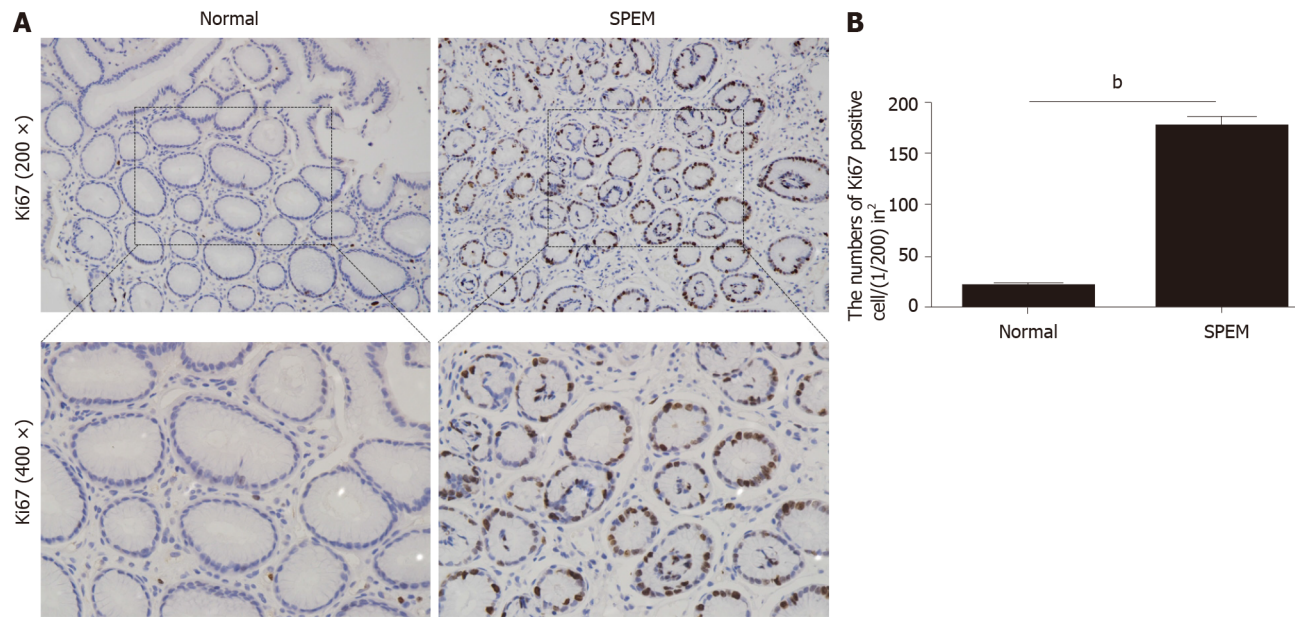
Figure 3 Immunohistochemical analysis of the expression of Ki67. A: Representative immunohistochemistry images of Ki67 in normal controls and spasmolytic polypeptide-expressing metaplasia (SPEM) (200 ×, 400 ×). B: The expression of Ki67 in spasmolytic polypeptide-expressing metaplasia is much higher than that in normal controls ($^bP < 0.01$). SPEM: Spasmolytic polypeptide-expressing metaplasia.

to inhibit SPEM. We induced SPEM in the mouse stomach by intraperitoneal administration of tamoxifen. The expression of Ki67 was higher in SPEM models than that in normal controls (Figure 6). Obviously, both YWXY decreased the expression of Ki67 compared to the model group ($P < 0.05$). These results imply that YWXY prevents the progression of precancerous lesions.

To address the hypothesis of the mechanism of YWXY inhibiting the progression of SPEM, we examined the expression of miR-7 with FISH and TFF2 with immunofluorescence. Our findings suggest that YWXY administration could restore the expression of miR-7. In addition, with high dosage of YWXY, there is an upward trend of miR-7 upregulation (Figure 7A). On the contrary, the expression of TFF2 was downregulated with YWXY administration compared to the healthy controls as measured by immunofluorescence (Figure 7B). The results show that the Chinese medicine YWXY has the ability to inhibit the development of SPEM, the mechanism of which might target miR-7 by mediating TFF2. In order to prove it, we compared the average fluorescence value of TFF2. Clearly, the expression of TFF2 in the model group was much higher than that in the control group ($P < 0.001$). Furthermore, intervening with YWXY could decrease the expression of TFF2 compared to the model ($P < 0.001$ in YWXY-H, $P < 0.05$ in YWXY-L) (Figure 8A). However, by reverse transcription-quantitative polymerase chain reaction, we did not find any difference in the relative mRNA expression of *TFF2* between the different groups (Figure 8B).

Expression of vascular endothelial growth factor-β and gastric intrinsic factor was restored with YWXY administration in the SPEM lesions

SPEM is a specific preneoplastic lesion, and cancer related pathways should be analyzed. Therefore, we detected the expression of VEGF-β with IHC, and the results showed that YWXY could restore the expression of VEGF-β, while statistical differences existed between the control and the model groups ($P < 0.05$) (Figure 9). Simultaneously, the cell proliferation was also detected with IHC. Unsurprisingly, the expression of gastric intrinsic factor (GIF) in the model group was scanty compared with the control. With the increased dosage of YWXY, the expression of GIF was restored (Figure 10).

DISCUSSION

Our research focused on the therapeutic effect of YWXY, and its mechanism of action for atrophy and IM have been interpreted[10,11]. However, it is conceivable that YWXY may play a role even in the prior neoplastic precursor, SPEM. Therefore, it was
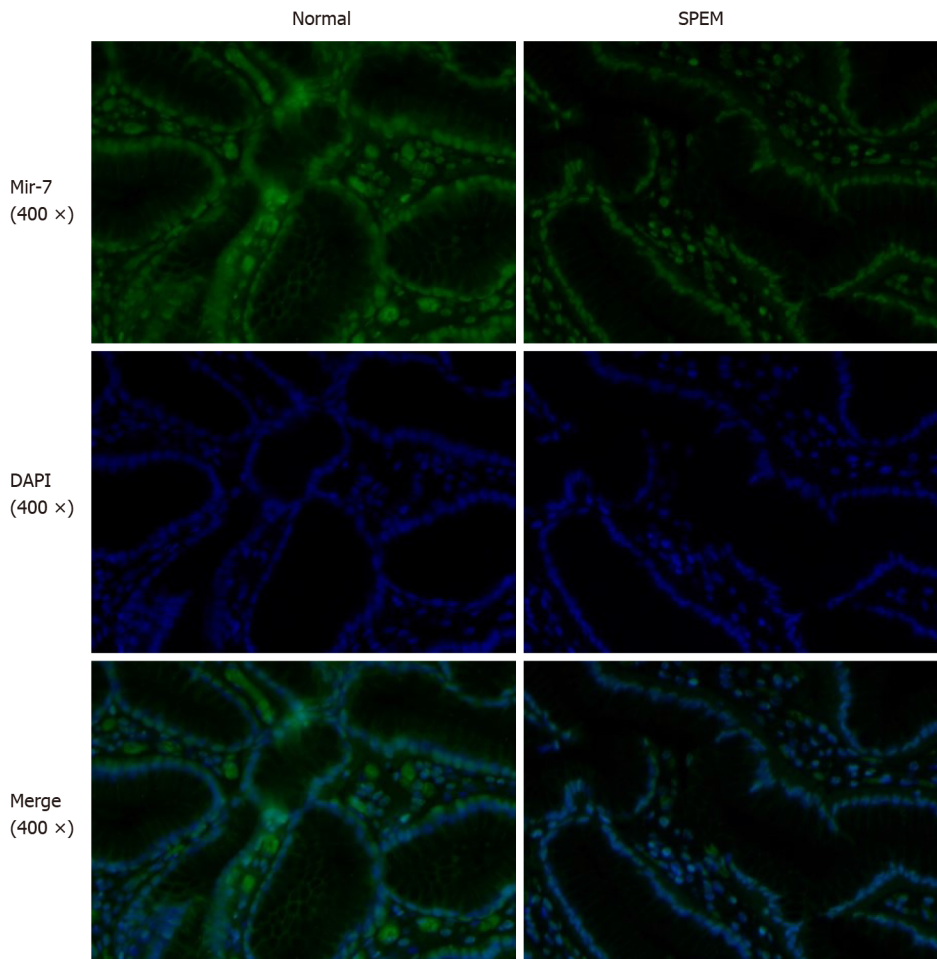
Figure 4 Fluorescence in situ hybridization analysis of microRNA-7 expression in spasmolytic polypeptide-expressing metaplasia and normal controls. The expression of microRNA-7 was downregulated in spasmolytic polypeptide-expressing metaplasia compared to in normal controls. SPEM: Spasmolytic polypeptide-expressing metaplasia; Mir-7: MicroRNA-7.

given to the tamoxifen-induced SPEM mice, and the therapeutic effect and underlying mechanisms were investigated.

It has been proven that two different SPEM mouse models induced by drug or chronic inflammation were identical. We chose a tamoxifen-induced reversible SPEM mouse model[22]. SPEM was confirmed by the coexpression of TFF2, Mucin 6 and GIF [23]. Clusterin was detected in all SPEM lineages, and it represented a specific marker of SPEM induction in the gastric oxyntic mucosa, whereas clusterin-positive IM cells do not express TFF2. Therefore, SPEM was identified with the positive coexpression of clusterin and TFF2 in our study[4,20].

In order to test the cell proliferation activity, Ki67 was detected. Interestingly, the cell proliferation at the interface between SPEM and intestinal metaplasia was more active than that in normal gastric mucosa, which was consistent with the results by Goldenring *et al*[20], implicating some evidence for the existence of IM emanating from SPEM. Recent studies have also highlighted the existence of SPEM and IM as useful biomarker for gastric cancer risk, and miR-7 has been identified as a tumor suppressor of gastric cancer[12,14]. Therefore, miR-7 was compared between the normal and SPEM gastric mucosa. It is particularly exciting to implicate that the expression of miR-7 in SPEM was inhibited obviously compared with that in normal gastric mucosa of CAG patients, supporting the hypothesis that miR-7 has the potential to represent earlier regulatory events in the cascade to gastric cancer.

Recently, several investigations have focused on the role of miRNAs in the development of stomach metaplasia[24-27]. The novelty of this study is the use of gastric specimens derived from CAG patients and healthy people. To our best knowledge, it is the first study to detect the expression of miR-7 and elucidate the potential mechanism of Chinese medicine mediated by microRNAs in SPEM. To test the idea that the profile of YWXY inhibiting activity from precursor to gastric malignancy was mediated by miR-7, the expression of Ki67 was discerned with a
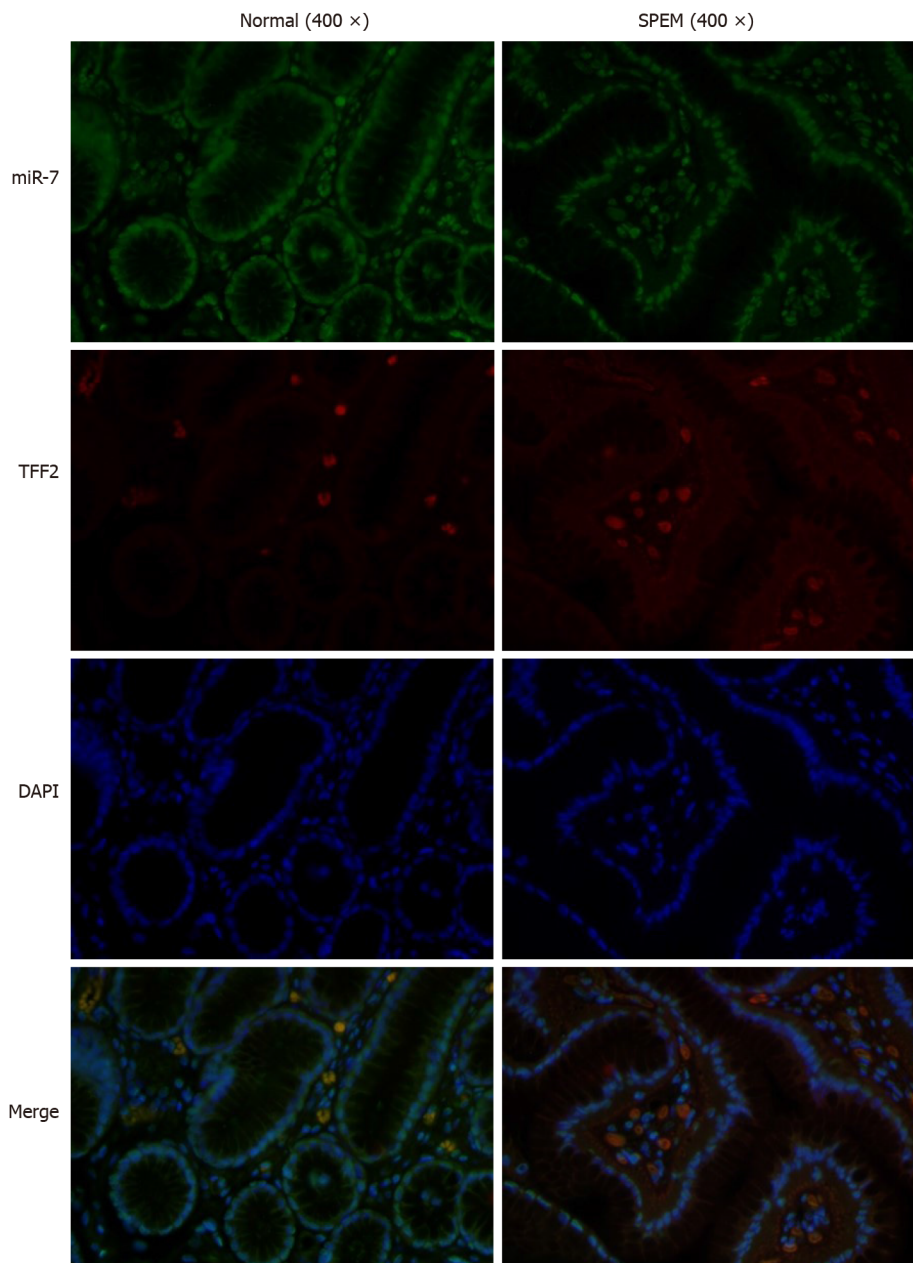
Figure 5 Representative fluorescence *in situ* hybridization images of microRNA-7 and trefoil factor 2 of human gastric mucosa (green: microRNA-7; red: trefoil factor 2; blue: DAPI). TFF2: Trefoil factor 2; SPEM: Spasmolytic polypeptide-expressing metaplasia; miR-7: MicroRNA-7.

tamoxifen-induced SPEM mouse model. We showed evidence that the expression of Ki67 was inhibited with YWXY compared to the control group. YWXY may inhibit cell proliferation to induce epigenetic modification of gastric mucosal genes. The expression of miR-7 was restored in the group with YWXY intragastric administration compared with the model group by FISH. On the other hand, downregulation of TFF2 was speculated in the YWXY group compared with the model group by immunofluorescence, whereas *TFF2* mRNA levels were not significantly changed by reverse transcription-quantitative polymerase chain reaction detection.

Studies revealed that TFF2 was a protective rapid response peptide coping with mucosal damage because of its motogenic effects *in vitro* and protective or healing effects *in vivo*[28,29]. Moreover, TFF2 was also considered to have protection against the progression of premalignant lesions in *Helicobacter pylori*-infected mice[30]. On the contrary, for those people who were *Helicobacter pylori*-infected gastric cancer relatives, there was no relation with the serum TFF2 levels and the presence of either IM or SPEM[31]. In our study, high levels of TFF2 were detected in the SPEM lesions of CAG patients and the mucosa of the SPEM mouse model. miR-7 might regulate the expression of TFF2 at the protein level but not the mRNA level.
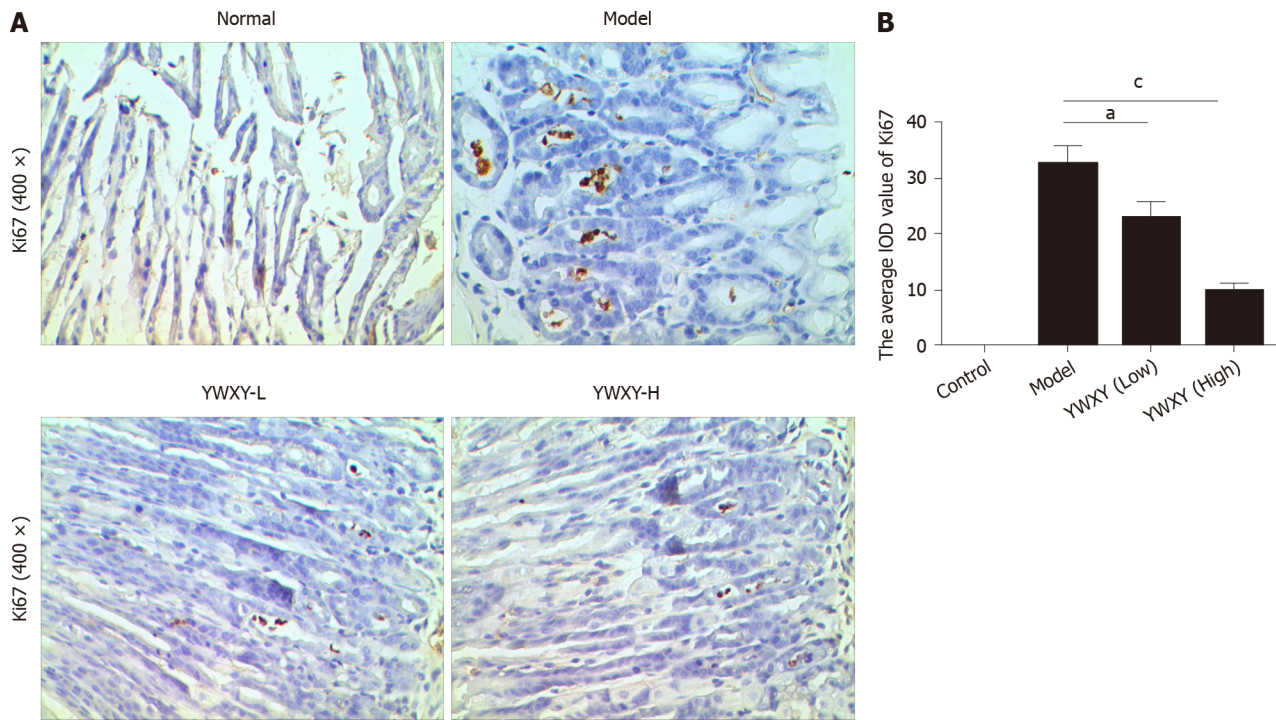
Figure 6 Expression of Ki67 in normal murine gastric mucosa and spasmolytic polypeptide-expressing metaplasia models. A: The expression of Ki67 was higher in the spasmolytic polypeptide-expressing metaplasia model than that in the normal control. B: Yiwei Xiaoyu granules had higher proliferative inhibition ability than spasmolytic polypeptide-expressing metaplasia models ([a]$P < 0.05$, [c]$P < 0.001$). YWXY-L: Low dose of Yiwei Xiaoyu granules; YWXY-H: High dose of Yiwei Xiaoyu granules.

VEGF-β as a parietal cell marker was illustrated to be downregulated in a tamoxifen-induced SPEM model at 3 d, whereas it recovered itself at 10 d and 21 d because of the rapid and reversible characteristics of the model[32,33]. Also, previous studies have reported that aging impairs angiogenesis and reduces expression of VEGF, which could illustrate the development of SPEM responsible for wound healing after ulcer injury[34-36]. In our study, with the YWXY intragastric administration for 10 d, the expression of VEGF-β was restored. Based on the acknowledgement of SPEM as a neoplastic precursor, it is conceivable that YWXY may inhibit the development and progression of SPEM by regulating VEGF-β.

Similarly, GIF was reported to locate in zymogenic chief cells at the base of control mice. The expression was downregulated significantly in the tamoxifen-induced mouse model[32,37]. In our study, scant GIF was discerned in the model group. However, with YWXY intragastric administration, the expression of GIF was restored. Thus, we speculated that SPEM lesion as a precursor to intestinal metaplasia and gastric adenocarcinoma could be treated by YWXY in gastric gland bases.

CONCLUSION

We showed evidence that miR-7 downregulation is an early event in the cascade from metaplasia to gastric cancer and that it contributes to the establishment of an intestinal expression profile through regulation of TFF2 both in human gastric mucosa and an *in vivo* model.

To the best of our knowledge, it is the first study to use the SPEM mouse model to uncover the effectiveness and the potential mechanism of Chinese medicine for the precursor of gastric adenocarcinoma. We performed a preliminary experiment to validate that YWXY had the ability to inhibit cell proliferation and restore the expression of miR-7 by mediating TFF2 in SPEM lesions. Nevertheless, the detailed mechanism of YWXY to prevent and inhibit the development and progression of SPEM lesions should be examined carefully in our future experiments.
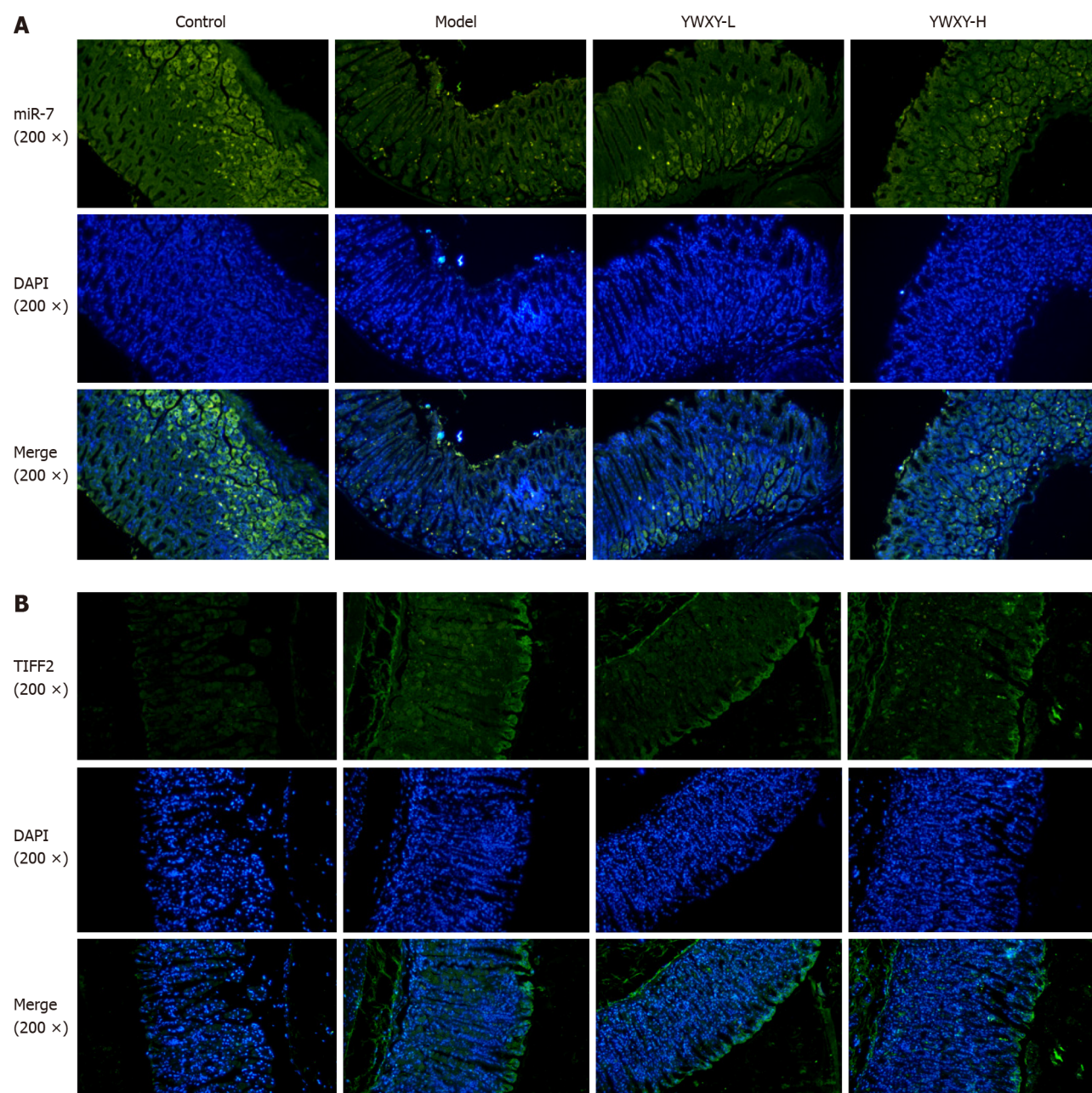
Figure 7 Expression of microRNA-7 and trefoil factor 2 in the murine gastric mucosa with Yiwei Xiaoyu granule administration. A: The expression of microRNA-7 for the spasmolytic polypeptide-expressing metaplasia mice model was restored with Yiwei Xiaoyu Granules (YWXY) administration (green: microRNA-7). B: The expression of trefoil factor 2 for the spasmolytic polypeptide-expressing metaplasia mice model was downregulated with Yiwei Xiaoyu administration. YWXY-H: High dose of Yiwei Xiaoyu granules; YWXY-L: Low dose of Yiwei Xiaoyu granules; TFF2: Trefoil factor 2; miR-7: MicroRNA-7.
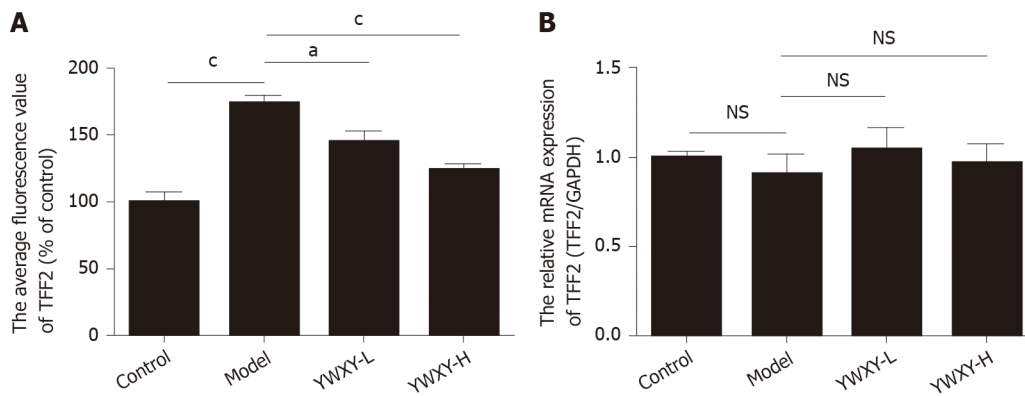
Figure 8 Yiwei Xiaoyu Granules administration could restore the expression of microRNA-7 by regulating trefoil factor 2 as measured by immunofluorescence but not reverse transcription-quantitative polymerase chain reaction. A: The expression of trefoil factor 2 in the model group was much higher than that in the control ($P < 0.001$). Intervening with Yiwei Xiaoyu granules (YWXY) could decrease the expression of trefoil factor 2 compared to the model (^c$P < 0.001$ in Yiwei Xiaoyu-High, ^a$P < 0.05$ in Yiwei Xiaoyu-Low); B: There was no difference in the relative mRNA expression of trefoil factor 2 in different groups by reverse transcription-quantitative polymerase chain reaction. YWXY-H: High dose of Yiwei Xiaoyu Granules; YWXY-L: Low dose of Yiwei Xiaoyu Granules; TFF2: Trefoil factor 2; NS: Not significant.
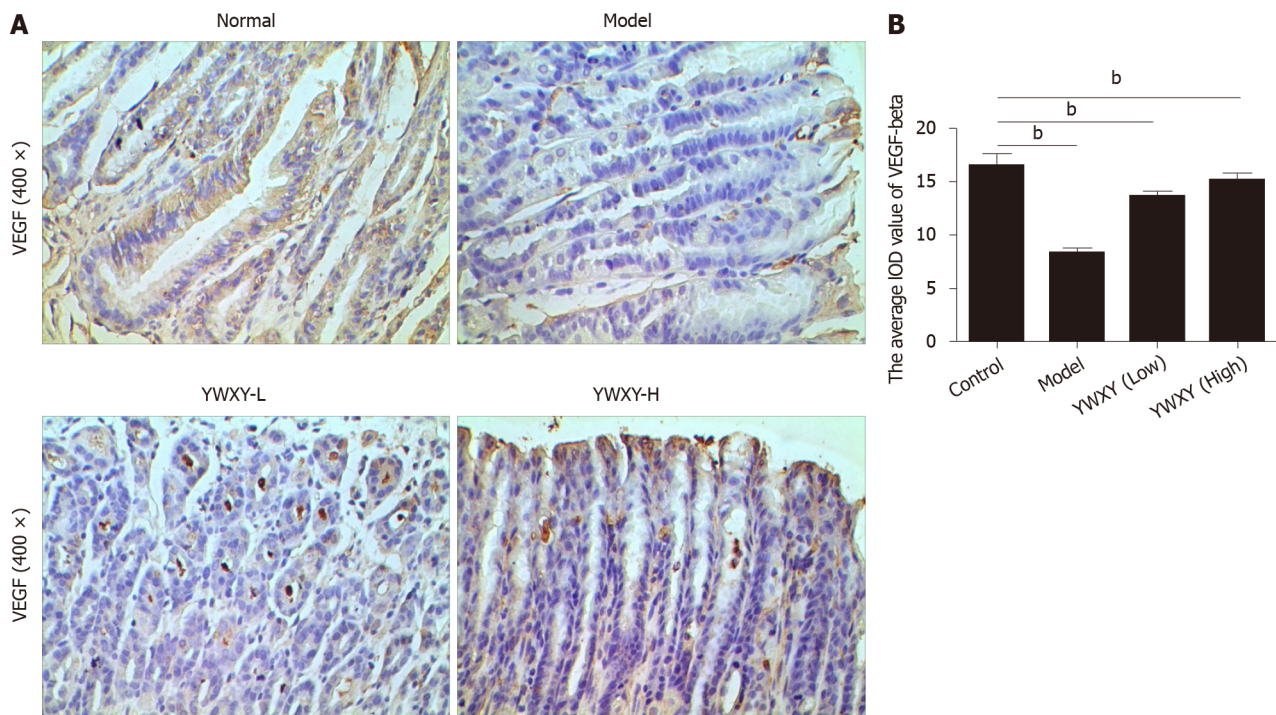

Figure 9 The expression of vascular endothelial growth factor-β was restored with Yiwei Xiaoyu granule administration in the spasmolytic polypeptide-expressing metaplasia lesions. A: The expression of vascular endothelial growth factor-β was upregulated with Yiwei Xiaoyu granules administration by immunohistochemistry measurement. B: Statistical differences existed between the control and the model (^b$P < 0.01$). YWXY-H: High dose of Yiwei Xiaoyu Granules; YWXY-L: Low dose of Yiwei Xiaoyu Granules; VEGF: Vascular endothelial growth factor.
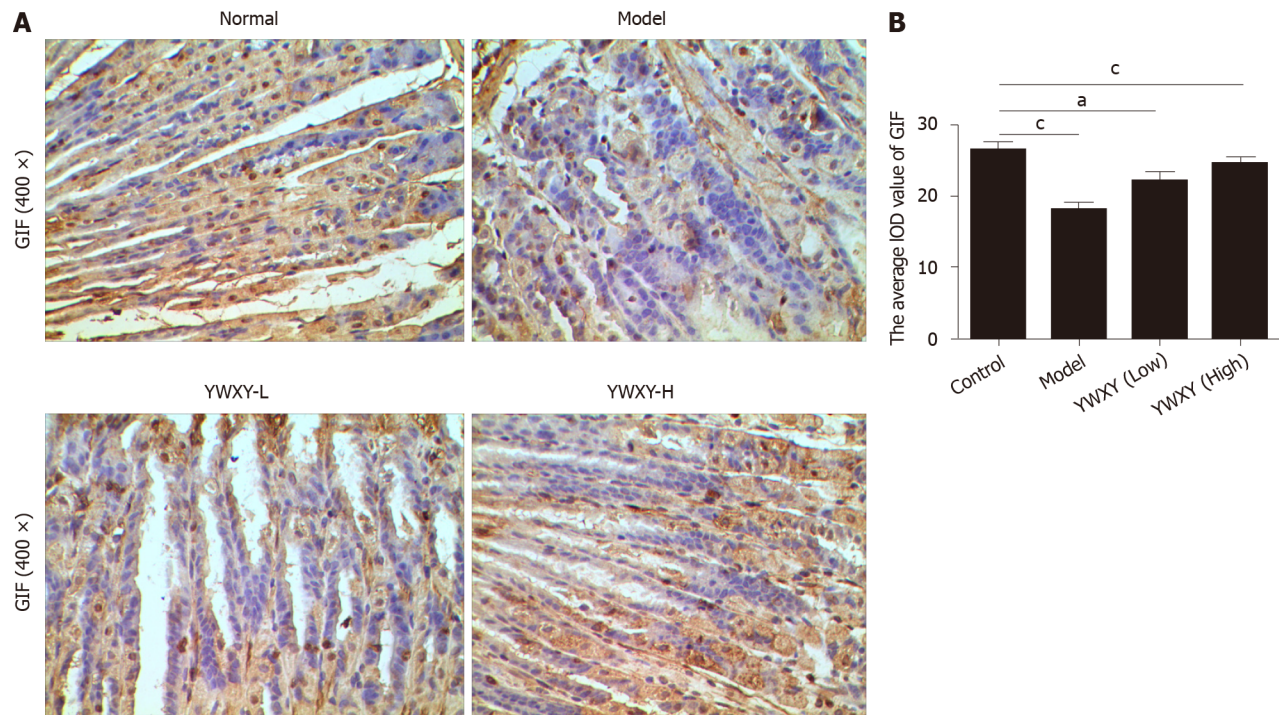
Figure 10 The expression of gastric intrinsic factor was restored with Yiwei Xiaoyu Granules administration in the spasmolytic polypeptide-expressing metaplasia lesions. A: The expression of gastric intrinsic factor was upregulated with Yiwei Xiaoyu Granules administration by immunohistochemistry. B: Statistics differences existed between the control and the model ($^aP < 0.05$, $^cP < 0.001$). YWXY: Yiwei Xiaoyu Granules; YWXY-H: High dose of Yiwei Xiaoyu Granules; YWXY-L: Low dose of Yiwei Xiaoyu Granules; GIF: Gastric intrinsic factor.

ARTICLE HIGHLIGHTS

Research background

Spasmolytic polypeptide-expressing metaplasia (SPEM) is the first metaplastic lesion to evolve and probably progresses to intestinal metaplasia.

Research motivation

Our group proved that Yiwei Xiaoyu granules (YWXY) could improve the mucosa atrophy, intestinal metaplasia and dysplasia of chronic gastric gastritis in a clinical trial, while the specific mechanism of YWXY still remains largely unknown.

Research objectives

To elucidate microRNA-7-mediated preventive and inhibitive effects of YWXY in SPEM lesions.

Research methods

Gastric mucosa biopsies were collected both in human and in a tamoxifen-induced SPEM mouse model. Then immunohistochemistry and immunofluorescence were performed to validate the SPEM lesions, and the potential mechanism was investigated. RNA transcripts were detected with reverse transcription-quantitative polymerase chain reaction.

Research results

We showed evidence that microRNA-7 downregulation was an early event in the cascade from metaplasia to gastric cancer and that it contributed to the establishment of an intestinal expression profile through regulation of trefoil factor 2 both in human gastric mucosa and an *in vivo* model. We validated that YWXY had the ability of inhibiting the cell proliferation and restoring the expression of microRNA-7 by mediating trefoil factor 2 in SPEM lesions.

Research conclusions

To the best of our knowledge, it is the first time to use the SPEM mouse model to uncover the effectiveness and potential mechanism of Chinese medicine for the

precursor of gastric adenocarcinoma.

Research perspectives

Nevertheless, the detailed mechanism of YWXY to prevent and inhibit the development and progression of SPEM lesions should be examined carefully in our next experiment. We believe it shows great potential for drug development to prevent and treat precancerous lesions.

ACKNOWLEDGEMENTS

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Basic Study

Effects of dietary zinc deficiency on esophageal squamous cell proliferation and the mechanisms involved

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Abstract

BACKGROUND
Dietary zinc deficiency has been shown to be associated with the development of esophageal cancer in humans, but the exact mechanism of action is not known

AIM
To observe the effects of dietary zinc deficiency on esophageal squamous cell proliferation.

METHODS
Thirty C57BL/6 mice were randomly divided into three groups: A zinc-sufficient (ZS) group, zinc-deficient (ZD) group, and zinc-replenished (ZR) group. For weeks 1–10, zinc levels in the mice diets were 30.66–30.89 mg/kg in the ZS group and 0.66–0.89 mg/kg in the ZD and ZR groups. During weeks 10–12, the ZR group was switched to the ZS diet; the other two groups had no changes in their diets. Changes in body weight, serum, and esophageal tissue zinc concentrations were assessed as well as differences in the expression of proliferating cell nuclear antigen (PCNA), mitogen-activated protein kinase p38 (p38MAPK), nuclear factor kappa B (NF-κB) p105, NF-κB p65, and cyclooxygenase (COX)-2 proteins in the esophageal mucosa.

RESULTS
The body weight and zinc concentration in the serum and esophageal mucosa were significantly lower in the ZD and ZR groups than in the ZS group ($P < 0.05$). In ZD mice, there was a marked proliferation of basal cells in the esophageal

mucosa, resulting in a disturbance in the arrangement of basal cells in layers 2–4, a thickening of the squamous layer, and a significant increase in the expression of the above-mentioned five proteins involved in proliferation and inflammation in the esophageal mucosa. Two weeks after switching to the ZS diet, the serum zinc concentration in the ZR group increased, and the expression of PCNA, NF-κB p105, and COX-2 decreased, but the concentration of zinc in the esophageal mucosa and the structure of the esophageal mucosa did not display any significant changes

CONCLUSION

The ZD diet decreased the growth rate and promoted the proliferation of esophageal squamous cells in mice. The mechanism of proliferation was related to the induced overexpression of COX-2, P38, PCNA, and NF-κB (p105 and p65), and the ZR diet reduced the expression of PCNA, NF-κB p105, and COX-2, thereby reversing this process.

Key Words: Zinc deficiency; Esophageal cancer; Esophageal squamous cell carcinoma; Esophageal squamous cells; Cell proliferation; Inflammatory response

Core Tip: Dietary zinc deficiency has been shown to be associated with the development of esophageal cancer in humans, but the exact mechanism of action is not known. The aim of this study was to observe the effects of dietary zinc deficiency on esophageal squamous cell proliferation. In addition, we investigated the pathway of zinc deficiency-induced esophageal squamous cell proliferation by detecting the expression of five predictive biomarkers. The results of the study showed that zinc-deficient diet decreased the growth rate and promoted the proliferation of esophageal epithelial squamous cells in mice. The mechanism was related to the induced overexpression of cyclooxygenase-2, P38, proliferating cell nuclear antigen, and nuclear factor kappa B (p105 and p65), and zinc replenishment reduced the expression of proliferating cell nuclear antigen, nuclear factor kappa B p105, and cyclooxygenase-2, thereby reversing this process.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), the predominant type of esophageal cancer, is a deadly disease with a 5-year survival rate of only 10%[1]. Because of the absence of early symptoms, patients with ESCC are usually diagnosed at a late stage and, therefore, have a poor prognosis. To improve the prevention and treatment of this deadly cancer, understanding its causes and discovering new early biomarkers are essential for chemoprevention and therapeutic options.

Risk factors for ESCC include alcohol and tobacco use, nutritional deficiencies, and exposure to environmental carcinogens such as *N*-nitrosomethylbenzylamine[2]. Epidemiological studies have shown an association between dietary zinc deficiency and the etiology of ESCC[3,4]. Studies from Linxian, China, an area of high ESCC prevalence, showed that zinc concentration in biopsy specimens was inversely associated with the risk of cancer development, providing the strongest evidence for an association between dietary zinc deficiency and the occurrence of esophageal cancer in humans[3]. It has been found that zinc deficiency can lead to the overexpression of various genes associated with immune response, apoptosis, cell proliferation, and transcriptional regulation. These overexpressed genes, such as pro-inflammatory cytokines interleukin-1β, interleukin-6, tumor necrosis factor alpha, and cyclic

nucleotide phosphodiesterases, may affect esophageal cancer development[5]. However, this is just one of many possibilities. Because of the multiple functions of this element, it can be assumed that the role of zinc in antitumor initiation and promotion is multi-pathway, although its mechanism of action is not fully understood.

The expression of five predictive biomarkers, proliferating cell nuclear antigen (PCNA), mitogen-activated protein kinase p38 (p38MAPK), nuclear factor kappa B (NF-κB) p105, NF-κB p65, and cyclooxygenase (COX)-2 proteins, was shown to be important in the development of esophageal cancer. PCNA is an indicator of cell proliferation[6]. NF-κB p105 and NF-κB p65 are members of the NF-κB family and play important roles in the transcriptional regulation of genes related to inflammation, cell proliferation, differentiation, apoptosis, immune response, and tumorigenesis[7]. Zinc can negatively regulate the NF-κB signaling pathway through numerous mechanisms[8]. COX-2 is an inducible enzyme that converts arachidonic acid to prostaglandins and is involved in inflammatory diseases and tumor development[9]. High COX-2 expression increases the risk of esophageal cancer in healthy people, and inhibition of COX-2 may be useful in the prevention and treatment of this cancer[10-12]. p38MAPK signaling has been linked to the development and progression of cancer, and altered p38MAPK expression has been associated with poor outcomes in patients with esophageal cancer[13]. Hence, the aim of this study was to investigate the effects of dietary zinc deficiency on the growth, development, and proliferation of esophageal squamous cells in mice. In addition, we examined the pathway of zinc deficiency-induced esophageal squamous cell proliferation by detecting the expression of the above-mentioned five predictive biomarkers.

MATERIALS AND METHODS

Chemicals and animal diets
Immunohistochemical detection kits purchased from Abcam Inc. (Cambridge, United Kingdom) were used, and immunostaining was performed according to the manufacturer's instructions. The D19410B egg white-based AIN-76A diet and D19401 egg white-based AIN-76A diet were purchased from Research Diets Inc. (New Brunswick, NJ, United States). These diets were assayed and found to contain 30.66–30.89 and 0.66–0.89 mg/kg zinc, respectively, and were accordingly used as zinc-sufficient (ZS) and zinc-deficient (ZD) diets (Table 1). This study was approved by the Institutional Research Ethics Committee of Beijing Shijitan Hospital. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

Experimental design
Thirty C57BL/6 mice (age, 3 wk; weaned) of specific-pathogen-free grade were procured from Sparford Laboratory Animal Technology Co. [Beijing, China; certificate number: SCXK (Beijing) 2011-0004]. The animals were housed in groups in stainless steel cages in a temperature-and humidity-controlled room with a 12 h light/dark cycle. The mice were randomized into three groups: ZS, ZD, and zinc-replenished (ZR) groups, with 10 mice in each group. For weeks 1–10, mice in the ZD and ZR groups were fed the ZD diet, and mice in the ZS group were fed the ZS diet. During weeks 10–12, the ZR group was switched to the ZS diet, and the other two groups were fed the same diets without change. All mice had free access to deionized water. The body weight and weight of the ingested feed for all mice were weighed weekly. All animals were euthanized after 12 wk of feeding. The changes in zinc concentrations in the serum and esophageal tissues, as well as differences in the expression levels of PCNA, p38MAPK, NF-κB p105, NF-κB p65, and COX-2 proteins in the esophageal mucosa, were assessed.

Collection and treatment of specimens
The animals were anesthetized with pentobarbital, which was provided by the animal room at our research facility, before euthanization. Blood was collected from the tail of each animal, and the serum was obtained and prepared for zinc analysis. Subsequently, whole esophagi were excised, opened longitudinally, and rinsed with normal saline. The esophageal mucosa was divided into three sections: One part for the detection of zinc concentration and the other two parts fixed in buffered formalin and embedded in paraffin. Cross-sections of the esophagus were cut to a thickness of 4 mm. Half of the sections were stained with hematoxylin and eosin and examined for histopathology under an optical microscope (Olympus, Tokyo, Japan) at 200 ×

Table 1 Effect of zinc deficiency and replenishment on body weight (g; *n* = 10)			
Age	ZS group	ZD group	ZR group
3 wk	19.06 ± 2.42	18.72 ± 2.89	18.38 ± 3.25
10 wk	27.80 ± 2.21	24.22 ± 2.47[a]	22.52 ± 2.89[a]
12 wk	30.55 ± 3.18	23.4 ± 2.76[b]	27.98 ± 3.16

[a]$P < 0.05$ *vs* zinc-sufficient (ZS) group.

[b]$P < 0.01$ *vs* ZS group.

Data are shown as mean ± SD. ZS group: Zinc-sufficient group; ZD group: Zinc-deficient group; ZR group: Zinc-replenished group.

magnification. The remaining tissue sections were dewaxed, hydrated, and analyzed with diluted primary antibodies (Abcam Inc.): PCNA (1:50; ab92552), P38 (1:50; ab31828), NF-κB p105 (1:50; ab32360), NF-κB p65 (1:50; ab16502), and COX-2 (1:50; ab15191).

Serum and esophageal mucosal zinc content was measured by inductively coupled plasma mass spectrometry (inductively coupled plasma mass spectrometry; model ELAN DRC II, Perkin Elmer Inc., Waltham, MA, United States). Prior to sample loading, serum samples were diluted 20 times, and esophageal mucosal samples were weighed, pretreated for microwave digestion, and diluted 20 times. The inductively coupled plasma mass spectrometry was set up with the following parameters: Nebulization gas flow rate, 0.98 L/min; auxiliary gas flow rate, 1.20 L/min; plasma gas flow rate, 15.0 L/min; retention time, 100 ms; sample absorption rate, 1 mL/min; scanning mode, single-point peak-jumping; resolution, 0.7–0.9 aum; ^{45}Sc; ^{166}Er; detection limit, Sc 0.03 ng/mL, Er 0.0003 ng/mL.

Statistical analysis

Statistical analyses were performed using SPSS software (version 22.0; IBM Inc., Armonk, NY, Unites States). Measurements conforming to a normal distribution were expressed as the mean ± SD. Differences between the groups were evaluated by one-way analysis of variance or the Kruskal–Wallis test for continuous variables. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of zinc deficiency and replenishment on body weight

As shown in Table 1, the body weight of mice at 3 wk of age did not differ significantly among the three groups. At 10 wk, the body weight of mice was significantly lower in the ZD and ZR groups than in the ZS group ($P < 0.05$). At 12 wk, the body weight of mice in the ZD group was significantly lower than that of mice in the ZS and ZR groups ($P < 0.05$). The body weight of mice in the ZR group at 12 wk was higher than that at 10 wk, but there was no significant difference in the body weight of mice in the ZS group, suggesting that dietary zinc replenishment remedied the decrease in body weight caused by zinc deficiency in mice.

Effect of zinc deficiency and replenishment on food intake

As shown in Table 2, the food intake of mice between the ages of 3 and 10 wk did not differ significantly among the three groups. At 12 wk, the food intake of mice in the ZD group was significantly lower than that of mice in the ZS group ($P < 0.01$), while there was no significant difference in food intake between the ZR and ZS groups.

Effect of zinc deficiency and replenishment on serum and esophageal mucosal zinc levels

As shown in Table 3, serum zinc levels of mice in the ZD group were significantly lower than those of mice in the ZS group ($P < 0.05$), while there was no significant difference between the serum zinc levels in the ZR and ZS groups. Esophageal mucosal zinc levels were significantly lower in the ZD group than in the ZS group ($P < 0.01$). Although esophageal mucosal zinc levels of mice in the ZR group increased, they were still significantly lower than those of mice in the ZS group ($P < 0.01$), suggesting that the improvement in the esophageal mucosal zinc level was

Table 2 Effect of zinc deficiency and replenishment on food intake (g/mouse/d/kg; *n* = 10)			
Age	ZS group	ZD group	ZR group
3 wk	386.25 ± 39.86	405.98 ± 40.24	396.47 ± 36.79
10 wk	358.49 ± 34.81	389.02 ± 36.24	380.11 ± 40.28
12 wk	309.72 ± 35.17	222.13 ± 28.48[b]	335.80 ± 38.13

[b]*P* < 0.01 *vs* zinc-sufficient group.

Data are shown as mean ± SD. ZS group: Zinc-sufficient group; ZD group: Zinc-deficient group; ZR group: Zinc-replenished group.

Table 3 Effect of zinc deficiency and replenishment on serum and esophageal mucosal zinc levels (*n* = 10)			
	ZS group	ZD group	ZR group
Serum zinc(μg/dL)	1.19 ± 0.14	0.97 ± 0.11[a]	1.29 ± 0.18
Mucosal zinc (mg/g)	32.80 ± 0.38	20.83 ± 0.24[b]	23.78 ± 0.29[b]

[a]*P* < 0.05 *vs* zinc-sufficient (ZS) group.

[b]*P* < 0.01 *vs* ZS group.

Data are shown as mean ± SD. ZS group: Zinc-sufficient group; ZD group: Zinc-deficient group; ZR group: Zinc-replenished group.

significantly lower than that in the serum zinc level.

Histopathological changes in the esophageal mucosa

Hematoxylin and eosin staining showed that the esophageal mucosa of mice in the ZS group consisted of a layer of basal cells that were arranged in an orderly manner. In ZD mice, there was apparent basal cell hyperplasia in the esophageal mucosa, which resulted in two to four layers of basal cells arranged in a disordered manner, along with a visible squamous layer thickening. However, columnar metaplasia, inflammation, or ulcers was not observed. Two weeks after zinc replenishment, the esophageal mucosa showed no obvious improvement (Figure 1).

Expression of PCNA, P38, NF-κB p105, NF-κB p65, and COX-2 in mouse esophageal mucosal tissue

Immunohistochemical staining showed that compared with mice in the ZS group, those in the ZD group showed increased expression levels of PCNA, P38, NF-κB p105, NF-κB p65, and COX-2 in the esophageal mucosa. Two weeks after zinc replenishment, the expression levels of PCNA, NF-κB p105, and COX-2 in the esophageal mucosa of ZR group decreased, while those of NF-κB, p65, and P38 showed no significant change (Figure 2).

DISCUSSION

Over the past several decades, zinc has been known to be an essential trace element that is widely distributed *in vivo* and plays a regulatory role in the immune system through its availability, which is tightly regulated by several transporters and regulators. As a functional component or activator of a variety of enzymes, zinc can promote human growth and development, augment nucleic acid and protein synthesis, and increase cell-mediated immune functions[14-16]. Zinc deficiency due to an improper diet is very common, as the human body cannot store zinc reserves[5]. Consequently, zinc deficiency is a global health problem, affecting approximately one-third of the world's population, predominantly in developing countries[17,18]. Dietary zinc intake is less than half the recommended dose in more than 10% of the population [5]. Acute zinc deficiency causes a decrease in innate and adaptive immunity, whereas chronic deficiency increases inflammation and the risk of cancer[19-22]. Many epidemiological studies have shown that zinc deficiency in humans is associated with an increased risk of developing ESCC, although the mechanism through which this occurs is not fully understood[3,23,24]. By using the ZD rat model, we designed this study to elucidate the effects of dietary zinc deficiency on esophageal squamous cells
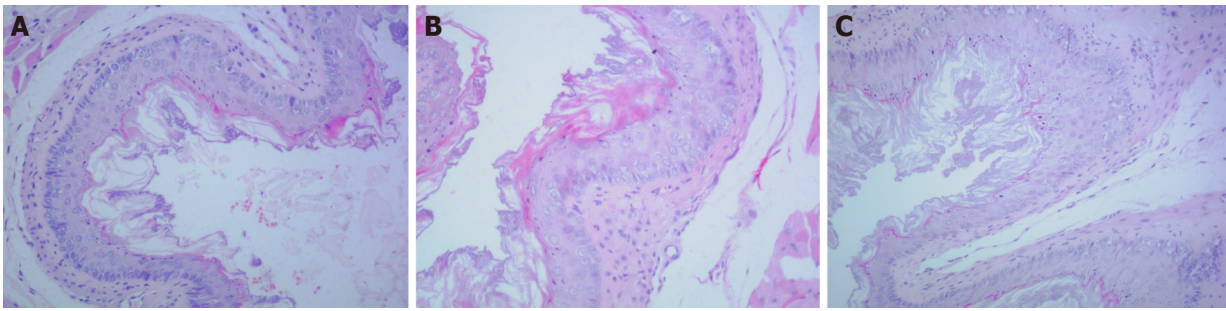
Figure 1 Hematoxylin and eosin staining of the esophageal mucosa (200 ×). A: Zinc-sufficient group; B: Zinc-deficient (ZD) group; C: Zinc-replenished group. The esophageal mucosa of mice in the Zinc-sufficient group (A) consisted of a layer of basal cells that were arranged in an orderly manner. In the ZD mice (B), there was apparent basal cell hyperplasia in the esophageal mucosa, that resulted in two to four layers of basal cells arranged in a disordered manner, along with a visible squamous layer thickening. The esophageal mucosa of mice in the Zinc-replenished group (C) showed no obvious improvement compared to that of mice in the ZD group.

and the possible mechanisms. In addition, the effect of zinc deficiency on the esophageal mucosa of ZD rats was observed.

Our results showed that after 12 wk of the ZD diet, food intake, body weight, and zinc content in the serum and esophageal mucosal tissues of ZD mice decreased significantly, which differed considerably from that in the ZS group. After 2 wk of zinc replenishment, the body weight and serum zinc content of the ZR mice increased, with no difference compared to the ZS group, while the esophageal mucosal zinc content was very low, indicating that the zinc content in the esophageal mucosal tissues recovered more slowly than the serum zinc content after zinc replenishment. In both animal and human studies, zinc has been shown to participate in the regulation of cell proliferation in several ways; therefore, zinc deficiency can restrict growth[25-26]. It is essential for enzyme systems to influence cell division and proliferation. In humans, an early symptom of zinc deficiency is diarrhea, followed by listlessness and depression [27]. Severe zinc deficiency can lead to hepatic encephalopathy, growth retardation, cell-mediated immune dysfunction, and cognitive impairment[25-28]. Zinc supplementation may be a crucial intervention for improving these clinical problems[29]. Furthermore, epidemiological studies have revealed an association between high circulating zinc concentrations and reduced risk of cancer[30-33]. The mean serum zinc levels in high-risk regions were significantly low. The elemental concentrations of zinc showed a significant difference ($P < 0.001$) in tumor and non-tumor tissues from the same individual[34]. Zinc deficiency is closely related to the risk of esophageal cancer in multiple populations[32,33]. Zinc is presumed to play a pivotal role in defending against the initiation and promotion of several malignancies, although the mechanism of this role is not fully known[21].

Since the genetic characteristics of mice are clearer than those of rats, mice are the best animal model for studying the development and progression of diseases. The pathological results of this study showed that esophageal mucosal basal cells of mice in the ZD group demonstrated obvious hyperplasia, disordered cell arrangement, and a thickened squamous layer. Previous studies have shown that dietary zinc deficiency may also lead to hyperplasia and hyperkeratosis of esophageal squamous cells in rats and that cell proliferation is directly related to the development of esophageal cancer [35]. Rats fed a low-zinc diet for 5 wk developed esophageal preneoplasia with unique genetic characteristics[36]. Feeding a low-zinc diet to rats for 23 wk resulted in increased expression of cancer-related inflammatory factors that could lead to the development of esophageal cancer when combined with a non-carcinogenic dose of the environmental carcinogen *N*-nitrosomethylbenzylamine[37-39], while replenishing zinc through a ZS diet reduced these effects in ZD rats. Studies from areas with a high incidence of esophageal cancer have shown that a higher total number of proliferating cells in the esophageal epithelium found in hyperplasia and dysplasia was associated with an increased risk of cancer[40]. Zinc is important for maintaining healthy esophageal epithelium, and zinc deficiency results in abnormal esophageal cell proliferation, promoting tumor development[41].

The expression of five predictive biomarkers, COX-2, NF-κB p65, NF-κB p105, PCNA, and P38, was examined *via* immunohistochemistry in this study. The results showed that dietary zinc deficiency could induce the overexpression of COX-2, P38, PCNA, NF-κB (p65 and p105), and other inflammatory factors, which may be related to the occurrence of ESCC. After 2 wk of ZR, the expression level of PCNA, NF-κB
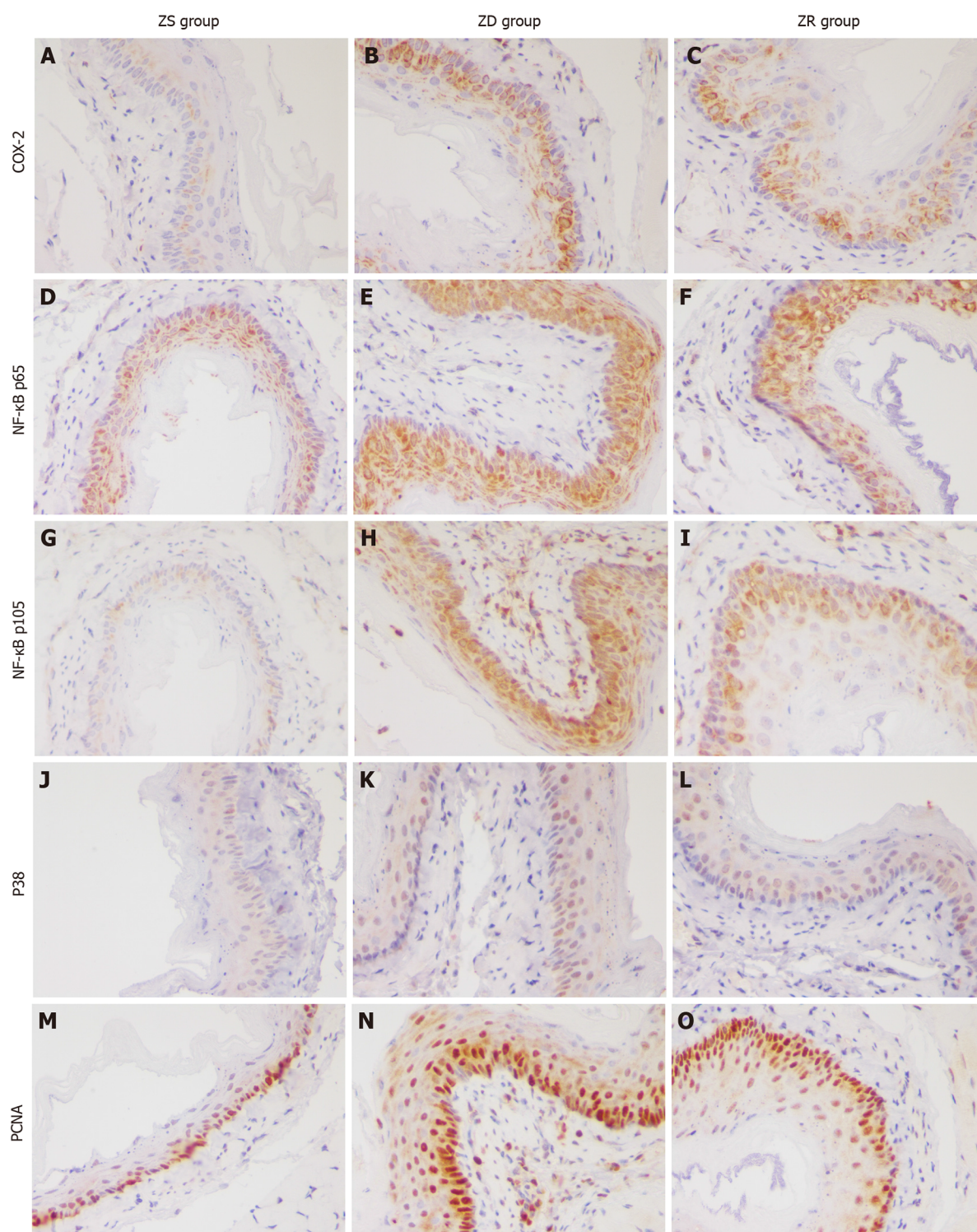
Figure 2 Immunohistochemical staining for cyclooxygenase-2, nuclear factor kappa B p65, nuclear factor kappa B p105, P38, and proliferating cell nuclear antigen in the esophageal mucosa (200 ×). A: Cyclooygenase (COX)-2 was not expressed or was minimally expressed in the cytoplasm of normal cells; B: COX-2 expression was significantly increased in the esophageal mucosa of mice in the zinc-deficient (ZD) group; C: Two weeks of zinc-replenished (ZR) group reduced the COX-2 expression level; D: Nuclear factor kappa B (NF-κB) p65 showed a low expression level in the cytoplasm of normal cells; E: NF-κB p65 expression level significantly increased in the esophageal mucosa of mice in the ZD group; F: Compared with mice in the ZD group, in those in the ZR group, NF-κB p65 expression level did not change significantly in the esophageal mucosa; G: NF-κB p105 showed a low expression level in the cytoplasm of normal cells; H: NF-κB p105 expression level significantly increased in the esophageal mucosa of mice in the ZD group; I: Two weeks of ZR group reduced the NF-κB p105 expression level; J: P38 was expressed in small amounts in the cytoplasm and nucleus of normal cells; K: P38 expression was increased in the nucleus of esophageal mucosal cells in the ZD group; L: Compared with mice in the ZD group, in those in the ZR group, P38 expression level did not change significantly in the esophageal mucosa; M: Proliferating cell nuclear antigen (PCNA) showed a low expression level in the cytoplasm of normal cells; N: PCNA expression level significantly increased in the esophageal mucosa of mice in the ZD group; Compared with mice in the ZD group, in those in the ZR group, 2 wk of ZR reduced the PCNA expression level. ZS: Zinc-sufficient; ZD: Zinc-deficient; ZR: Zinc-replenished; COX-2: Cyclooxygenase-2; NF-κB: Nuclear factor kappa B; PCNA: Proliferating cell nuclear antigen.

p105, and COX-2 in the esophageal mucosa decreased. This finding is consistent with the results of Fong *et al*[42], who found that zinc deficiency also affected cancer development by regulating the expression of NF-κB p65, COX-2, and leukotriene A4 hydrolase. Zinc deficiency significantly increased the incidence of esophageal cancer by inducing the overexpression of inflammatory factors, while zinc supplementation reversed this process[37]. Zinc deficiency-induced inflammation is a critical factor in ESCC development. Furthermore, Taccioli *et al*[36] found that short-term zinc deficiency could induce the overexpression of proinflammatory genes *S100A8* and *S100A9* in the esophageal mucosa. Chronic inflammation has been implicated in the pathogenesis of ESCC[37]. Zinc deficiency upregulates oncogenic *miR-21*, *miR-31*, and *miR-223* and downregulates the tumor suppressor gene *miR-375*, all of which are accompanied by the dysregulation of their target genes in esophageal cancer[42-44].

CONCLUSION

In conclusion, dietary zinc deficiency can inhibit growth and promote the proliferation of esophageal epithelial squamous cells in mice. The mechanism may be related to the induced overexpression of COX-2, P38, PCNA, NF-κB (p65 and p105), and other tumor-related factors. Zinc replenishment reversed this process by reducing the expression of PCNA, NF-κB p105, and COX-2. Because China has a high incidence of esophageal cancer, it may be beneficial to prevent the occurrence of esophageal cancer by promoting an increase in the intake of foods rich in zinc, such as fish, seafood, meat, fresh vegetables, and fruits.

ARTICLE HIGHLIGHTS

Research background

Zinc is an element with multiple functions. Zinc deficiency can lead to overexpression of several genes related to immune response, apoptosis, cell proliferation, and transcriptional regulation. Dietary zinc deficiency has been shown to be associated with the development of esophageal cancer in humans, but the exact mechanism of action is not known.

Research motivation

Esophageal squamous cell carcinoma is a deadly disease with a 5-year survival rate of only 10%. Because of the absence of early symptoms, patients with esophageal squamous cell carcinoma ESCC are usually diagnosed at a late stage and, therefore, have a poor prognosis. To improve the prevention and treatment of this deadly cancer, understanding its causes and discovering new early biomarkers are essential for chemoprevention and therapeutic options.

Research objectives

The aim of this study was to investigate the effects of dietary zinc deficiency on the growth, development, and proliferation of esophageal squamous cells in mice. In addition, we investigated the pathway of zinc deficiency-induced proliferation of esophageal squamous cells by detecting the expression of five predictive biomarkers, namely proliferating cell nuclear antigen (PCNA), mitogen-activated protein kinase p38 (p38MAPK), nuclear factor kappa B (NF-κB) p105, NF-κB p65, and cyclooxygenase (COX)-2 protein.

Research methods

Thirty C57BL/6 mice were randomly divided into three groups: a zinc-sufficient (ZS) group, zinc-deficient (ZD) group, and zinc-replenished (ZR) group. For weeks 1–10, zinc levels in the mice diets were 30.66–30.89 mg/kg in the ZS group and 0.66–0.89 mg/kg in the ZD and ZR groups. During weeks 10–12, the ZR group was switched to the ZS diet; the other two groups had no changes in their diets. Changes in body weight, serum, and esophageal tissue zinc concentrations were assessed as well as differences in the expression of PCNA, p38MAPK, NF-κB p105, NF-κB p65, and COX-2 proteins in the esophageal mucosa.

Research results

The body weight and zinc concentration in the serum and esophageal mucosa were significantly lower in the ZD and ZR groups than in the ZS group. In ZD mice, there was a marked proliferation of basal cells in the esophageal mucosa, resulting in a disturbance in the arrangement of basal cells in layers 2–4, a thickening of the squamous layer, and a significant increase in the expression of the above-mentioned five proteins involved in proliferation and inflammation in the esophageal mucosa.

Research conclusions

The results indicated that the ZD diet decreased the growth rate and promoted the proliferation of esophageal squamous cells in mice. The mechanism of proliferation was related to the induced overexpression of COX-2, P38, PCNA, and NF-κB (p105 and p65), and the ZR diet reduced the expression of PCNA, NF-κB p105, and COX-2, thereby reversing this process.

Research perspectives

In this study, all five proteins were detected by immunohistochemistry staining, which is a semi-quantitative method. In future studies, we will try to increase the sample size and use a quantitative approach to make the results more meaningful.

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ORIGINAL ARTICLE

Case Control Study

Genetic variation of *TGF-BR2* as a protective genotype for the development of colorectal cancer in men

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Abstract

BACKGROUND
The role of transforming growth factor beta (TGF-β) signaling, including both the cytokine and their receptors, in the etiology of colorectal cancer (CRC) has been of particular interest lately.

AIM
To investigate the association between promoter polymorphism in TGF-β receptor 2 TGF-BR2[-875]A with a CRC risk in a cohort of Bulgarian patients using a case-control gene association study approach, as well as the protein levels of TGF-β1 in the peripheral blood.

METHODS
A cohort of 184 CRC patients and 307 sex and age-matched healthy subjects were recruited in the study. A genotyping of the TGF-BR2G[-875]A (rs3087465) polymorphism was performed by primer-introduced restriction analyses-polymerase chain reaction approaches.

RESULTS
The frequency of TGF-BR2G[-875]A genotype was decreased in male patients with CRC than in healthy men (31.3% *vs* 44.8%; *P* = 0.058). Among males, the TGF-BR2G[-509]G genotype was related to a significantly increased risk of CRC

development (OR = 1.820, 95%CI: 0.985-3.362, P = 0.055) than the GA + AA genotype. Also, TGF-BR2[-875]*A-allele itself was rarer in men with CRC than healthy men (19.1% *vs* 26.9%, P = 0.086) and was associated with a protective effect (OR = 0.644; 95%CI: 0.389-1.066; P = 0.086). Regarding the genotypes, we found that TGF-β1 serum levels were higher in GG genotype in healthy persons above 50 years than the CRC patients [36.3 ng/mL interquartile range (IQR) 19.9-56.5 *vs* 22.4 ng/mL IQR 14.8-29.7, P = 0.014]. We found significant differences between higher levels of TGF-β1 serum levels in healthy controls above 50 years (GG genotype) and CRC patients (GG genotype) at the early stage (36.3 ng/mL IQR 19.9-56.5 *vs* 22.8 ng/mL IQR 14.6-28.6, P = 0.037) and advanced CRC (36.3 ng/mL IQR 19.9-56.5 *vs* 21.6 ng/mL IQR 15.9-33.9, P = 0.039).

CONCLUSION

In summary, our results demonstrated that TGF-BR2 AG and AA genotypes were associated with a reduced risk of CRC, as well as circulating levels of TGF-β could prevent CRC development in a gender-specific manner. Notably, male carriers of TGF-BR2 -875A allele genotypes had a lower risk of CRC development and progression, suggesting that TGF-BR2 -875A/G polymorphism significantly affects the protective biological factors that also impact the risk of colon and rectal carcinogenesis.

Key Words: Colorectal carcinoma; Cytokine; *TGF-BR2* gene; TGF-BR2G[-875]A; Single nucleotide polymorphism

Core Tip: Disruptions in transforming growth factor beta (TGF-β)-associated cancer mechanisms are essential in early-stage tumor development, whereas activation of TGF-β-signaling can encourage invasion and metastasis of cancer. Our findings from this case-control study suggested that the highest risk for developing colorectal neoplasia was found for the GG genotype. The increased risk for colorectal cancer (CRC) development was associated with male CRC patients homozygous for the GG genotype. In contrast, male carriers of TGF-BR2 -875A allele genotypes of *TGF-BR2* had a lower risk of CRC development and progression. No other studies for this polymorphism and CRC association from the literature are available to the best of our knowledge.

INTRODUCTION

The role of transforming growth factor beta (TGF-β) signaling in the colon and rectal cancer etiology has been intensively studied for the last decades. It is thought that disruptions in TGF-β-associated cancer mechanisms are essential in early-stage tumor development. In contrast, activation of TGF-β-signaling can encourage invasion and metastasis of cancer[1]. In addition, its involvement in the tumor microenvironment control typically includes inhibition of the tumor-specific immune cells and facilitation of the cancer cells survival. This again emphasizes that the TGF-β signaling in cancer exerts multi-directional functions between cancer cells encouragement and tumor micro-environment inhibition[2].

Furthermore, it was shown that TGF-β-signaling has dual roles in developing and progressing gastrointestinal tumors - as both a tumor promoter and tumor suppressor [3]. Although the mechanism by which TGF-β converts its inhibitory into stimulating growth effect is not well known, it is thought that the cytokine enhances many

mitogenic growth factors, including TGFα, FGF, and EGF, and oncogenic pathways, such as Ras/MAPK pathway, JNK pathway, and PI3 kinase/Akt pathway, *etc*[4]. Thus, many mechanisms of TGF-β are involved in the proliferation of colorectal cancer (CRC) cells. Nevertheless, TGF-β also promotes angiogenesis and immunosuppression. This is especially valid for CRC. In the last decade, the role of TGF-β1 was recognized in colorectal tumorigenesis. There is an increasing amount of proof that TGF-β-signaling modifications induced by TGF-β1 or SMAD mutations or polymorphisms contribute to the development and progression of CRC[5].

Inflammation also plays a significant role in the support and promotion of CRC growth. Indeed, dysregulated immune response in CRC patients involved different immune cell types, leading to the release of a range of cytokines, chemokines, and growth factors that control both inflammation and carcinogenesis[6]. Furthermore, colonic epithelial cells are simultaneously producers and respondents to cytokines [interleukins (IL), and chemokines], signals that modulate the behavior of epithelial cells by influencing their proliferation, migration, and survival[7]. In such a way, cytokines carry out the cross-talk between cells of the immune system and the CRC cells, forming networks with anti-tumor (interferon-γ, IL-12, IL-15, IL-17F, and IL-18), pro-tumor (IL-4, IL-6, IL-8, IL-11, IL-17A, IL-22, IL-23, IL-33, tumor necrosis factor, TGF-β, and vascular endothelial growth factor) or bivalent or unclear properties to intestinal cancer (IL-1, IL-9 IL-10, IL-21, and granulocyte-macrophage colony-stimulating factor)[8-10].

Amongst these mediators, TGF-β was shown to exert functions such as induction of reactive oxygen species, inflammation-associated epithelial-mesenchymal transition, angiogenesis, and metastasis[7,11]. TGF-β1 serves its functions by binding to two receptors-type I and type II receptors, *i.e.*, *TGF-BR1* and *TGF-BR2*, respectively. Through binding to *TGF-BR2*, downstream signaling involving cell-cycle checkpoint genes [*e.g.*, CDKN1A (p21), CDKN1B (p27), and CDKN2B (p15)] is initiated. As a result, cell growth is arrested[12]. Therefore, TGF-β1 exerts tumor-suppressing effects in the intestines' epithelium by inhibiting cell proliferation and inducing apoptosis. In line with this, many colorectal cancers can avoid the suppressive effects of TGF-β1 that make them resistant to TGF-β-induced growth inhibition[5].

Many mutations in different genes were associated with CRC development, such as the *APC* gene, mutation of *KRAS* and *TP53*, deletion on chromosome 18q, germline mutation in DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*). Furthermore, mutations in microsatellites of certain tumor-suppressor genes (*e.g.*, TGF-BR2, BAX, E2F4, and IGFR2, have also been identified in colorectal tumors[5].

The polymorphic variant in the promoter region of TGF-BR2 (rs3087465) due to a G to A transition was reported previously in patients with Lynch syndrome and other cancer types[1,5,13]. However, there are still many unanswered questions. For example, TGF-BR2 polymorphisms were not explored extensively as a genetic protective factor for CRC. Furthermore, since TGF-β signaling is altered in CRC, targeting this intracellular pathway may represent a potential therapeutic approach. However, it is difficult to efficiently discover and administer therapeutic products for the treatment of cancer. This is because the simple blockage of TGF-β signaling could enhance immunity in the tumor microenvironment but may lead to the development of more aggressive cancer phenotypes[1].

Since TGF-β1 exerts its effects by these receptors and given the importance of the TGF-β1 signaling pathway in CRC development, we could hypothesize that genetic polymorphisms in the TGF-β1 gene and genes for TGF-β receptors may also play a role in CRC susceptibility. Previously, we reported the role of circulating TGF-β1 and the -509C/T functional promoter polymorphism (rs1800469) within the TGF-β1 gene (TGF-B1) in the susceptibility, progression, and prognosis of CRC among Bulgarian patients in a gender-dependent manner[14].

Therefore, we aimed to investigate the association between another promoter polymorphism (in TGF-BR2G[-875]A) with a CRC risk in a cohort of Bulgarian patients using a case-control gene association study approach. We also estimated the role of this polymorphism at different stages of the disease, defined as early and advanced in men and women. Additionally, we were interested in the TGF-β1 in the peripheral blood associated with the different genotypes as a non-invasive marker for CRC development and progression and the relationship between this polymorphism and serum levels of TGF-β1 in patients and healthy people.

MATERIALS AND METHODS

CRC patients

A total of 184 patients with CRC at a mean ± SD age of 65 ± 10 years, obtained in the University Hospital and Trakia Hospital, Stara Zagora, during 2011-2017, were included in the study. The diagnosis was made by employing standard clinical, laboratory, endoscopic, histopathological, and radiological criteria. CRC patients underwent curative surgical resection of the tumors. Previous diagnosis of inflammatory bowel disease or other autoimmune diseases or individual or family history of any known hereditary cancer syndromes were considered exclusion criteria for the patients and controls. Additionally, patients did not receive any neo-adjuvant chemotherapy or radiation therapy prior to surgery.

Tumor, node and metastasis classification was used for tumor grading and staging. Accordingly, CRC patients were divided into two groups: 88 patients with early CRC (1st stage + 2nd stage) and 96 patients with advanced CRC (3rd + 4th stages). The CRC group was composed of 115 males (62.5%) and 69 females (37.5%). There were no significant differences between the mean age of males and females ($P = 0.65$).

A total of 307 healthy volunteers were included in the study, matched with patients by age and gender. The healthy control group consisted of 240 females and 67 males, with a mean ± SD age 42 ± 13 years.

The blood concentrations of TGF-β1 in CRC patients were compared to matched controls over 50 years, where the gender distribution was considered similar ($\chi^2 = 0.055$; $P = 0.814$).

The demographic and clinical characteristics of the CRC populations of patients and control subjects were summarized previously in our paper investigating the TGF-β1 gene promoter-509C/T polymorphism[14](https://doi.org/10.1371/journal.pone.0201775.t001) .

Ethical considerations

According to the Helsinki Declaration and local Ethics Committee's ethical guidelines, all participants gave written informed consent for the study. All patients were informed about the purpose of the study.

Specimen collection and preparation

At least 6 mL of peripheral venous blood from the CRC patients and healthy controls were collected in sterile tubes. Plasma samples were obtained and frozen at -80°C before use to determine the protein level of TGF-β1. The genomic DNA from 200 μl peripheral blood was extracted using Gene Matrix Purification Kit (EURx, Poland) following the manufacturer's instructions and stored at -80°C until use. We measured the DNA samples' concentration and purity spectrophotometrically at 260/280 nm using a GeneQuant 1300 spectrophotometer (GE Healthcare Life Sciences, Switzerland).

Genotyping of TGF-BR2G[-875]A promoter polymorphism

We performed the genotyping of the *TGF-BR2*G[-875]A (rs3087465) by primer-introduced restriction analysis-polymerase chain reaction (PCR) assay[15]. The primer sequences were the following: forward primer- 5'-GCAAGAAAGGAAATTTGAAAGTTTGT-3' and reverse primer 5'-TCACCTGAATGCTTGTGCTTTT-3'. The PCR amplification was accomplished as follows: (1) denaturation at 94°C/5 min; (2) 30 cycles at 94°C/45 s, 57°C/45 s and 72°C/45 s; and (3) final extension cycle at 72°C/7 min). Rsal (10 U/μL) restriction enzyme was used to digest the 124bp PCR products at 37°C overnight. Then, we electrophoresed the final products on 3.5% agarose gel and visualized them directly with ethidium bromide staining. Two fragments of 99bp and 25bp resulted from the *TGF-BR2*[-875]*A allele, while *TGF-BR2*[-875]*G allele produced a fragment of 124bp.

GeneAmp PCR System 9700 (Applied Biosystems) was used to perform all PCR reactions by using Thermo Fisher Scientific (United States) and Metabion GmbH (Germany) PCR reagents and primers.

Measurement of TGF-β1 in the plasma samples

Enzyme-linked immunosorbent assay (ELISA) method was performed to measure the protein level of latent acid-activated TGF-β1 protein in the participants' plasma samples (Quantikine ELISA Kits, R&D systems, Minneapolis, MN, United States).

Latent serum TGF-β1 was firstly activated by acid (1N HCl) and neutralized by 1.2N NaOH/0.5M HEPES, following the manufacturer protocol. The activated Serum samples from the patients and controls were stored in a fridge (2-6°C) for less than 16 h. Then we analyzed them together in the same analytic batch. A 4-point parametric standard curve calculated the results using the manufacturer`s standards within the range from 0-2000 ng/mL and expressed as ng/mL. The minimum detectable levels ranged from 1.7–15.4 ng/mL.

Statistical analysis

Statistical analysis of the data was performed using the Statistical software package (StatSoft v. 12.0, Inc., United States). We calculated the sample size by the GAS Power Calculator, given the significance level α=0.05, prevalence = 0.1, the anticipated effect size (Cohen's d = 0.5), and desired statistical power level 1-β = 0.8, which determined the sample size of cases *n* = 184 and controls *n* = 307.

$χ^2$ test was used to determine the statistical differences in the distribution of genotype and allele frequencies between CRC patients and healthy controls. The Hardy-Weinberg equilibrium was tested by comparing the observed genotype frequencies to the expected frequencies for cases and controls by $χ^2$ test.

When the observed frequencies were a smaller group, we used the Fisher exact test and Yates' corrected *P*-value (c). The association between TGF-BR2 genotypes and risk of CRC was evaluated by calculating the odds ratios (ORs) and 95%CI using the StatPages.net website (http://statpages.org/index.html).

Results were considered significant at $P ≤ 0.05$.

RESULTS

Association of TGF-BR2G[-875]A polymorphisms with CRC susceptibility

The genotype distribution for TGF-BR2G[-875]A (rs3087465 polymorphism) demonstrated no deviation from Hardy-Weinberg equilibrium in the cases ($χ^2$ = 0.2, *P* = 0.905) and controls ($χ^2$ = 0.122, *P* = 0.940).

The different genotype and allele frequencies of the TGF-BR2G[-875]A promoter polymorphisms in patients with CRC and controls are presented in Table 1. However, we did not observe differences between cases and controls regarding the distribution of the studied polymorphism ($χ^2$ = 1.38, *P* = 0.50). When we stratified the data according to the study participants' sex, we obtain the following results. The frequency of TGF-BR2G[-875]A genotype was decreased in male patients with CRC than in healthy men (31.3% *vs* 44.8%; *P* = 0.058).

Moreover, male subjects with the GG genotype exhibited a higher risk of CRC development than the GA + AA genotype (OR = 1.820, 95%CI: 0.985-3.362, *P* = 0.055). In contrast, the GA genotype was associated with a lower risk compared with the GG + AA genotype in men (OR = 0.562, 95%CI: 0.302-1.047, *P* = 0.068). Additionally, the TGF-BR2G[-875]A genotype was associated with a reduced risk of CRC development (OR = 0.544; 95%CI: 0.289-1.023; *P* = 0.058) referred to the TGF-BR2G[-875]G genotype among men.

However, TGF-BR2[-875]*A-allele itself was rarer in men with CRC than healthy men (19.1% *vs* 26.9%, *P* = 0.086) and was associated with a protective effect (OR = 0.644; 95%CI: 0.389-1.066; *P* = 0.086).

The observed genotype distribution of TGF-BR1 rs4743325 polymorphism in men was similar among women with CRC and healthy women but did not reach statistical significance.

Association of TGF-BR2G[-875]A polymorphism with the stage of CRC

After stratification of CRC patients into those with early CRC (I stage + II stage) and advanced CRC (III stage + IV stage), we did not find differences in genotype distribution among patients with early *vs* advanced stage (Table 2).

Besides, no differences between genotype distribution among patients with early CRC and healthy controls were observed (Table 3).

When we compared advanced CRC cases with healthy controls, we found that male carriers of the A allele (GA/GA + AA genotypes) had a significantly decreased risk of advanced CRC (OR = 0.459, 95%CI: 0.217-0.969, *P* = 0.039; OR = 0.466, 95%CI: 0.226-0.961, *P* = 0.037, respectively) (Table 4).

Additionally, TGF-BR2[-875]*A-allele alone was also a protective factor for advanced CRC in men compared to healthy men (17.2% *vs* 26.9%; OR = 0.566, 95%CI:

Table 1 Difference in genotype distribution and allele frequencies of *TGFBR2* rs3087465 polymorphism between the case (colorectal cancer patients) and control (healthy persons) groups, females and males, respectively

Genotype	CRC, *n* (%)	Healthy controls, *n* (%)	OR (95%CI)	*P* value
n	184 (100)	307 (100)		
GG	117 (63.6)	193 (62.9)	Reference	
GA	58 (31.5)	102 (33.2)	0.938 (0.631-1.393)	0.751
AA	9 (4.9)	12 (3.9)	1.237 (0.506-3.026)	0.640
GA + AA	67 (36.4)	114	0.969 (0.664-1.416)	0.873
G allele	292 (79.3)	488 (79.5)	Reference	
A allele	76 (20.7)	126 (20.5)	1.008 (0.732-1.387)	0.961
Female	69	240		
GG	42 (40.9)	159 (66.3)	Reference	
GA	22 (31.9)	72 (30)	1.157 (0.664-2.079)	0.626
AA	5 (7.2)	9 (3.7)	2.103 (0.669-6.608)	0.195
GA + AA	27 (39.1)	81 (33.8)	1.262 (0.726-2.193)	0.409
GG *vs* GA + AA	42 *vs* 27	159 *vs* 81	0.792 (0.456-1.377)	0.409
GA *vs* GG + AA	22 *vs* 47	72 *vs* 168	1.092 (0.614-1.944)	0.764
G allele	106 (76.8)	390 (81.3)	Reference	
A allele	32 (23.2)	90 (18.7)	1.346 (0.794-2.281)	0.269
Male	115	67		
GG	75 (65.2)	34 (50.7)	Reference	
GA	36 (31.3)	30 (44.8)	0.544 (0.289-1.023)	0.058
AA	4 (3.5)	3 (4.5)	0.604 (0.128-2.850)	0.521
				c 0.823
GA + AA	40 (34.8)	33 (49.3)	0.549 (0.297-1.015)	0.055
GG *vs* GA + AA	75 *vs* 40	34 *vs* 33	1.820 (0.985-3.362)	0.055
GA *vs* GG + AA	36 *vs* 79	30 *vs* 37	0.562 (0.302-1.047)	0.068
G allele	186 (80.9)	98 (73.1)	Reference	
A allele	44 (19.1)	36 (26.9)	0.644 (0.389-1.066)	0.086

c: Corrected; CRC: Colorectal cancer.

0.309÷1.037, *P* = 0.064).

The frequency of the TGF-BR2G[-875]G genotypes was overrepresented among male patients with advanced CRC *vs* healthy men compared to GA + AA genotype (OR = 2.146, 95%CI: 1.041-4.422, *P* = 0.037). On the contrary, the GA genotype in men was protective regarding advanced CRC compared to the GG + AA genotype (OR = 0.477, 95%CI: 0.228-0.997, *P* = 0.047).

Analyses in female patients with early or advanced CRC did not show a significant association of genotypes with the cancer stage.

Serum levels of TGF-β1 in relation to the TGF-BR2 genotypes

As we previously established, the mean serum levels of TGF-β1 were significantly lower in CRC patients than healthy persons above 50 years (24.72 ± 10.77 ng/mL *vs* 34.54 ± 27.06 ng/mL; *P* = 0.005–*t*-test) (23.1 ng/mL IQR 18.2-29.3 *vs* 24.9 ng/mL IQR 13.5-50.9; *P* = 0.436-U-test)[14].

When we further stratified the CRC patients and healthy controls above 50 by sex, we found a significant increase in TGF-β1 in healthy men above 50 years compared to male patients with CRC (51.9 ng/mL IQR 42.4-65.3 *vs* 23.2 ng/mL IQR 18.4-28.1, *P* = 0.000377) and healthy women (51.9 ng/mL IQR 42.4-65.3 *vs* 16.9 ng/mL IQR 10.9-31.1,

Table 2 Genotype distributions and allele frequencies of *TGFBR2* rs3087465 polymorphism in the case group divided into advanced and early colorectal cancer groups (and females and males, respectively)

TGFBR2 -875G/A (rs3087465)	Advanced CRC, *n* (%)	Early CRC, *n* (%)	OR (95%CI)	*P* value
Total *n*	96 (52.2)	88 (47.8)		
GG	64 (66.7)	53 (60.2)	Reference	
GA	29 (30.2)	29 (33)	0.828 (0.441-1.556)	0.557
AA	3 (3.1)	6 (6.8)	0.414 (0.099-1.735)	0.216
				c 0.373
GA + AA	32 (33.3)	35 (39.8)	0.757 (0.415-1.382)	0.365
G allele	157 (81.8)	135 (76.7)	Reference	
A allele	35 (18.2)	41 (23.3)	0.734 (0.442-1.218)	0.230
Female	35 (36.5)	34 (38.6)		
GG	22 (62.9)	20 (58.8)	Reference	
GA	12 (34.3)	10 (29.4)	1.091 (0.388-3.071)	0.869
AA	1 (2.9)	4 (11.8)	0.227 (0.023-2.207)	0.171
				c 0.370
GA + AA	13 (37.1)	14 (41.2)	0.844 (0.321-2.222)	0.731
G allele	56 (80)	50 (73.5)	Reference	
A allele	14 (20)	18 (26.5)	0.694 (0.313-1.539)	0.368
Male	61 (63.5)	54 (61.4)		
GG	42 (68.9)	33 (61.1)	Reference	
GA	17 (27.9)	19 (35.2)	0.703 (0.317-1.561)	0.386
AA	2 (3.3)	2 (3.7)	0.786 (0.105-5.878)	0.814
				c 1.000
GA + AA	19 (31.1)	21 (38.9)	0.711 (0.329÷1.535)	0.384
GG *vs* GA + AA	42 *vs* 19	33 *vs* 21	1.407 (0.651-3.038)	0.384
G allele	101 (82.8)	85 (78.7)	Reference	
A allele	21 (17.2)	23 (21.3)	0.768 (0.398÷1.484)	0.432

c: Corrected; CRC: Colorectal cancer.

P = 0.000482) (Figure 1).

We obtained similar results when we include the healthy controls above 50 years genotyped for TGF-BR2 -875G/A (n = 48) (not shown).

Regarding the genotypes, we found that TGF-β1 serum levels were higher in GG genotype in healthy persons above 50 years than the CRC patients (36.3 ng/mL IQR 19.9-56.5 *vs* 22.4 ng/mL IQR 14.8-29.7, P = 0.0143). On the contrary, TGF-β1 Levels were higher in CRC patients with GA + AA genotype than healthy persons above 50 years who possessed the same genotype. However, this observation did not reach significance (24.04 ng/mL IQR 19.2-28.2 *vs* 16.3 ng/mL IQR 10.05-42.3, P = 0.171) (Figure 2A).

However, TGF-β1 serum levels were comparable in CRC patients with both GG and GA + AA genotypes (22.4 ng/mL IQR 14.8-29.7 *vs* 24.04 ng/mL IQR 19.2-28.2, P = 0.397). TGF-β1 serum levels were enhanced in healthy controls with GG genotypes in comparison with GA + AA genotype (36.3 ng/mL IQR 19.9-56.5, *vs* 16.3 ng/mL IQR 10.05-42.3, P = 0.057).

When subdivided groups by gender, we found a significant difference between higher TGF-β1 serum levels in female CRC patients with GA + AA genotype compared to healthy women above 50 years with the same genotype (27.2 ng/mL IQR 18.5-31.6 *vs* 14.3 ng/mL IQR 8.4-25.9, P = 0.04) (Figure 2B). However, TGF-β1 serum levels were significantly higher in healthy men above 50 years with the GG genotype

Table 3 TGFBR2G[-875]A rs3087465 polymorphism distribution in early CRC cases compared to healthy controls

TGFBR2 -875G/A (rs3087465)	Early CRC, *n* (%)	Healthy controls, *n* (%)	OR (95%CI)	*P* value
Total *n*	88 (47.8)	307 (100)		
GG	53 (60.2)	193 (62.9)	Reference	
GA	29 (33)	102 (33.2)	1.035 (0.620-1.728)	0.894
AA	6 (6.8)	12 (3.9)	1.821 (0.653-5.080)	0.246
GA + AA	35 (39.8)	114	1.118 (0.688-1.817)	0.652
G allele	135 (76.7)	488 (79.5)	Reference	
A allele	41 (23.3)	126 (20.5)	1.176 (0.788-1.756)	0.427
Female	34 (38.6)	240		
GG	20 (58.8)	159 (66.3)	Reference	
GA	10 (29.4)	72 (30)	1.104 (0.492-2.478)	0.810
AA	4 (11.8)	9 (3.7)	3.533 (0.996-12.535)	0.039
				c 0.103
GA + AA	14 (41.2)	81 (33.8)	1.374 (0.660-2.861)	0.394
G allele	50 (73.5)	390 (81.3)	Reference	
A allele	18 (26.5)	90 (18.7)	1.560 (0.869-2.802)	0.134
Male	54 (61.4)	67		
GG	33 (61.1)	34 (50.7)	Reference	
GA	19 (35.2)	30 (44.8)	0.653 (0.309-1.379)	0.262
AA	2 (3.7)	3 (4.5)	0.687 (0.108-4.378)	0.690
				c 1.000
GA + AA	21 (38.9)	33 (49.3)	0.656 (0.317-1.357)	0.254
GG *vs* GA + AA	33 *vs* 21	34 *vs* 33	1.525 (0.737-3.156)	0.254
G allele	85 (78.7)	98 (73.1)	Reference	
A allele	23 (21.3)	36 (26.9)	0.737 (0.405-1.340)	0.316

c: Corrected; CRC: Colorectal cancer.

than the male CRC patients (50.9 ng/mL IQR 42.4-74 *vs* 22.6 ng/mL IQR 15.9-28.4, *P* = 0.00022) (Figure 2C).

When we compared TGF-β1 levels among early and advanced CRC cases, and healthy controls, we did not find differences in the levels (Figure 3).

Putting together all data on TGF-β1 serum levels in regards to early CRC (*n* = 39) and advanced CRC (*n* = 34) and healthy people above 50 years (*n* = 48), and their genotypes, we found significant differences between higher levels of TGF-β1 serum levels in healthy controls above 50 years (GG genotype) and CRC patients (GG genotype) at the early stage (36.3 ng/mL IQR 19.9-56.5 *vs* 22.8 ng/mL IQR 14.6-28.6, *P* = 0.037) and advanced CRC (36.3 ng/mL IQR 19.9-56.5 *vs* 21.6 ng/mL IQR 15.9-33.9, *P* = 0.039) (Figure 4A). Additionally, TGF-β1 serum levels were increased in healthy persons above 50 years with homozygous TGF-BR2G[-875]G genotype compared to GA + AA genotype (36.3 ng/mL IQR 19.9-56.5 *vs* 16.3 ng/mL IQR 10-42.3, *P* = 0.058) (Figure 4A).

Depending on the sex of the patients and healthy controls above 50 years, the highest levels of the cytokine occurred in healthy males above 50 years in comparison with CRC patients at early or advanced stages of the disease (51.9 ng/mL IQR 42.4-65.3 *vs* 23.3 ng/mL IQR 16.5-27.1, *P* = 0.00078; and 51.9 ng/mL IQR 42.4-65.3 *vs* 22.9 ng/mL IQR 18.7-30.5, *P* = 0.00039, respectively) (Figure 4B).

No differences in TGF-β1 serum levels in female CRC patients and healthy persons were observed.

Table 4 TGFBR2G[-875]A rs3087465 polymorphism distribution in advanced CRC cases compared to healthy controls

TGFBR2 -875G/A (rs3087465)	Advanced CRC, *n* (%)	Healthy controls, *n* (%)	OR (95%CI)	*P* value
Total *n*	96 (52.2)	307 (100)		
GG	64 (66.7)	193 (62.9)	Reference	
GA	29 (30.2)	102 (33.2)	0.857 (0.520-1.414)	0.546
AA	3 (3.1)	12 (3.9)	0.754 (0.206-2.756)	0.668
				c 0.940
GA + AA	32 (33.3)	114	0.846 (0.522-1.373)	0.499
GA *vs* GG + AA	29 *vs* 67	102 *vs* 205	0.870 (0.530-1.429)	0.582
G allele	157 (81.8)	488 (79.5)	Reference	
A allele	35 (18.2)	126 (20.5)	0.863 (0.570÷1.308)	0.488
Female	35 (36.5)	240		
GG	22 (62.9)	159 (66.3)	Reference	
GA	12 (34.3)	72 (30)	1.205 (0.565-2.567)	0.629
AA	1 (2.9)	9 (3.7)	0.803 (0.097-6.647)	0.839
				c 1.000
GA + AA	13 (37.1)	81 (33.8)	1.160 (0.556-2.421)	0.693
G allele	56 (80)	390 (81.3)	Reference	
A allele	14 (20)	90 (18.7)	1.083 (0.578-2.032)	0.803
Male	61 (63.5)	67		
GG	42 (68.9)	34 (50.7)	Reference	
GA	17 (27.9)	30 (44.8)	0.459 (0.217-0.969)	0.039
AA	2 (3.3)	3 (4.5)	0.540 (0.085-3.417)	0.507
				c 0.841
GA + AA	19 (31.1)	33 (49.3)	0.466 (0.226-0.961)	0.037
GG *vs* GA + AA	42 *vs* 19	34 *vs* 33	2.146 (1.041-4.422)	0.037
GA *vs* GG + AA	17 *vs* 44	30 *vs* 37	0.477 (0.228-0.997)	0.047
G allele	101 (82.8)	98 (73.1)	Reference	
A allele	21 (17.2)	36 (26.9)	0.566 (0.309-1.037)	0.064

c: Corrected; CRC: Colorectal cancer.

DISCUSSION

CRC is globally the second- to third largest cancer-associated cause of death[16].

Although recent improvements in diagnostics and surgical treatment options were demonstrated, as well as the CRC mortality rate is gradually decreasing in some western countries. Still, the prognosis is unfavorable in metastasized cases[17].

TGF-β signaling may further contribute to these unfavorable outcomes. In line with this, a more profound understanding of the role of TGF-β and its receptors for tumor cells and the CRC tumor environment is crucial. Moreover, this can widen the therapeutic strategies, especially in novel target therapy development[16].

It was shown that TGF-β possesses a double role in the development and progression of the various gastrointestinal tumor, acting as both a tumor suppressor and tumor promoter[3].

However, beyond the recognized role of TGF-β in colorectal tumorigenesis, there is growing evidence that mutations or polymorphisms of TGF-β receptors could also contribute to CRC development[5]. It was also demonstrated that inactivated or absent TGF-BR2 might be a factor causative for CRC transformation[18,19].
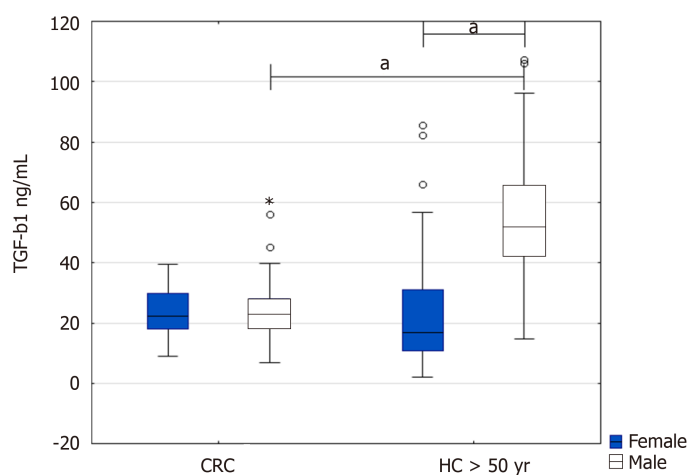
Figure 1 Serum levels of transforming growth factor beta 1 in colorectal cancer patients compared to healthy controls above 50 years.
Results are presented as median and interquartile range. [a]$P < 0.001$, *extremes, ∘outliers.
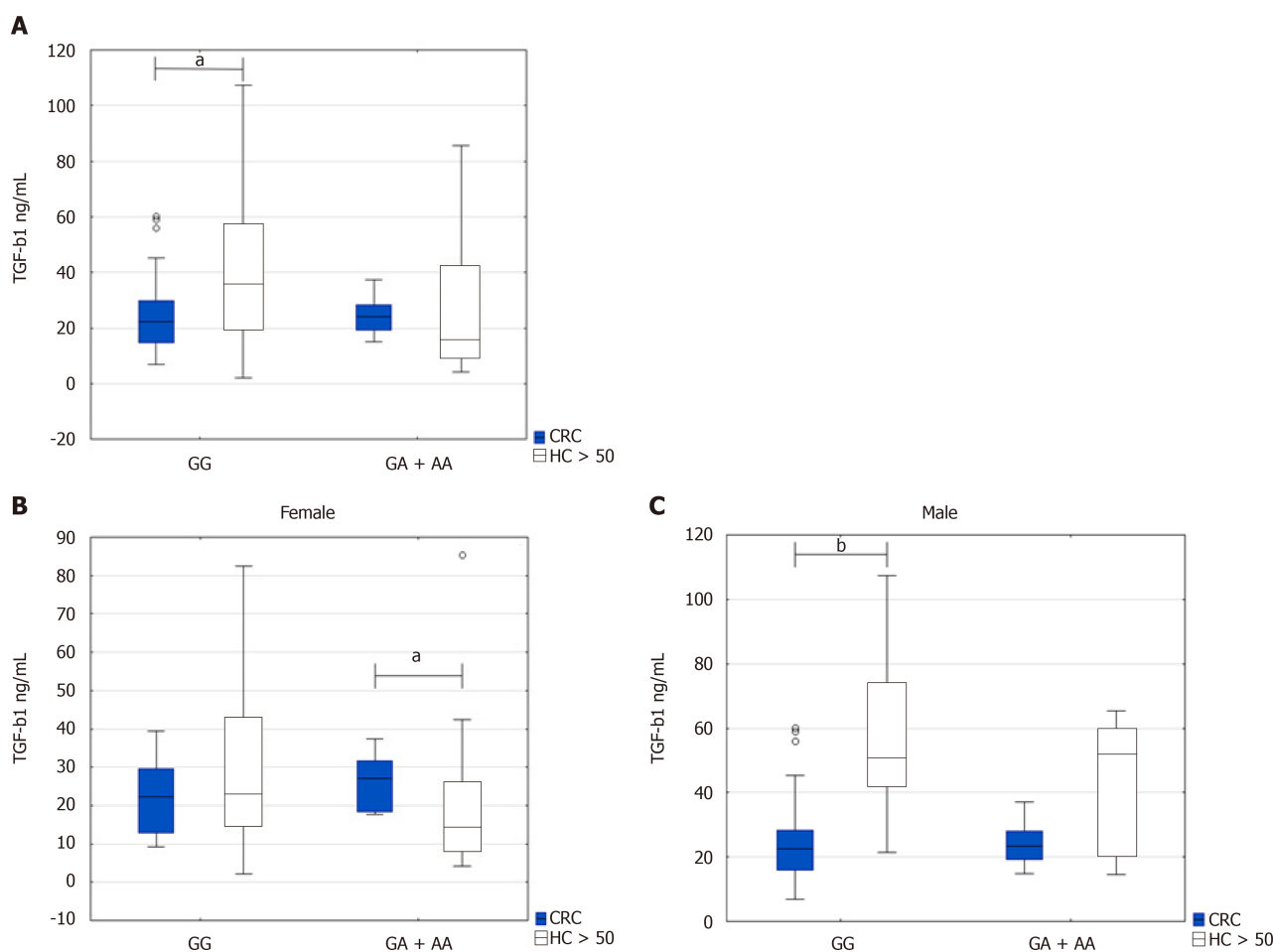
Figure 2 Transforming growth factor beta serum levels in colorectal cancer patients and healthy controls above 50 years. A: Depending on the *TGF-BR2* -875G/A genotype; B: Depending on the female CRC patients and controls; C: Depending on the male CRC patients and controls. Results are presented as median and interquartile range. [a]$P < 0.05$, [b]$P < 0.001$, ∘outliers.

This gene inactivation due to mutation of TGF-BR2 occurs in about 30% of CRC and > 90% in microsatellite unstable CRC[20-22]. Thus, primary attention is paid to accumulated mutations of TGF-BR2 in the context of microsatellite instability in CRC. However, despite frameshift mutations in the *TGF-BR2* gene, the mutated gene still can produce a functional protein[23,24].

Figure 3 Transforming growth factor beta 1 serum levels in early and advanced colorectal cancer and healthy persons above 50 years-genotyped for the *TGF-BR2* -875G/A. Results are presented as median and interquartile range. ∗Extremes, ◦outliers.
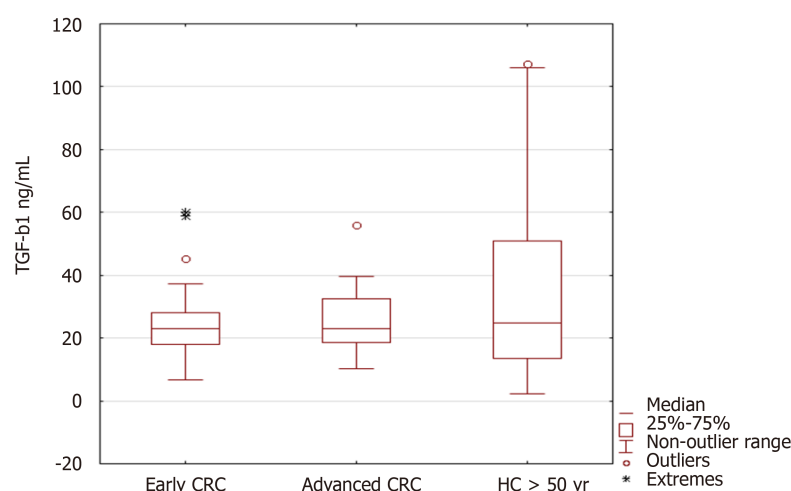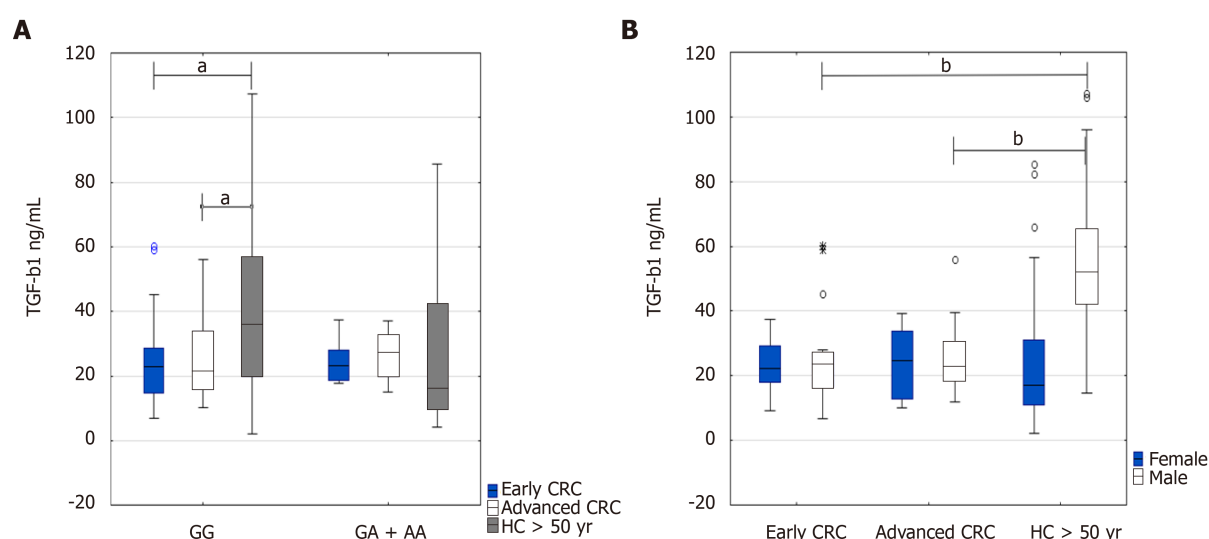

Figure 4 Transforming growth factor beta 1 serum levels in early and advanced colorectal cancer and healthy persons above 50 years. A: Regarding genotype; B: Regarding sex of the patients. Results are presented as median and interquartile range. [a]$P < 0.05$, [b]$P < 0.001$, ∗extremes, ◦outliers.

More research is devoted to a polymorphic allele of the type I receptor. Different polymorphisms of TGF-BR2 donate to CRC development are still unclear[5]. Previously, we found decreased TGF-β1 in serum samples of male CRC patients significantly associated with CC genotype, indicating that -509C/T functional promoter polymorphism (rs1800469) within the TGF-β1 gene (*TGF-B1*) increased the cancer risk, particularly for advanced stages[14]. A noteworthy finding of the study of Xu *et al*[25] was that TGF-B1 -509C/T and TGF-BR2 -875A/G gene polymorphisms acted synergistically on the decreased risk of gastric cancer. The risk of gastric cancer was notably lower among subjects who carried both the TGF-B1 -509C and TGF-BR2 -875A allele genotypes. We also demonstrated that a combination of TGF-B1T[-509]T with the TGF-BR2G[-875]A genotype might be a protective factor against relapsing-remitting multiple sclerosis development in men[15].

Additionally, TGF-BR2 polymorphisms were observed in various conditions and diseases, such as hypospadias[26], thyroid carcinoma[27], cardiovascular diseases[28], *etc*.

In the present case-control study, we evaluated the distribution of the allele and genotype frequencies of TGF-BR2 -875 G/A polymorphism in Bulgarian CRC patients and assessed CRC development risk and the association of serum TGF-β protein expression in a gender-dependent manner.

Our findings from this case-control study suggested that the highest risk for developing colorectal neoplasia was found for the GG genotype. The increased risk for CRC development was associated with male CRC patients homozygous for GG genotype, whereas the lowest risk–with GA genotype. To the best of our knowledge, no other studies for this polymorphism and CRC association from the literature are available.

A study that investigated the TGF-BR2 -875 GG genotype found a significantly decreased risk of gastric cancer development in the Chinese carriers of the A allele (AA/AG genotypes) (OR = 0.58; 95%CI: 0.62-0.91; *P* < 0.001)[25]. Additionally, a combination of the TGF-B1 -509 C and TGF-BR2 -875 A alleles were further related to a decreased risk of gastric cancer development (OR = 0.42; 95%CI: 0.32-0.57, *P* < 0.001). The authors also speculated that these findings elucidated the possible biological mechanisms and the underline tumor heterogeneity.

In our study, TGF-BR2[-875]*A-allele itself was associated with a protective effect regarding CRC development. When we further stratified the healthy controls by age, we found a significant increase in serum TGF-β1 in healthy men above 50 years compared to male patients with CRC, especially those carrying the GG genotype. Still, the highest serum TGF-β levels were observed in healthy men with GA + AA genotype.

After stratifying CRC patients into those with early and advanced, we found that male carriers of the A allele (GA/GA + AA genotypes) had a significantly decreased risk of advanced CRC.

The reasons for these disparities in the distribution of genetic polymorphisms in populations stratified by gender are not fully understood. However, recently developed gene-sequencing technologies have elucidated some of the possible mechanisms. It is well-known that TGF-β acts as a tumor promoter in the advanced stages of CRC carcinogenesis. Higher expression of TGF-β was related to recurrence and decreased survival rate of CRC patients[29,30]. Furthermore, prolonged expression of TGF-β in the intestines stimulates the neoplastic transformation, invasion, and metastasis[31,32].

On the contrary, TGF-β usually inhibits tumor progression in premalignant epithelial cells. However, in TGF-β pathway dysregulation, signal reprogramming occurs that promotes survival and spreading of cancer cells[33,34].

Stanilova *et al*[14] demonstrated for the first time the role of TGF-β in CRC depending on the gender of the patients. Their findings emphasized the significance of TGF-β and its functional polymorphism in CRC development in both male and female patients.

We did not find significant differences in the present study when we compared TGF-β1 serum levels among early and advanced CRC cases and healthy controls. However, when put together all data on TGF-β1 serum levels in regards to early and advanced CRC and healthy people above 50 years, we found significant differences between higher levels of TGF-β1 serum levels in healthy controls above 50 years and CRC patients at the early and advanced CRC, significant differences were calculated only for the GG genotype.

Additionally, TGF-β1 serum levels were also increased in healthy persons above 50 years with homozygous TGF-BR2G[-875]G genotype compared to GA + AA genotype.

Our study enlarges the data with a new entry regarding the effect of the TGF-BR2 gene -875G/A promoter polymorphism on serum acid-activated latent TGF-β1 quantities in serum samples of healthy persons and CRC patients. This study first examined serum TGF-β1 levels associated with the TGF-BR2 -875G/A polymorphism in a large group of Bulgarian healthy control subjects. We generally observed significant differences in serum TGF-β1 quantities depending on age and gender combined with genotype in the healthy control group, where the highest levels of the cytokine occurred in healthy males above 50 years.

One can suggest that decreased TGF-β combined with TGF-BR2 -875GG genotype might be connected with uncontrolled chronic inflammation, including in the gastrointestinal tract. The hypothesis for altered levels of cancer-associated cytokines, *i.e.*, decreased TGF-β1 and IL-10 in peripheral blood, together with cancer-associated reprogramming of gene expression in blood cells, was supported by many investigators, including us[35,36]. Thus, we believe that normal concentrations of circulating TGF-β1 may suppress tumorigenesis by controlling the systemic and local gut inflammation. Furthermore, TGF-β may suppress tumor growth by inhibiting IL-6 trans signalling in CRC[9,36].

Moreover, a CRC cancer-specific overall survival has been shown to correlate with high TGF-β and low TGF-BR1 and TGF-BR2[37].

No differences in TGF-β1 serum levels in female CRC patients and healthy persons were found in our study, but the trend was similar; however, without reaching significance. Additional studies should further explain the observed discrepancies.

CONCLUSION

In conclusion, our results demonstrated that TGF-BR2 -875AG and AA genotypes were associated with a reduced risk of CRC, as well as circulating levels of TGF-β could prevent CRC development in a gender-specific manner. Notably, male carriers of TGF-BR2 -875A allele genotypes had a lower risk of CRC development and progression. Suggesting that TGF-BR2 -875A/G polymorphisms significantly affect protective biological factors, influencing the risk of colon and rectal carcinogenesis.

Although there is growing evidence that TGF-β signaling alterations (both mutations and polymorphisms of TGF-β receptors, *etc.*) contribute to CRC development and progression, there are still many unknown mechanisms. However, our data could help determine the greater risk for CRC development, especially in those patients that possess at least one C allele in their genotype. Ongoing advances in understanding TGF-β's role in the pathogenesis and development of CRC may enable new approaches to CRC prevention and treatment.

ARTICLE HIGHLIGHTS

Research background

The role of transforming growth factor beta (TGF-β) signaling, which includes both the cytokine and its receptors, in the etiology of colorectal cancer (CRC) has been invest-igated recently. TGF-β-associated cancer pathways must be disrupted in the early stages of tumor growth, while TGF-β activation can promote cancer invasion and metastasis.

Research motivation

Given the importance of the TGF-β1 signaling pathway in CRC production and the fact that TGF-β1 exerts its effects through these receptors, we could hypothesize that genetic polymorphisms in the TGF-β1 gene and genes for TGF-β receptors may also play a role in CRC susceptibility. Previously, we recorded that circulating TGF-β1 and the -509C/T functional promoter polymorphism (rs1800469) within the TGF-β1 gene (TGF-B1) plays a gender-dependent role in the resistance, development, and prognosis of CRC in Bulgarian patients. Therefore, we were interested in gender-associated differences in the frequency of TGF-BR2G[-875]A promoter polymorphism and CRC risk.

Research objectives

We performed a case-control gene association research approach to examine the association between TGF-β receptor 2 TGF-BR2G[-875]A promoter polymorphism and CRC risk in a cohort of Bulgarian patients, as well as TGF-β1 protein levels in the peripheral blood. We also estimated the role of this polymorphism at different stages of the disease, defined as early and advanced in men and women.

Research methods

One hundred eighty-four CRC patients and 307 sex and age-matched stable participants were recruited in the study. Primer-introduced restriction analysis-polymerase chain reaction methods were used for genotyping the TGF-BR2G[-875]A (rs3087465) polymorphism.

Research results

The GG genotype was shown to have the greatest chance of developing colorectal neoplasia in this case-control study. Male CRC patients who were homozygous for the GG genotype had an elevated risk of developing CRC. Male carriers of TGF-BR2 -875A allele genotypes, on the other hand, had a lower chance of CRC growth and progression. TGF-β1 serum levels were higher in the GG genotype in people over 50 years old than in CRC patients.

Research conclusions

TGF-BR2 AG and AA genotypes were associated with a lower risk of CRC in our study. Besides, circulating TGF-β levels could inhibit CRC production in a gender-specific manner.

Research perspectives

Since we documented that male carriers of TGF-BR2 -875A allele genotypes had a lower risk of CRC formation and progression, we can imply that the TGF-BR2 -875A/G polymorphism has a direct effect on the protective biological factors that influence the risk of colon and rectal carcinogenesis.

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ORIGINAL ARTICLE

Clinical Trials Study

Induction chemotherapy with albumin-bound paclitaxel plus lobaplatin followed by concurrent radiochemotherapy for locally advanced esophageal cancer

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Institutional review board statement: The study was reviewed and approved by the First Medical Center of the Chinese People's Liberation Army (PLA) General Hospital Institutional Review Board (Approval No. S2016-099-02).

Clinical trial registration statement: This study is registered at Chinese Clinical Trial Registry. The registration identification number is ChiCTR1900025080.

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Abstract

BACKGROUND
Albumin-bound paclitaxel (ABP) has been used as second- and higher-line treatments for advanced esophageal cancer, and its efficacy and safety have been well demonstrated. Lobaplatin (LBP) is a third-generation platinum antitumor agent; compared with the first two generations of platinum agents, it has lower toxicity and has been approved for the treatment of breast cancer, small cell lung cancer, and chronic granulocytic leukemia. However, its role in the treatment of esophageal cancer warrants further investigations.

AIM
To investigate the efficacy and safety of induction chemotherapy with ABP plus LBP followed by concurrent radiochemotherapy (RCT) for locally advanced esophageal cancer.

METHODS
Patients with pathologically confirmed advanced esophageal squamous cell carcinoma (ESCC) at our hospital were enrolled in this study. All patients were treated with two cycles of induction chemotherapy with ABP plus LBP followed by concurrent RCT: ABP 250 mg/m^2, ivgtt, 30 min, d1, every 3 wk; and LBP, 30 mg/m^2, ivgtt, 2 h, d1, every 3 wk. A total of four cycles were scheduled. The dose of the concurrent radiotherapy was 56-60 Gy/28-30 fractions, 1.8-2.0 Gy/fraction, and 5 fractions/wk.

RESULTS
A total of 29 patients were included, and 26 of them completed the treatment protocol. After the induction chemotherapy, the objective response rate (ORR) was 61.54%, the disease control rate (DCR) was 88.46%, and the progressive

disease (PD) rate was 11.54%; after the concurrent RCT, the ORR was 76.92%, the DCR was 88.46%, and the PD rate was 11.54%. The median progression-free survival was 11.1 mo and the median overall survival was 15.83 mo. Cox multivariate analysis revealed that two cycles of induction chemotherapy followed by concurrent RCT significantly reduced the risk of PD compared with two cycles of chemotherapy alone ($P = 0.0024$). Non-hematologic toxicities were tolerable, and the only grade 3 non-hematologic toxicity was radiation-induced esophagitis (13.79%). The main hematologic toxicity was neutropenia, and no grade 4 adverse event occurred.

CONCLUSION
Induction chemotherapy with ABP plus LBP followed by concurrent RCT is effective in patients with locally advanced ESCC, with mild adverse effects. Thus, this protocol is worthy of clinical promotion and application.

Key Words: Esophageal squamous cell carcinoma; Esophagus cancer; Induction chemotherapy; Concurrent radiochemotherapy; Radiotherapy; Chemotherapy; Albumin-bound paclitaxel; Lobaplatin

Core Tip: This study aimed to investigate the efficacy and safety of induction chemotherapy with albumin-bound paclitaxel (ABP) plus lobaplatin followed by concurrent radiochemotherapy (RCT) for locally advanced esophageal cancer. A total of 29 patients were included, and 26 of them completed the treatment protocol. Induction chemotherapy with ABP plus lobaplatin followed by concurrent RCT is effective in patients with locally advanced esophageal squamous cell carcinoma, with mild adverse effects. Thus, this protocol is worthy of clinical promotion and application.

INTRODUCTION

Esophageal cancer is one of the most common forms of cancer worldwide, with an estimated 572000 new cases and 509000 deaths in 2018[1,2]. The most common type of esophageal cancer in China is esophageal squamous cell carcinoma (ESCC), which accounts for 89% of all esophageal cancer cases[3]. The 5-year survival rate of Chinese ESCC patients is 20%-30% overall[4]. The preferred treatment modality for esophageal cancer is surgery, but 80% of patients are no longer eligible for radical surgery upon diagnosis[5,6]. Concurrent chemoradiotherapy has been found to yield better overall survival than radiotherapy[7-9]. While definitive radiochemotherapy (RCT) remains the mainstay of treatment for locally advanced esophageal cancer[10], the treatment modalities have long been controversial. Several clinical trials have explored and evaluated the multidisciplinary treatments for advanced unresectable esophageal cancer, but there is still no standardized treatment protocol. In the COSMOS trial[11], the 1-year survival rate of patients with esophageal cancer treated by surgery after induction chemotherapy with docetaxel plus cisplatin and 5-fluorouracil (DCF) regimen was 67.9%, which confirmed the efficacy of induction chemotherapy followed by definitive RCT in treating esophageal cancer. Another multicenter randomized controlled trial (JCOG1510)[12] further confirmed that induction chemotherapy + surgery or induction chemotherapy + concurrent definitive RCT was superior to concurrent definitive RCT in terms of overall survival (OS) in patients with locally advanced unresectable ESCC. Paclitaxel combined with carboplatin is one of the standard chemotherapy regimens recommended in guidelines, but there is limited

evidence for other taxanes and platinum agents. Nanoparticle albumin-bound (nab)-paclitaxel has been shown to provide significant efficacy and safety benefits over paclitaxel and is currently approved for the treatment of breast cancer, lung cancer, pancreatic cancer, and melanoma. In recent years, albumin-bound paclitaxel (ABP) has been used as second- and higher-line treatments for advanced esophageal cancer, and its efficacy and safety have been well demonstrated. Lobaplatin (LBP) is a third-generation platinum antitumor agent; compared with the first two generations of platinum agents, it has lower toxicity and has been approved for the treatment of breast cancer, small cell lung cancer, and chronic granulocytic leukemia. However, its role in the treatment of esophageal cancer warrants further investigations.

Thus, we conducted the present prospective study to investigate the efficacy and safety of induction chemotherapy with ABP plus LBP followed by concurrent RCT in the treatment of locally advanced esophageal cancer.

MATERIALS AND METHODS

Subjects

ESCC patients attending our hospital were included according to the following inclusion criteria: (1) Patients voluntarily participated in this study, had good compliance, could cooperate with the trial requirements during the observation and follow-up periods, and signed an informed consent form; (2) Patients with histopathologically confirmed stage III or IVA unresectable advanced ESCC, which had not been treated with anti-tumor drugs other than the study drug within the past 4 wk; and the patient could receive the specialized anti-tumor treatment; (3) Patients with at least one measurable lesion [≥ 1 cm on computed tomography (CT) or ≥ 2 cm on other imaging modes]; (4) Patients with an Eastern Cooperative Oncology Group score of ≤ 2 and having indications for chemotherapy; (5) Patients who lost ≤ 10% of the body weight in the last 6 mo and could tolerate radiotherapy; (6) Males or females aged 18-76 years; (7) Patients with the following laboratory-confirmed bone marrow, liver, kidney, and heart functions within 7 d before the first dose: White blood cell count ≥ 3000/µL, absolute neutrophil count ≥ 1500/µL, platelet count ≥ 100000/µL, and hemoglobin ≥ 9.0 g/dL; aspartate aminotransferase and alanine aminotransferase ≤ 2.5 times upper limit of normal (ULN), and alkaline phosphatase ≤ 4 times ULN, and total bilirubin ≤ 1.5 times ULN; serum creatinine ≤ 1.5 times ULN and blood urea nitrogen ≤ 2.5 times ULN; prothrombin time and/or international normalized ratio or partial thromboplastin time ≤ 1.5 times ULN; and left ventricular ejection fraction ≥ 60% as assessed by Doppler ultrasound, and electrocardiographic findings were basically normal; and (8) Females must use contraception during the treatment and within 6 mo upon the completion of the treatment, and they should not be pregnant or lactating; and males must take birth control measures during the treatment and within 6 mo upon the completion of the treatment.

The exclusion criteria were: (1) Patients with severe acute infection, purulent/chronic infection, or protracted wound healing; (2) Patients with esophageal perforation (*e.g.*, with existing or possible tracheoesophageal fistula), which had shown obvious symptoms and multiple distant metastases; (3) Patients who had abnormal coagulation and/or bleeding tendency (*e.g.*, active peptic ulcers) or were receiving a thrombolytic or anticoagulant therapy; (4) Patients with pre-existing severe cardiac disease, including: Congestive heart failure, uncontrollable high-risk arrhythmia, unstable angina pectoris, myocardial infarction within 6 mo, severe heart valve disease, and resistant hypertension; (5) Patients with uncontrollable neurological or psychiatric diseases or mental disorders; the patients had poor compliance and were unable to follow the treatment protocol or describe their treatment responses; and (6) Patients with severe cirrhosis and/or severe renal insufficiency.

Treatments

All patients were treated with two cycles of induction chemotherapy with ABP (Keaili, produced by CSPC Ouyi Pharmaceutical Co., Ltd) combined with LBP: ABP 250 mg/m², ivgtt, 30 min, d1, every 3 wk; and LBP, 30 mg/m², ivgtt, 2 h, d1, every 3 wk. Concurrent RCT was given after the induction chemotherapy. Intensity-modulated radiation therapy (56-60 Gy/28-30 fractions, 1.8-2.0 Gy/fraction, 5 fractions/wk) was applied as the radiotherapy. Two cycles of chemotherapy was applied on days 1 and 21 of radiotherapy, and the chemotherapy regimen was the same as that in the induction chemotherapy.

Evaluation of efficacy and adverse events

Chest CT, magnetic resonance imaging, and positron emission tomography-CT (if necessary) were performed after induction chemotherapy, 1 mo after concurrent RCT, and at each follow-up visit. The efficacy was evaluated using the benchmarks of Response Evaluation Criteria in Solid Tumors version 1.1, which included complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD); the objective remission rate (ORR) was calculated using the following formula: ORR = (CR + PR)/total cases × 100%.

The adverse events were graded according to the US NCI Common Terminology Criteria for Adverse Events version 3.0. Adverse events occurring during the study period were recorded continuously.

After the completion of treatment, all patients were followed by telephone or outpatient visits.

Primary and secondary outcome measures

The primary outcome measure was progression-free survival (PFS). The secondary outcome measures included OS, ORR after induction chemotherapy, ORR after concurrent RCT, correlations with prognostic factors, and adverse events.

Statistical analysis

Examination indicators at baseline were described. Count data are presented with the number and percentage of cases; for measurement data, the mean, standard deviation, median, and maximum and minimum values were calculated. For the efficacy indicators, ORR is presented using the number and percentage of cases and PFS and OS are described using Kaplan-Meier curves; the median and 95% confidence interval (CI) were calculated, and factors affecting disease progression were explored using COX multivariate regression, in which hazard ratio (HR) and 95%CI are listed. The types and severity of adverse events during the trial were described, and the incidence of adverse events was calculated (presented as number and percentage of cases). All statistical analyses were performed using SPSS 19.0 software package.

RESULTS

Treatment completion

A total of 29 patients with locally advanced ESCC were included in this study between April 2019 and October 2020, and the baseline data of these patients are shown in Table 1. Three patients withdrew from the study because of surgery (n = 1), PD after enrollment without treatment (n = 1), and loss to follow-up (n = 1). All patients (100%) completed the induction chemotherapy. The completion rate of the entire study protocol (2 cycles of induction chemotherapy + 2 cycles of concurrent RCT) was 65.38% (n = 17). Five patients (19.23%) underwent two cycles of induction chemotherapy + one cycle of concurrent RCT, and the reasons for not completing the entire protocol were grade III radiation-induced esophagitis in three cases and grade III myelosuppression in two. Four (13.79%) patients underwent two cycles of induction chemotherapy only, among whom three experienced disease progression and one refused to receive concurrent radiotherapy.

Short-term efficacy

After two cycles of induction chemotherapy, ORR was 61.54% (n = 16); the disease control rate (DCR) was 88.46% (n = 23), among which PR was achieved in 16 (61.54%) cases and SD in 7 (26.92%); PD was noted in three (11.54%) cases, including *in situ* progression (n = 1), mediastinal lymph node progression (n = 1), and esophagotracheal fistula (n = 1). Among patients who completed induction chemotherapy + concurrent RCT, the evaluated ORR was 76.92% (n = 20) and the DCR was 88.46% (n = 23), among which PR was achieved in 20 (76.92%) cases and SD in 3 (11.54%); PD was noted in three (11.54%) cases (Table 2).

Follow-up and long-term outcomes

By October 2020, 29 patients had been followed for a median of 15.28 (5-28) mo. Disease progression occurred in 14 (48.28%) patients, including four (17.24%) cases of *in situ* progression, two (6.90%) cases of lymph node metastasis, and seven (24.14%) cases of distant metastasis [including two (6.90%) cases of liver metastasis, one (3.45%) case of brain metastasis, three (10.34%) cases of lung metastasis, and one (3.45%) case

Table 1 Baseline characteristics of patients

Feature	n (%)
Gender	
Male	29 (100)
Female	0 (0)
Age [yr; median (range)]	62 (56, 65)
Clinical stage	
III	15 (51.7)
IV	14 (48.3)
Tumor location	
Upper thoracic esophagus	6 (20.7)
Upper part of middle thoracic esophagus	3 (10.3)
Middle thoracic esophagus	6 (20.7)
Lower part of middle thoracic esophagus	8 (27.6)
Lower thoracic esophagus	6 (20.7)

Table 2 Short-term efficacy, *n* (%)

	After induction chemotherapy	After induction chemotherapy + concurrent radiochemotherapy
ORR	16 (61.54)	20 (76.92)
PR	16 (61.54)	20 (76.92)
SD	7 (26.92)	3 (11.54)
PD	3 (11.54)	3 (11.54)

ORR: Objective response rate; PR: Partial response; SD: Stable disease; PD: Progressive disease.

of spleen and kidney metastases]. Thirteen patients died. The median PFS was 11.1 mo, the median OS was 15.83 mo, and the 1-year OS was 42% (Figure 1).

Adverse events and safety

The incidence of post-treatment hematologic toxicities is as follows. After the treatment, the incidence of grade 3 anemia, leukopenia, neutropenia, and thrombocytopenia was 0%, 10.35%, 6.9%, and 0%, respectively. The incidence of non-hematologic toxicities including decreased appetite, fatigue, radiation-induced esophagitis, decreased body weight, and abnormal liver function was 13.79%, 13.79%, 34.48%, 3.45%, and 3.45%, respectively. These non-hematologic toxicities were generally grade 1 or 2; the only grade 3 non-hematologic toxicity was radiation-induced esophagitis, and no grade 4 toxicity was noted (Table 3).

Results of multivariate Cox analysis

The number of chemotherapy cycles was a statistically significant factor affecting the prognosis ($P = 0.0024$). Patients with esophageal cancer who received three (HR = 0.0555; 95%CI: 0.0066-0.4668) or four cycles of chemotherapy (HR = 0.0043; 95%CI: 0.0002-0.0992) had a significantly lower risk of disease progression compared to those who received only two cycles of induction chemotherapy.

The prognostic impact of the overall nutritional score was not statistically significant ($P = 0.0826$); however, compared to patients with an initial score of 5, patients with an initial score of 1 (HR = 0.0037; 95%CI: 0.0001-0.2715), 3 (HR = 0.0077; 95%CI: 0.0001-0.6176), and 4 (HR = 0.0131; 95%CI: 0.0002-0.7049) had better prognoses (Figure 2).

Table 3 Adverse events (*n* = 29)

	Grade 1 or 2 (%)	Grade 3 (%)
Hematologic toxicities		
Anemia	3.85	0
Leukopenia	69.23	10.35
Neutropenia	61.54	6.9
Thrombocytopenia	15.4	0
Non-hematologic toxicities		
Decreased appetite	13.79	0
Fatigue	13.79	0
Radiation-induced esophagitis	34.48	13.79
Decreased body weight	3.45	0
Abnormal liver function	3.45	0

DISCUSSION

The efficacy of induction chemotherapy followed by definitive RCT in treating esophageal cancer has been proved in recent years. In the COSMOS trial[11], the 1-year survival rate of patients with esophageal cancer treated by surgery after induction chemotherapy with DCF regimen reached 67.9%. Another multicenter randomized controlled trial (JCOG1510)[12] further confirmed that induction chemotherapy + surgery or induction chemotherapy + concurrent definitive RCT was superior to concurrent definitive RCT in terms of OS in patients with locally advanced un-resectable ESCC. In the current study, the ORR after two cycles of induction chemotherapy with ABP combined with LBP was 61.54%, with a DCR of 88.46%; 100% of our subjects completed the induction therapy without severe hematologic toxicities or chemotherapy-induced nausea and vomiting. Twenty-two patients further received concurrent RCT; 65.38% of them completed induction chemotherapy plus concurrent RCT, while 19.23% received one cycle of concurrent RCT due to radiation-induced esophagitis or myelosuppression. The ORR was 76.92% and disease control rate was 88.46% after the completion of induction chemotherapy followed by concurrent RCT, which were comparable to those of previous studies; the median PFS was 11.1 mo and the median OS was 15.83 mo; notably, the 1-year PFS rate was 49.45% and the 1-year OS rate was 64.96%. Cox multivariate analysis of the efficacy and prognosis concluded that two cycles of induction chemotherapy followed by concurrent RCT significantly reduced the risk of disease progression compared with two cycles of chemotherapy only (P = 0.0024), suggesting that induction chemotherapy combined with definitive RCT is an efficacious and well-tolerated treatment modality in patients with esophageal cancer. With fewer and milder toxicities, it enhances chemotherapy tolerability and prolongs survival.

Wang *et al*[13] demonstrated that weekly nab-paclitaxel plus cisplatin with concurrent definitive radiotherapy is an effective and well-tolerated treatment option for ESCC. In addition, Wang *et al*[14] compared the values of nanoparticle albumin-bound paclitaxel plus cisplatin (nab-TP) *vs* solvent-based paclitaxel plus cisplatin (sb-TP) and found that nab-TP demonstrated a higher ORR (50% *vs* 30%, P = 0.082) and disease control rate (81% *vs* 65%, P = 0.124) than sb-TP, as well as a longer median PFS (6.1 mo, 95%CI: 5.3-6.9) (P = 0.029). In contrast, LBP used in our current study is a new-generation platinum drug. Many studies have shown that LBP has therapeutic efficacy in the treatment of advanced esophageal cancer, with tolerable side effects[15-17]. Among patients who received induction therapy plus concurrent RCT, the incidence of grade 3 leukopenia and neutropenia was 10.35% and 6.9%, respectively. The non-hematologic toxicities including decreased appetite, fatigue, weight loss, and abnormal liver function were generally grade 1 or 2; the only grade 3 non-hematologic toxicity was radiation-induced esophagitis, and no grade 4 toxicity was noted. Thus, ABP combined with LBP has acceptable adverse effects and can achieve good long-term survival in patients with locally advanced ESCC when used either as an induction chemotherapy regimen or as a concurrent chemotherapy regimen.
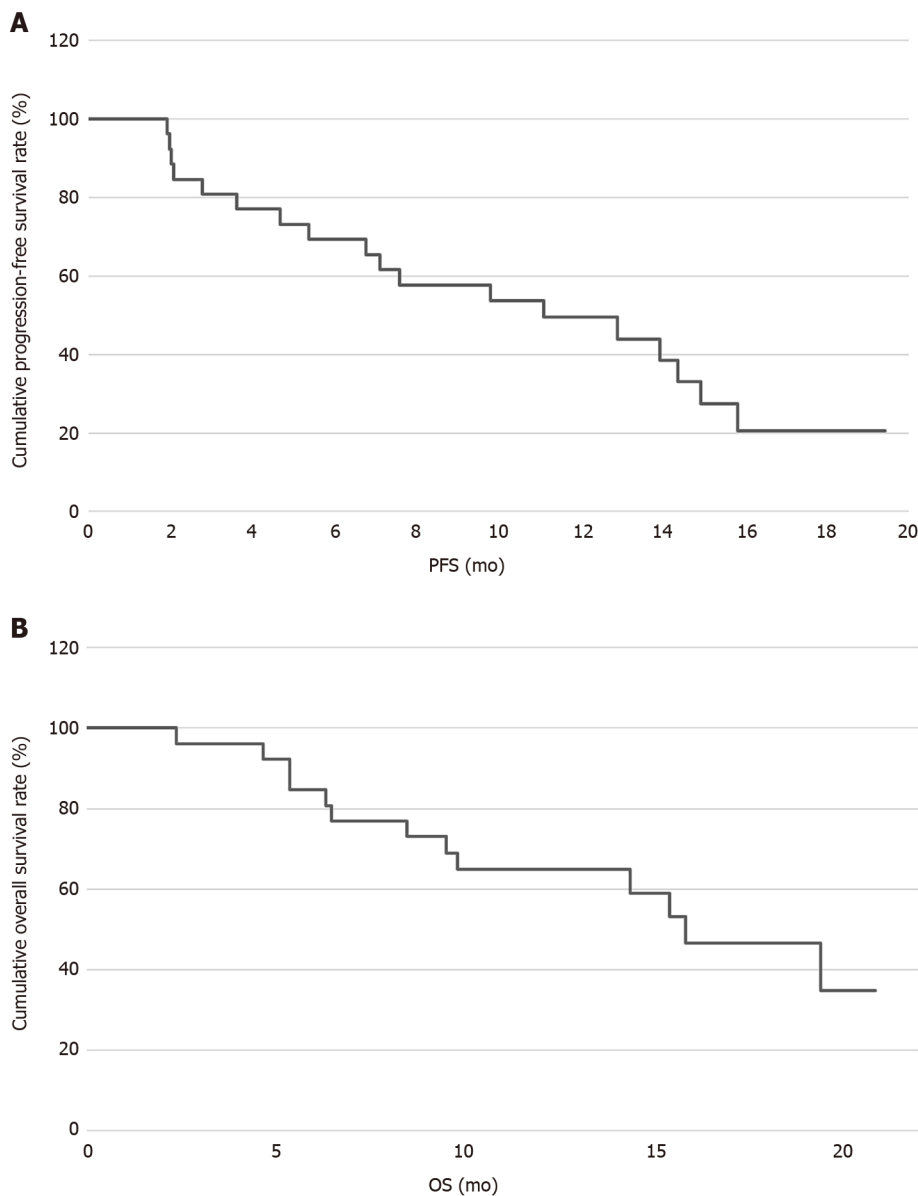
Figure 1 Cumulative progression-free survival and overall survival. A: 1-year progression-free survival; B: 1-year overall survival. PFS: Progression-free survival; OS: Overall survival.

However, although RCT is the main treatment modality for patients with stage II-III esophageal cancer who refuse surgery or has a contraindication for surgery and in patients with locally advanced unresectable (stage IVa) esophageal cancer[18,19], different patterns of recurrence and metastasis still occur. Sudo *et al*[20] reported the types of recurrence after definitive chemoradiotherapy: The incidence of luminal relapse, regional relapse, distant metastasis, new cancer diagnosed by esophago-gastroduodenoscopy (NC-E), and new cancer other than NC-E (NC-O) was 14%, 6%, 19%, 17%, and 8%, respectively. In the present study, disease progression occurred in 14 (48.28%) patients, including five (17.24%) cases of *in situ* progression, two (6.90%) cases of lymph node metastasis, and eight (27.59%) cases of distant metastasis [including two (6.90%) cases of liver metastasis, one (3.45%) case of brain metastasis, three (10.34%) cases of lung metastasis, and one (3.45%) case of spleen and kidney metastases]. The results of this study also suggested that nutritional scores before and during treatment had an impact on prognosis, which needs to be further verified in studies with larger sample sizes.

CONCLUSION

In conclusion, induction chemotherapy with ABP plus LBP followed by concurrent

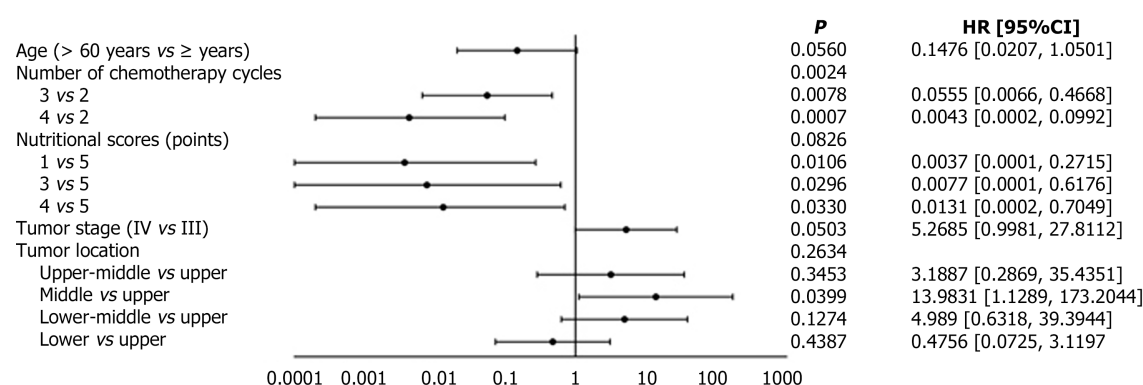|	P	HR [95%CI]
Age (> 60 years *vs* ≥ years)	0.0560	0.1476 [0.0207, 1.0501]
Number of chemotherapy cycles	0.0024	
3 *vs* 2	0.0078	0.0555 [0.0066, 0.4668]
4 *vs* 2	0.0007	0.0043 [0.0002, 0.0992]
Nutritional scores (points)	0.0826	
1 *vs* 5	0.0106	0.0037 [0.0001, 0.2715]
3 *vs* 5	0.0296	0.0077 [0.0001, 0.6176]
4 *vs* 5	0.0330	0.0131 [0.0002, 0.7049]
Tumor stage (IV *vs* III)	0.0503	5.2685 [0.9981, 27.8112]
Tumor location	0.2634	
Upper-middle *vs* upper	0.3453	3.1887 [0.2869, 35.4351]
Middle *vs* upper	0.0399	13.9831 [1.1289, 173.2044]
Lower-middle *vs* upper	0.1274	4.989 [0.6318, 39.3944]
Lower *vs* upper	0.4387	0.4756 [0.0725, 3.1197

Figure 2 Multivariate Cox analysis.

RCT is effective in patients with locally advanced ESCC, with mild adverse effects. Thus, this protocol is worthy of clinical promotion and application. However, as an interim report, this study was limited by its short follow-up intervals, and patients' survival and tumor recurrence/metastasis need to be further investigated. In addition, the induction chemotherapy regimen as well as the optimal dose and fractionation schedule of the definitive RCT for ESCC deserves further clinical research.

ARTICLE HIGHLIGHTS

Research background

The most common type of esophageal cancer in China is esophageal squamous cell carcinoma (ESCC), which accounts for 89% of all esophageal cancer cases. The 5-year survival rate of Chinese ESCC patients is 20%-30% overall. The preferred treatment modality for esophageal cancer is surgery, but 80% of patients are no longer eligible for radical surgery upon diagnosis.

Research motivation

We conducted the present prospective study to investigate the efficacy and safety of induction chemotherapy with albumin-bound paclitaxel (ABP) plus lobaplatin (LBP) followed by concurrent radiochemotherapy (RCT) in the treatment of locally advanced esophageal cancer.

Research objectives

This study aimed to investigate the efficacy and safety of induction chemotherapy with ABP plus LBP followed by concurrent RCT for locally advanced esophageal cancer.

Research methods

Patients with pathologically confirmed advanced ESCC were enrolled in this study. All patients were treated with two cycles of induction chemotherapy with ABP plus LBP followed by concurrent RCT. A total of four cycles were scheduled.

Research results

Cox multivariate analysis revealed that two cycles of induction chemotherapy followed by concurrent RCT significantly reduced the risk of progressive disease compared with two cycles of chemotherapy alone. Non-hematologic toxicities were tolerable, and the only grade 3 non-hematologic toxicity was radiation-induced esophagitis. The main hematologic toxicity was neutropenia, and no grade 4 adverse event occurred.

Research conclusions

Induction chemotherapy with ABP plus LBP followed by concurrent RCT is effective in patients with locally advanced ESCC, with mild adverse effects. Thus, this protocol is worthy of clinical promotion and application.

Research perspectives

As an interim report, this study was limited by its short follow-up intervals, and patients' survival and tumor recurrence/metastasis need to be further investigated.

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SYSTEMATIC REVIEWS

Colorectal cancer in Arab world: A systematic review

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Abstract

BACKGROUND

The incidence of colorectal cancer (CRC) is increasing among young individuals in the Arab world as well as in other regions of the world.

AIM

To explore the incidence and prevalence of CRC in the Arab world.

METHODS

The PubMed, Scopus, Web of Science, EBSCO and Wiley databases were searched to retrieve relevant articles irrespective of the language or the publication year. The search terms were "("colon OR rectum OR sigmoid OR rectal OR colonic OR colorectal") AND ("cancer OR malignancy OR malignant OR neoplasm") AND ("Jordan" OR "United Arab Emirates" OR "Bahrain" OR "Tunisia" OR "Algeria" OR "Djibouti" OR "Saudi Arabia" OR "Sudan" OR "Syria" OR "Somalia" OR "Iraq" OR "Oman" OR "Palestine" OR "Qatar" OR "Comoros" OR "Kuwait" OR "Lebanon" OR "Libya" OR "Egypt" OR "Morocco" OR "Mauritania" OR "Yemen"). Reviews, meta-analyses, and articles containing nonoriginal data were excluded. Retrieved articles were screened, and relevant data were extracted. Descriptive statistics were used for data analysis.

RESULTS

Nine studies were included. Five of the studies provided information regarding the prevalence of CRC. The prevalence of CRC was 0.72% in Saudi Arabia and 0.78% in the United Arab Emirate, while in Egypt, it ranged from 0.4% to 14%. Four studies showed information regarding the incidence. The annual incidence rate of CRC in Qatar was 7.5/100000/year. In Egypt, the crude incidence rate (CIR) in males was 3.1 for colon cancer and 1 for rectal cancer, while in females, it was 2.3 for colon cancer and 0.8 for rectal cancer. The age-standardized rate for CRC incidence in 2003 was 36.90 for males, 26.50 for females, and 30.49 for both sexes in Saudi Arabia. In 2016, the CIRs in Saudi Arabia were 3.6 and 2.1 in females for colon cancer and rectal cancer, respectively, while in males, it was 3.3 and 2.8 for colon cancer and rectal cancer, respectively. One study in Egypt revealed that 25% of CRC cases occurred among individuals younger than 40 years old.

CONCLUSION

There is a considerable prevalence of CRC in some Arab countries. More studies are needed to explore the incidence and prevalence of CRC in the rest of the Arab world.

Key Words: Colorectal cancer; Incidence; Prevalence; Arab world

Core Tip: Colorectal cancer (CRC) represents the third most common cause of cancer globally. Although only a few studies have addressed the prevalence and incidence of CRC in the Arab world, this systematic review found that there is a considerable prevalence of CRC in Egypt, Saudi Arabia, Qatar and the United Arab Emirate. More studies are needed to explore the incidence and prevalence of CRC in the rest of the Arab world.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer (10.0%), and it is the second leading cause of cancer deaths worldwide (9.4%)[1].

In the past decade, an increase in the incidence of CRC has been observed worldwide. Additionally, there is increase in the prevalence of CRC in the younger population, and new cases are expected to increase among the younger population aged 20–49 years by 2030[2,3].

In particular, the prevalence of CRC is increasing among young individuals in the Middle East and other regions in the world[4,5]. These changes in the incidence and epidemiology of the disease presentation have also been observed in the Arab world [2]. The influence of Western lifestyles on the Arab population has led to an increase in the prevalence of CRC and affected younger populations[2].

To our knowledge, there has been no systematic review on CRC prevalence and/or incidence in the Arab World.

The primary aim of this review was to explore the prevalence and/or incidence of CRC in the Arab world by reviewing the available literature studies from Arab countries.

MATERIALS AND METHODS

Literature search

The PubMed, Scopus, Web of Science, EBSCO and Wiley databases were searched using the following search terms: "("colon OR rectum OR sigmoid OR rectal OR colonic OR colorectal") AND ("cancer OR malignancy OR malignant OR neoplasm") AND ("Jordan" OR "United Arab Emirates" OR "Bahrain" OR "Tunisia" OR "Algeria" OR "Djibouti" OR "Saudi Arabia" OR "Sudan" OR "Syria" OR "Somalia" OR "Iraq" OR "Oman" OR "Palestine" OR "Qatar" OR "Comoros" OR "Kuwait" OR "Lebanon" OR "Libya" OR "Egypt" OR "Morocco" OR "Mauritania" OR "Yemen"), to retrieve relevant articles irrespective of the language or the publication year of the articles. For non-English articles, all relevant data were taken from the English abstract, and two reviewers translated the full text to English to retrieve all other data of interest. Reviews, meta-analyses, and all other articles containing nonoriginal data were excluded from our review. All retrieved articles were screened and selected by three independent authors. Relevant data were extracted into a standardized data collection sheet by four independent authors. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow chart is shown in Figure 1. This systematic review was registered in the PROSPERO registry (CRD42021226703).

Statistical analysis

Descriptive statistics were used for data analysis.

RESULTS

At the time of this review, a total of nine studies containing information about the prevalence and/or incidence of CRC in the Arab world were included[6-14] (Table 1). Five studies provided information regarding the prevalence of CRC in Arab Worlds [one from Saudi Arabia[6], 3 from Egypt[9,11,12], and one from the United Arab Emirate (UAE)]13.

The prevalence of CRC was 0.72% in Saudi Arabia[6] and 0.78% in the UAE [13, while Egypt reported different prevalence rates of 0.4%[11], 9.4%[12] and 14%[9].

Among these studies, four showed information regarding the incidence of CRC in the Arab world[7,8,10,14]. In their retrospective analysis of Qatar's area, Rasul *et al*[7] reported an average annual incidence rate of 7.5/100000/year. A retrospective study in Egypt (from 2008 to 2011) revealed that the crude rate in males was 3.1 for colon cancer and 1 for rectal cancer, while in females, it was 2.3 for colon cancer and 0.8 for rectal cancer[10].

The age-standardized rate for CRC incidence in 2003 was 36.90 for males, 26.50 for females, and 30.49 for both sexes in Saudi Arabia, as reported by Ibrahim *et al*[8]. However, another retrospective analysis of Saudi Arabia Ministry of Health Registry data including 13013 participants from general population was conducted in 2016; the crude incidence rates (CIRs) for colon and rectal cancer among females were 3.6 and

Table 1 Summary of included studies

Ref.	Year	Country	Study type	Number of participants	Population	Age	Male	Female	Prevalence	Incidence	Diagnostic test	Period of assessment	Affected colon segment
Salih *et al*[6]	2014	Saudi Arabia	Retrospective case–control study	1600	General	49 (32-62)	No data		0.72% (12/1600)	No	Colonoscopy and biopsy	No data	No data
Rasul *et al*[7]	2001	Qatar	Retrospective analysis	45	CRC patients attended to Hamad General Hospital	Mean 57.1, Range 33-83	26	19	No data	24pts/year. Average annual incidence 7.5/100000/year	Biopsy	1994 to 1998	Descending 55.5% and rectum 24%
Ibrahim *et al*[8]	2008	Saudi Arabia	Retrospective	No data	No data	No data	No data	No data	No data	Age-standardized rate for incidence in 2003 is 36.90 for males, 26.50 for females, and 30.49 for both sexes	No data	1994 to 2003	No data
Gado *et al*[9]	2014	Egypt	Descriptive cross-sectional hospital-based study.	412	Colonoscopies for symptomatized patients	Mean 51. Range 16–80	No data	56% of patient	57 (14%) Peak frequencies were in the 5th and 7th. Decade, 25% of cancers occurred in patients aged less than 40 yr	No data	Colonoscopy and biopsy	2000-2012	(53%) in the left colon (sigmoid colon, descending colon and splenic flexure) and (16%) in the rectum, (32%) in the proximal colon (cecum, ascending colon, hepatic flexure and transverse colon, Synchronous tumors in (2%)
Ibrahim *et al*[10]	2014	Egypt	Retrospective	No data	Colonoscopies	No data	No data	No data	No data	Crude rate in males is: 3.1 for colon and 1 for Rectal cancer. While in females: 2.3 for colon cancer and 0.8 for rectal cancer	No data	2008 to 2011	No data
Elwassief *et al*[11]	2015	Egypt	Questionnaire	547	Relatives of CRC patients	49 ± 9	335	212	2 (0.4%)	No data	Colonscopy and biopsy	No data	Distal
Gado *et al*[12]	2016	Egypt	Retrospective	286	Colonoscopies, 96.5% of cases had symptoms	25.1 ± 22	153	133	27 (9.4%)	No data	Colonscopy and biopsy	2010-2014	No data
Fayadh *et al*[13]	2019	United Arab Emerate (UAE)	8 yr observational study	7540	Colonoscopies	Average age (53), 46% of cancers below age 50 and 14% below the age of 40 years	No data	No data	69 (0.78%)	No data	Colonoscopy	2012-2019	No data
Almatroudi	2020	Saudi	Retrospective	13013	General	No data	7116	5897	No data	In 2016 CIR in females	No data	2006 to 2016	Rectum, colon

| [14] | Arabia | analysis of Saudi MOH registry data | | (4157 colon cancer and 2959 rectal cancer) | for colon cancer is 3.6 and for rectal cancer is 2.1 while in males is 3.3 for colon cancer and 2.8 for rectal cancer |

MOH: Ministry of health; CIR: Crude incidence rate.

2.1, respectively, and the CIRs for colon and rectal cancer among males were 3.3 and 2.8, respectively[14].

Regarding the age of CRC patients, there was predominance in the fourth or fifth decade of life[6,7,11,13]. However, Gado *et al*[9] in Egypt reported two peak frequencies in the fifth and seventh decades; 25% of CRC occurred in patients aged less than 40 years.

DISCUSSION

The updated CRC burden according to the latest GLOBOCAN 2020 estimates demonstrated that CRC ranks third among frequently newly diagnosed cancers, with almost 1.9 million new cases (10.0%), and second leading cause of death worldwide, with approximately 935000 deaths in 2020 (9.4%)[1]. The incidence rates are 4-fold higher in countries with developed economies, mainly in European regions, Australia/New Zealand, and Northern America. Furthermore, the overall CRC trends are increasing for incidence and decreasing for mortality almost all over European countries, with some national and regional variability attributed to differing levels of healthcare expenditure and the resulting quality of screening, diagnosis, and treatment [15,16]. Despite the rising trends of CRC, there is a paucity of data reporting the incidence and/or prevalence of CRC in Arabian countries. The retrieved 9 studies were mostly retrospective data analyses, with only four studies providing information regarding the incidence of CRC in the Arab world[7,8,10,14].

CRC incidence has always been known as an indicator of higher levels of socioeconomic development and is dominant in countries undergoing major economic transition. This is well demonstrated in higher incidence in Europe, Australia and Northern America[17-19]. Additionally, Almatroudi[14], in his large epidemiological study of CRC in Saudi Arabia, showed that there was a markedly increasing incidence of CRC from 2006 to 2016. He attributed that increase to the large-scale screening program that increased the case detection rate and the change toward more unhealthy lifestyles with higher incidence in large cities, such as the regions of Riyadh, Makkah, and Eastern Province, where westernized lifestyles and flourishing industries are more evident. A hospital-based case-control study in Kuwait concluded that CRC risk is
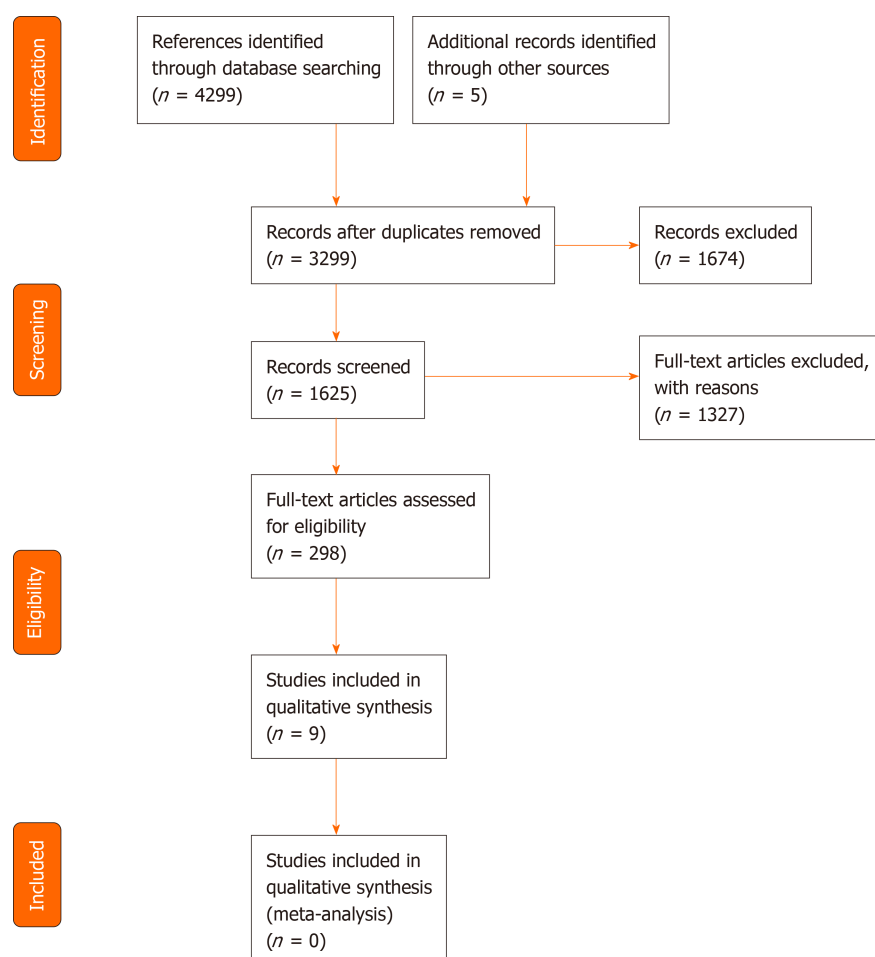
Figure 1 PRISMA 2009 flow diagram.

strongly attributed to higher body mass index, excessive red and processed meat consumption and decreased fruit/vegetable consumption[20].

The rising trend of CRC despite the screening programs adopted in many countries was disappointing. This was partly justified by the favorable outcomes of screening, and a decline in incidence within older age groups was not able to overcome the rising incidence of CRC in a younger population[21-23]. This was in accordance with Fayadh *et al*[13] in their single center experience of CRC screening in UAE from 2012 to 2019, which demonstrated increasing trends in CRC with an average age of 53 years. Of note, 46% of CRC cases were below the age of 50. Furthermore, another single center experience from Egypt reported that approximately 25% of CRC cases occurred in individuals younger than 40 years of age[9].

Limitations of our study

Our study has some limitations. There were few studies that met our inclusion criteria, and many Arab countries were not represented due to the lack of suitable studies for our review. There were not enough data to examine some questions of interest, such as regional differences in the prevalence, epidemiology and risk factors for CRC in Arab countries and the lack of programmed screening and/or surveillance strategies for CRC in most Arab countries.

CONCLUSION

In conclusion, there is a considerable prevalence of CRC in some Arab countries. More studies are needed to explore the incidence and prevalence of CRC in the rest of the Arab world.

Recommendations: Based on the available literature, it is recommended that multicenter prospective studies be conducted to assess the actual prevalence and incidence of CRC in different Arab countries and in different age groups. Proper

utilization of retrospective data emerging from currently running CRC screening programs in some countries and establishment of new screening programs in other countries will guide decisions in management and prevention strategies to contain the rising incidence of CRC in the Arab world. Proper awareness about CRC and early screening among the population represents the initial step to prevent morbidity and mortality resulting from CRC.

ARTICLE HIGHLIGHTS

Research background
Morbidity and mortality of colorectal cancer (CRC) is increasing globally. There is a particular concern about the rising incidence of CRC in young people in different parts of the world.

Research motivation
It is crucial for each country/region to know the actual prevalence, incidence, and predisposing factors for CRC to help in adequate planning for screening programs, preventive measures, and proper allocation of health care resources.

Research objectives
The main objective of this study was to explore and summarize the available evidence about prevalence and/or incidence of CRC in the Arab world.

Research methods
A systematic review of available literature was done to retrieve articles containing original data about CRC in the Arab world. Available data were extracted and summarized.

Research results
Nine studies including data about CRC in 5 Arab countries were found. Reported prevalence of CRC in Saudi Arabia was 0.72%, in United Arab Emirates was 0.78% and in Egypt ranged from 0.4%-14%. Qatar reported an average annual incidence rate of 7.5/100000/year. Egypt reported a crude rate of 3.1 in males and 2.3 in females. In Saudi Arabia, the crude incidence rate for CRC was 3.6 and 3.3 among females and males respectively. CRC tends to occur in the fourth or fifth decade of life, however, 25% of CRC patients were less than 40 years.

Research conclusions
Some Arab countries have a considerable prevalence of CRC. More data are expected to arise from the currently running CRC screening programs.

Research perspectives
Multicenter prospective trials and proper utilization of retrospective data are needed to assess the actual prevalence and incidence of CRC in different Arab countries.

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Cell-free DNA liquid biopsy for early detection of gastrointestinal cancers: A systematic review

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Abstract

BACKGROUND

Gastrointestinal tumors are among the most common cancer types, and early detection is paramount to improve their management. Cell-free DNA (cfDNA) liquid biopsy raises significant hopes for non-invasive early detection.

AIM

To describe current applications of this technology for gastrointestinal cancer detection and screening.

METHODS

A systematic review of the literature was performed across the PubMed database. Articles reporting the use of cfDNA liquid biopsy in the screening or diagnosis of gastrointestinal cancers were included in the analysis.

RESULTS

A total of 263 articles were screened for eligibility, of which 13 articles were included. Studies investigated colorectal cancer (5 studies), pancreatic cancer (2 studies), hepatocellular carcinoma (3 studies), and multi-cancer detection (3 studies), including gastric, oesophageal, or bile duct cancer, representing a total of 4824 patients. Test sensitivities ranged from 71% to 100%, and specificities ranged from 67.4% to 100%. Pre-cancerous lesions detection was less performant with a sensitivity of 16.9% and a 100% specificity in one study. Another study using a large biobank demonstrated a 94.9% sensitivity in detecting cancer up to 4 years before clinical symptoms, with a 61% accuracy in tissue-of-origin identification.

CONCLUSION

cfDNA liquid biopsy seems capable of detecting gastrointestinal cancers at an early stage of development in a non-invasive and repeatable manner and screening simultaneously for multiple cancer types in a single blood sample. Further trials in clinically relevant settings are required to determine the exact

place of this technology in gastrointestinal cancer screening and diagnosis strategies.

Key Words: Cell-free DNA; Tumor DNA; Liquid biopsy; Next-generation sequencing; Cancer genomics; Pancreatic cancer; Colorectal cancer; Hepatocellular carcinoma; Multi-cancer detection; Cancer screening; Public health; Precision oncology

Core Tip: Liquid biopsy cell-free DNA represents a promising non-invasive method for detecting various gastrointestinal cancers at an early stage of development. The current literature suggests a high-performance profile for this technology and the potential to improve the global course of gastrointestinal cancers currently diagnosed at an advanced stage, such as pancreatic cancer. Prospective validation studies in relevant clinical settings are required to determine the applicability and added value of these new diagnostic and screening tests in global cancer care.

INTRODUCTION

Tumors developing from the gastrointestinal tract are among the most common cancer types, colorectal and stomach cancer, counting for 19.5% and 11.1% respectively worldwide in 2020[1]. Risk factors notably include smoking, obesity, poor diet, genetic factors, and infections with hepatitis B virus or Helicobacter pylori bacteria[2]. Early detection and diagnosis represent a crucial component to allow effective treatment and improve survival. Nowadays, different screening strategies have been developed, such as colonoscopy for colorectal cancer or blood testing for alpha-fetoprotein (AFP) or magnetic resonance imaging in high-risk patients for liver cancer, but other types of tumors often lack screening strategies and non-invasive testing. For instance, so far, no efficient screening methods are available for pancreatic cancer; most patients experience their first symptoms at advanced and metastatic stages, explaining the 5-year survival rate of only 5% to 10%[3].

These past few years, researchers have focused their attention on a new promising diagnostic method, liquid biopsy, which uses biomarkers such as circulating cell tumor, RNA fragments, or cell-free DNA (cfDNA). Unlike tissue samples obtained by invasive methods like needle biopsies or endoscopies, biomarkers can be detected in body fluids, mostly blood[4], and address limitations of tissues biopsies not only in diagnosis and screening, but also in diagnosis and screening the treatment response and follow-up[5-7]. Among liquid biopsy options, cfDNA raises the most significant hopes in early cancer detection. Historically, it was first reported in 1948 by Mandel *et al* among healthy patients. In 1977, Leon *et al* described elevated levels of cfDNA in the serum of cancerous patients for the first time[4,8,9]. CfDNA is continuously released in the bloodstream through different mechanisms such as apoptosis, necrosis, and active secretion by the tumor cell. When originating from a cancer cell, cfDNA is called circulating tumor DNA (ctDNA)[4]. Concentration levels seem to correlate with the cancer stage and size; advanced-stage cancer patients show a higher concentration of cfDNA[8,9]. While cfDNA quantification in the bloodstream might indicate the presence or absence of cancer, sequencing and analyzing the mutation patterns of this cfDNA goes one step further: mutational profiling might give the researchers clues on the tumor's tissue of origin, providing information to target further specific investig-ations[9]. Recent progress in genomic technology also provides highly sensitive detection of low-prevalence mutations, even in high signal-to-noise configurations, thus theoretically enabling very early cancer diagnosis. The ability to run repeatable, non-invasive, multi-cancer early detection tests would bring significant advantages in

the global care of frequently hardly reachable cancer locations, such as gastrointestinal cancers.

The present systematic review of the literature aims to describe the current state of developing cfDNA liquid biopsies as a means of early gastrointestinal cancer detection and screening.

MATERIALS AND METHODS

A systematic review of the literature was performed following the PRISMA guidelines [10]. All articles written in English from January 2010 to January 2021 were searched on January 19th, 2021, through the PubMed database using the following research algorithm: (liquid biopsy OR cfdna) AND (multiple OR gastrointestinal OR colon OR colorectal OR gastric OR oesophag* OR liver OR hepatocellular OR pancreatic) AND (cancer OR tumor OR tumour) AND (screening OR diagnos* OR detect*) AND early AND (blood OR venous OR plasma) NOT review.

After a first selection based on titles for screening, eligible articles were selected based on abstract analysis. Then, full-text analysis of the eligible articles searched for criteria of the finally included articles. Two investigators (I Uhe, J Douissard) independently assessed the articles for eligibility and inclusion. Discordances in study inclusions were solved by re-evaluation between the two reviewers.

All relevant articles reporting human studies investigating cfDNA liquid biopsy as a screening method or diagnosis method for newly discovered untreated primary gastrointestinal cancers were included. Studies investigating multiple cancer screening, including gastrointestinal but not limited to them, were also included. Excluded articles were studies investigating cfDNA as a follow-up method after cancer treatment, minimal residual disease detection, studies investigating cfDNA as a prognosis method only, reviews, meta-analyses, theoretical papers, and biological studies not reporting clinical outcomes. Studies reporting cancer patients who were already treated, surgically or medically, have also been excluded. To improve the present review's clinical relevance, only the total number of participants in the papers' validations cohorts were considered. If available, test performances were reported in terms of sensitivity (Se), specificity (Sp), positive and negative predictive values, or area under the curve (AUC).

Literature search and studies characteristics

A total of 263 articles were identified through the PubMed search. Two articles were not written in English, 11 were not original publications, and 119 did not involve cfDNA. Thirty-five articles did not mention gastrointestinal cancer, and 44 did not investigate cfDNA as a screening or diagnosis method, leaving 52 articles. After full-text reading, thirteen studies were ultimately included for analysis, representing a total of 4824 patients (Table 1, Figure 1). The largest study included blood samples from 1194 participants[11], while the smallest study included samples of 130 participants[12]. Six studies took place in China[11,13-17], three in the United States[9, 18,19], and four in Europe[12,20-22]. Five were multicentric[9,11,16,18,19], four monocentric[13,14,17,22] and four studies did not mention the information. Five studies focused on colorectal cancer (CRC)[9,12,17,20,22], three on various cancer types [14,19,21] of which two included gastric cancers[14,19], three on hepatocellular carcinoma (HCC)[11,15,16] and two on pancreatic ductal adenocarcinoma (PDAC)[13, 18]. All studies compared cancer and non-cancer individuals. Five of them also included in their analysis a group of patients with pre-cancerous lesions, such as colorectal adenoma or hyperplasia, liver cirrhosis, or chronic hepatitis B virus infection [11,12,15,16,22] (Table 2).

Risk of bias of included studies

The risk of bias of included studies was determined using the ROBINS-I tool (2016) [23]. Except for one study with an overall low risk of bias[16], all included studies were at moderate risk (Table 3).

Extraction and sequencing methods

All studies collected cfDNA from plasma samples. Kits used for cfDNA extraction from plasma samples can be found in Table 4. The QIAamp circulating nucleic acid kit was the most employed, a spin column-based kit (*n* = 7/13). A large majority of studies used next-generation sequencing (NGS) (*n* = 9/13), two used real-time polymerase chain reaction (RT-PCR), one digital droplet PCR, and one multiplex

Table 1 Characteristics of included studies

Ref.	Year	Country	Mono/multicentric	Type of cancer	Total number of patients in validation cohort	Type of groups analyzed
Li *et al*[13]	2020	China	Monocentric	PDAC	208	Cancer *vs* healthy
Chen *et al* [14]	2020	China	Monocentric	Gastric, esophagus, colorectal, lung or liver	418	Cancer diagnosed *vs* healthy; Pre-diagnosed patients *vs* healthy
Guler *et al* [18]	2020	United States	Multicentric	PDAC	228	Cancer *vs* healthy
Junca *et al* [12]	2020	France	NA	Colorectal	130	Cancer *vs* healthy *vs* advanced-adenoma *vs* non-advanced adenoma and/or hyperplastic polyp(s)
Tao *et al* [15]	2020	China	NA	HCC	175	HBV-related HCC *vs* cancer-free HBV patients
Cristiano *et al*[19]	2019	United States	Multicentric	Breast, colorectal, lung, ovarian, pancreatic, gastric, bile duct	423	Cancer *vs* healthy
Li *et al*[17]	2019	China	Monocentric	Colorectal	140	Cancer *vs* healthy
Qu *et al*[16]	2019	China	Multicentric	HCC	331	HBsAg1 positive without cancer based on screening with serum AFP and ultrasonography
Cai *et al*[11]	2019	China	Multicentric	HCC	1194	Cancer *vs* healthy *vs* 392 LC/HB *vs* BLL
Wan *et al* [9]	2019	United States	Multicentric	Colorectal	817	Cancer *vs* healthy
Jensen *et al* [20]	2019	Denmark	NA	Colorectal	234	Cancer *vs* healthy
Nunes *et al* [21]	2018	Portugal	NA	Breast, colorectal, lung	356	Cancer *vs* healthy
Perrone *et al*[22]	2014	Italy	Monocentric	Colorectal	170	Cancer *vs* healthy *vs* premalignant lesion (adenoma/hyperplasia)

PDAC: Pancreatic ductal adenocarcinoma; HCC: Hepatocellular carcinoma; LC/HB: Liver cirrhosis/hepatitis B; BLL: Benign liver lesions; HBV: Hepatitis B virus; AFP: Alpha-fetoprotein.

methylation-specific PCR. Various mutational patterns and genomic profiling strategies were investigated (Table 4). Most studies focused on methylation variations (*n* = 7/13), while others investigated specific mutation locations such as *KRAS* and *BRAF* or more complex mutational patterns.

Tests performance

Overall test performances for each cancer subgroup are described in Table 5.

RESULTS

CRC

Clinically relevant sensitivities and specificities to detect colorectal adenocarcinoma were achieved in three studies[9,20,21], Li *et al*[17] and Jensen *et al*[20] focusing on tumor-specific methylations. In contrast, Wan *et al*[9] investigated complex cfDNA mutational patterns using a machine-learning-based model. Sensitivities ranged from 74% to 85%, while specificities ranged from 85% to 99%. In a fourth study, Perrone *et al* [22] reported an AUC of 0.709 when discriminating CRC from healthy patients. However, for premalignant lesions, the performance was lower, with an AUC of 0.535 [22]. Similarly, investigating adenomas and adenocarcinomas through cfDNA *KRAS* and *BRAF* mutations, Junca *et al*[12] found a mean sensitivity of 16.9% for a 100% specificity reflecting a still lower sensitivity in premalignant lesions detection but allowing a high level of precision.

Table 2 Number of patients in each group

Ref.	Total patients in validation cohort	Nbr patient cancer group	Nbr patient healthy group	Nbr patient additional group 1	Nbr patient in aditionnal group 2
Li *et al*[13]	208	101	107	-	-
Chen *et al*[14]	418	113	2071	98 pre-diagnosed patients	-
Guler *et al*[18]	228	23	205	-	-
Junca *et al*[12]	130	20	40	39 advance adenoma	31 non-advance adenoma
Tao *et al*[15]	175	89	86	-	-
Cristiano *et al* [19]	423	208	215	-	-
Li *et al*[17]	140	74	66	-	-
Qu *et al*[16]	331	-	-	HBsAg (+)	-
Cai *et al*[11]	1194	809	256	129 LC/CHB	-
Wan *et al*[9]	817	546	271	-	-
Jensen *et al* [20]	234	143	91	-	-
Nunes *et al* [21]	356	253	103	-	-
Perrone *et al* [22]	170	34	63	73 adenoma/hyperplasia	-

LC: Liver cirrhosis, CHB: Chronic hepatitis B virus infection. 90 GC patients without surgery and 110 who had undergone surgery.

Pancreatic cancer

Examining methylation patterns in cfDNA, Li *et al*[13] described eight methylation markers in patients suffering from PDAC; SIX3, TRIM73, MAPT, FAM150A, EPB41L3, MIR663, LOC100130148, and LOC100128977. These markers identified PDAC patients efficiently, with a sensitivity of 93.2% and a specificity of 95.2% (AUC = 0.943). By investigating 5-hydroxymethylcytosine (5hmC) changes in circulating cfDNA, Guler *et al*[18] achieved similar performance with an AUC of 0.921.

Hepatocellular carcinoma

Cai *et al*[11] found promising results using a mutational pattern of 32 gene markers to discriminate HCC patients from healthy individuals, with a sensitivity and specificity of 82.7% and 76.4%, respectively. Furthermore, when comparing HCC patients with cancer-free high-risk patients (chronic hepatitis B or liver cirrhosis), the model performed similarly with an 82.7% sensitivity and 67.4% specificity[11].

Comparing HCC patients with cancer-free asymptomatic HBV patients based on cfDNA mutational pattern of specific locations, Qu *et al*[16] achieved a sensitivity and specificity of 100% and 94%, respectively. Further, using somatic copy number aberration in cfDNA as an alternative to methylation or specific mutations analysis, Tao *et al*[15] investigated the possibility of discriminating HBV-related HCC from cancer-free chronic HBV patients. Their predictive model performed appropriately, showing a high level of precision in two validation cohorts, with an AUC of 0.92 and 0.81.

Multi-cancer detection

Nunes *et al*[21] investigated the possibility to diagnose lung, breast, and colorectal cancer patients simultaneously from healthy individuals by detecting aberrant methylations on specific locations. They achieved an overall specificity of 73.5% and a sensitivity of 74.2%. For colorectal cancer, specificity was 69.9%, and sensitivity was 78.4%[21].

With a comparable strategy targeting five cancers (gastric, oesophageal, lung, liver, and colorectal), Chen *et al*[14] demonstrated the potential ability of cfDNA liquid biopsy to achieve multicancer detection several years before the actual diagnosis. Based on blood samples from a large biobank, they analyzed samples from 3 groups. The post-diagnosis group included patients with a newly discovered and untreated

Table 3 Risk of bias of included studies, determined using the ROBINS-I tool (2016)

Ref.		Entry	Judgement	Support for judgement
Li *et al*[13]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Chen *et al* [14]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Guler *et al* [18]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Junca *et al* [12]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention

	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analysesconsistent with a priori plan
Tao *et al*[15]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants in the supplementary materials
	C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants in the supplementary materials
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Cristiano *et al*[19]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Li *et al*[17]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Qu *et al*[16]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Low risk	Pre-registered protocol available (NCC201709011)

Cai *et al*[11]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Wan *et al*[9]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Jensen *et al* [20]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Nunes *et al* [21]	A	Bias due to confounding	Low risk	No confounding factors
	B	Bias in selection of participants into the study	No information	No information about the start of follow up and intervention for the participants
	C	Bias in classification of interventions	No information	No information about the start of follow up and intervention for the participants
	D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions
	E	Bias due to missing data	Low risk	All data were reported
	F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention
	G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan
Perrone *et al* [22]	A	Bias due to confounding	Low risk	No confounding factors

B	Bias in selection of participants into the study	Low risk	Information provided about the start of follow up and intervention for the participants	
C	Bias in classification of interventions	Low risk	Information provided about the start of follow up and intervention for the participants	
D	Bias due to deviationsfrom intended interventions	Low risk	No deviations from the planned interventions	
E	Bias due to missing data	Low risk	All data were reported	
F	Bias in measurement of outcomes	Low risk	Comparable methods of outcome assessment in the groups, intervention received in each group unlikely to influence the outcome measure, any error in measuring the outcome is unrelated to intervention	
G	Bias in selection of the reported result	Moderate risk	No pre-registered protocol available; outcome measurements and analyses consistent with a priori plan	

Table 4 Details of extraction and sequencing methods used in each of the included studies

Ref.	Source of cfDNA	Focus in cfDNA	Extraction method (used kit)	Sequencing method	Sequencing method details
Li *et al*[13]	Plasma	Methylated markers	QIAamp Circulating Nucleic Acid Kit (Qiagen, 55114)	NGS	Illumina HiSeq 2000 platform
Chen *et al* [14]	Plasma	Cancer-specific methylation signatures	QIAamp Circulating Nucleic Acid kit (Qiagen, 55114)	NGS	APA Library Quantification Kit for Illumina (KK4844) and sequenced on an Illumina NextSeq 500
Guler *et al* [18]	Plasma	5hmC modifications	QIAamp Circulating Nucleic Acid Kit (QIAGEN, Germantown, MD)	NGS	NextSeq550 instrument with version 2 reagent chemistry (Illumina, San Diego, CA).
Junca *et al* [12]	Plasma	*KRAS* and *BRAF* mutational status	QIAamp Circulating Nucleic Acid kit (Qiagen, Hilden, Germany)	RT-PCR	Q24 PyroMark system (Qiagen, Hilden, Germany)
Tao *et al* [15]	Plasma	Somatic copy number aberration	QIAamp CirculatingNucleic Acid Kit (Qiagen)	NGS	Next generation sequencing (Illumina)
Cristiano *et al*[19]	Plasma	Fragmentation size	Qiagen Circulating Nucleic Acids Kit (Qiagen GmbH)	NGS	NEBNext DNA Library Prep Kit for Illumina
Li *et al*[17]	Plasma	Aberrant DNA hypermethylation of CpGislands	DNeasy Blood & TissueKit (Qiagen)	NGS	Methylated CpG tandem ampli-fication and sequencing
Qu *et al*[16]	Plasma	Specific mutations	ARCHITECT i2000SR Chemical luminescence immunity analyzer	NGS	Next generation sequencing
Cai *et al*[11]	Plasma	5hmC modifications	NA	NGS	5hmC-Seal
Wan *et al*[9]	Plasma	cfDNA mutations patterns	MagMAX cfDNA Isolation Kit	NGS	Illumina NovaSeq 6000 Sequencing System
Jensen *et al* [20]	Plasma	Tumour-specific DNA methylation	Gentra Puregene Tissue Kit (Qiagen)	DD-PCR	Bisulfite sequencing and methylation-specific droplet digital PCR
Nunes *et al* [21]	Plasma	Aberrant DNA methylation	QIAamp MinElute ccfDNA (Qiagen, Hilden, Germany)	qMSP	qMSP
Perrone *et al*[22]	Plasma	*KRAS* mutated cfDNA	Qiamp DNA Blood Extraction Kit (Qiagen)	RT-PCR	RT-PCR

NGS: Next-generation sequencing; RT-PCR: Real-time polymerase chain reaction; qMSP: Multiplex methylation-specific polymerase chain reaction.

malignancy at the time of sampling. The pre-diagnosis group included patients with no known malignancy at the sampling time but who developed cancer within four years after sampling (pre-diagnosis). Finally, the control group included healthy individuals who were still free of malignant disease four years after sampling. Their model achieved an overall detection specificity of 96% when comparing healthy individuals to pre-diagnosis and post-diagnosis groups. Overall sensitivity was 87.5% for the post-diagnosis group, ranging from 75% in colorectal cancer to 96% in lung cancer. It reached 94.9% in the pre-diagnosis group, ranging from 91% in oesophageal cancer to 100% in liver cancer[14].

Table 5 Sensibility and sensitivity of included studies

	Ref.	Group of validation cohorts	Sensitivity	Specificity	Positive predictive value	Negative predictive value	AUC
PDCA	Li *et al*[13]	Cancer *vs* healthy	93.2	95.2	NA	NA	0.943
	Chen *et al*[14]	Cancer *vs* healthy	NA	NA	NA	NA	0.921
HCC	Guler *et al*[18]	HBV-related HCC *vs* cancer-free HBV group 1	18	97.4	NA	NA	0.92
		HBV-related HCC *vs* cancer-free HBV group 2	29	95.6	NA	NA	0.81
	Junca *et al*[12]	HCC *vs* cancer-free HBV	100	94	17	100	NA
	Tao *et al*[15]	HCC *vs* healthy	82.7	76.4	NA	NA	0.884
		HCC *vs* high risk (HBV and cirrhosis)	82.7	67.4	NA	NA	0.846
Various cancer types	Cristiano *et al* [19]	Pre-diagnosis *vs* healthy	84.9	96.1	NA	NA	NA
		Post-diagnosis *vs* healthy	87.5	96.1			
	Li *et al*[17]	All cancer *vs* healthy	80	95	NA	NA	0.94
			73	98			
		Gastric cancer *vs* healthy	81	95			
			81	98			
		Colorectal cancer *vs* healthy	81	95			
			70	98			
		Bile duct cancer *vs* healthy	88	95			
			81	98			
		Pancreatic cancer *vs* healthy	71	95			
			65	98			
	Qu *et al*[16]	All cancer *vs* healthy	74.2	73.5	87.1	52.1	NA
		Colorectal cancer *vs* healthy	78.4	69.9	48.3	90	
Colorectal	Cai *et al*[11]	Cancer/adenoma *vs* healthy	16.9	100	100	59.2	NA
	Wan *et al*[9]	Cancer *vs* healthy	74	90	NA	NA	0.887
	Jensen *et al* [20]	Cancer *vs* healthy	85	85	NA	Na	0.92
	Nunes *et al* [21]	Cancer *vs* healthy	85	99	NA	NA	NA
	Perrone *et al* [22]	Cancer *vs* healthy	NA	NA	NA	NA	0.709
		Adenomas *vs* healthy	NA	NA	NA	NA	0.535

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; PDAC: Pancreatic ductal adenocarcinoma.

In contrast to these two studies focused on cfDNA methylations, Cristiano *et al*[19] explored a multi-cancer detection model analyzing cfDNA fragmentation patterns, including gastric, bile duct, colorectal and pancreatic cancers. Their model reached an overall detection sensitivity of 80% for a specificity of 95%, or a sensitivity of 73% for a specificity of 98%, and a global AUC of 0.94. Furthermore, enhanced by a machine-learning algorithm, they were able to identify the tissue of origin of cancer samples with a 61% accuracy[19]. Detailed performances per cancer type of this model can be found in Table 3.
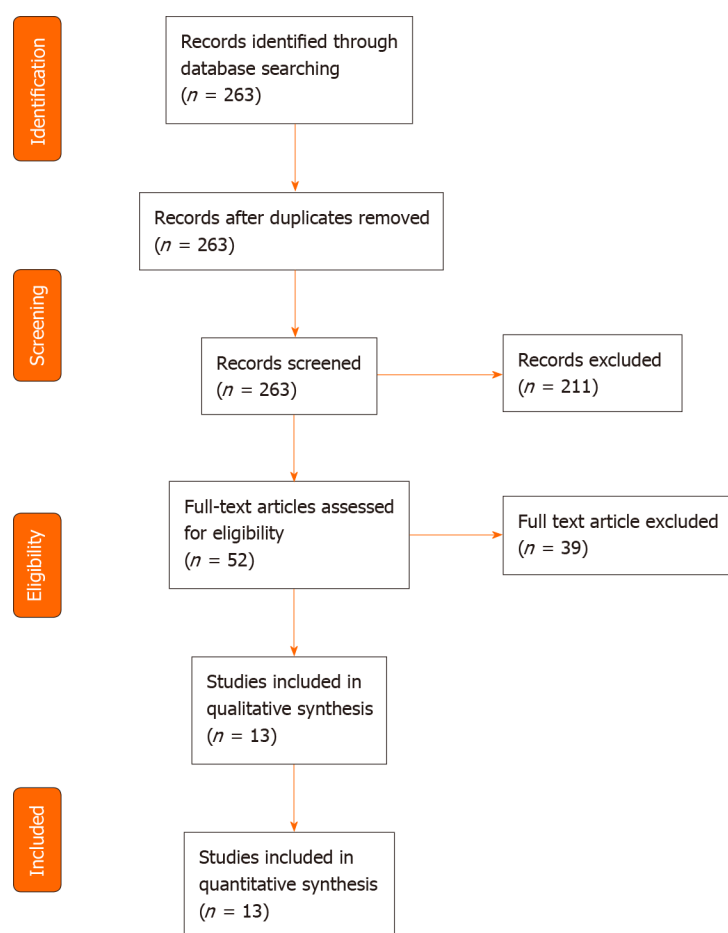
Identification

Records identified through database searching
(*n* = 263)

Screening

Records after duplicates removed
(*n* = 263)

Records screened
(*n* = 263)

Records excluded
(*n* = 211)

Eligibility

Full-text articles assessed for eligibility
(*n* = 52)

Full text article excluded
(*n* = 39)

Studies included in qualitative synthesis
(*n* = 13)

Included

Studies included in quantitative synthesis
(*n* = 13)

Figure 1 PRISMA flow diagram summarizing the search strategy.

DISCUSSION

Liquid biopsy appears as a promising non-invasive method for the initial screening and diagnosis of various gastrointestinal cancers. High levels of sensitivity and specificity described in the included studies seem within acceptable ranges for eventual clinical use. In the case of HCC, cfDNA tests demonstrated better detection performances when compared to the standard surveillance of high-risk patients combining AFP dosage and ultra-sound monitoring. It also appears to be a viable solution regarding the challenge of pancreatic cancer screening; due to the paucity of symptoms in the early phases and the absence of acceptable screening strategies even for high-risk groups, this type of cancer remains frequently detected at metastatic or locally advanced and unresectable stages. Conversely, colorectal cancer is one of the few cancers with a standardized and efficient large-scale screening strategy based on the colonoscopy and the fecal occult blood test. Still, there is room for improved and more cost-effective strategies. Of note, cfDNA liquid biopsy's ability to detect several cancer types simultaneously appears as a potential paradigm shift in global cancer care, and studies investigating such application achieved a high level of performance. Further, as demonstrated by Chen *et al*[13], this technology bears the potential to predict cancer several years before the onset of clinical symptoms and identify or direct investigations towards specific tissues of origin.

The central role of early cancer detection in improving oncologic and public-health outcomes is well established. However, it is a challenge for liquid biopsy since smaller and earlier-stage tumors tend to release lower levels of ctDNA[24]. The signal-to-noise ratio of ctDNA is thus meager compared to non-cancer-derived cfDNA, with a detection percentage ranging from 0 to 11.7%[25,26]. The extraction method plays a critical role in improving detection performance. Different procedures have been developed, the more widespread being column-based, polymer-based, phenol-chloroform, or magnet-based[9,27]. These methods are efficient and allow to reach a high DNA concentration but remain expensive and time-consuming[9,27]. In this context, some authors proposed plasma processing methods without the need for

DNA extraction. Breitbach *et al*[28] notably used quantitative RT-PCR to measure cfDNA concentration in plasma. Not only did the method showed great feasibility with higher levels of cfDNA found among cancer patients, but it also proved to be more time effective and more efficient than the eluate of the QIAamp DNA Blood Mini Kit, for example, with levels of cfDNA in unpurified plasma 2.79 fold higher[28].

Regarding the sequencing method, some authors focused their attention on specific mutations while others analyzed the whole genome searching for non-specific mutational patterns, most of them using NGS methods. Different factors can explain the apparent predominance of NGS over other PCR methods such as RT-PCR in the published studies. Although more technically demanding and expensive, NGS is a hypothesis-free approach that carries a higher discovery power of new mutational patterns, in addition to a higher sensitivity to rare variants[29,30]. Further, its superior multiplex capabilities tend to improve the workflow when studying a large number of locations and samples. These high throughput and detection sensitivity capabilities might be valuable in a screening configuration for early cancer detection, which deals with lower levels of mutation than advanced stage cancers and aims at testing a high volume of patients.

As the field is at an early stage of clinical exploration, there is still a high variability in trial designs and reporting methods, thus undermining the global quality of tests' performance analysis. Of note, biocomputational trials based on biobank samples often report higher levels of sensitivity and specificity but are less likely to translate into clinically relevant performances as prospective trials would. Applicability to real-life clinical applications is thus the most awaited step to achieve for the scientific validation of this technology, and upcoming clinical trials will need to address many questions, such as the appropriate balance between sensitivity and specificity in a screening purpose, the timing of screening tests, patient selection, socio-economic parameters and dealing with the uncertainty around tissues of origin in positive tests.

CONCLUSION

Liquid biopsy cfDNA represents an efficient, non-invasive, and promising method for detecting various gastrointestinal cancers at an early stage of development. These tools could improve the global prognosis of cancers currently diagnosed at an advanced stage due to the lack of effective screening and diagnostic methods, such as pancreatic cancer. Allowing early detection of several types of cancers and reducing the burden of multiple screening tests, cfDNA liquid biopsies could change the course of gastrointestinal cancers care for a significant number of patients and induce a paradigm shift in cancer-related public health policies, provided that they can demonstrate their clinical relevance in future studies.

ARTICLE HIGHLIGHTS

Research background

Liquid biopsy cell-free DNA (cfDNA) represents a promising non-invasive method for detecting various gastrointestinal cancers at an early stage of development.

Research motivation

Various and recent literature is available on this topic, with exponentially growing interest.

Research objectives

To review the current state of development of cfDNA liquid biopsy in the field of gastrointestinal cancer early detection.

Research methods

A systematic review of the literature according to the PRISMA guidelines.

Research results

The current literature suggests a high-performance profile for this technology and the potential to improve the global course of gastrointestinal cancers currently diagnosed at an advanced stage, such as pancreatic cancer.

Research conclusions

cfDNA liquid biopsy showed high potential in the diagnosis of early gastrointestinal cancers and simultaneous screening of multiple cancer types.

Research perspectives

Further trials in clinically relevant settings are required to determine the exact place of this technology in future diagnosis strategies.

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Atezolizumab plus bevacizumab versus sorafenib or atezolizumab alone for unresectable hepatocellular carcinoma: A systematic review

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Abstract

BACKGROUND

Despite the use of current standard therapy, the prognosis of patients with unresectable hepatocellular carcinoma (HCC) is poor, with median survival times of 40 mo for intermediate HCC (Barcelona Clinic Liver Cancer [BCLC] stage B) and 6–8 mo for advanced HCC (BCLC stage C). Although patients with early-stage HCC are usually suitable for therapies with curative intention, up to 70% of

patients experience relapse within 5 years. In the past decade, the United States Food and Drug Administration has approved different immunogenic treatment options for advanced HCC, the most common type of liver cancer among adults. Nevertheless, no treatment is useful in the adjuvant setting. Since 2007, the multi-kinase inhibitor sorafenib has been used as a first-line targeted drug to address the increased mortality and incidence rates of HCC. However, in 2020, the IMbrave150 trial demonstrated that combination therapy of atezolizumab (anti-programmed death-ligand 1 [PD-L1]) and bevacizumab (anti-vascular endothelial growth factor [VEGF]) is superior to sorafenib, a single anti-programmed death 1/PD-L1 antibody inhibitor used as an anti-cancer monotherapy for HCC treatment.

AIM
To conduct a systematic literature review to evaluate the evidence supporting the efficacy and safety of atezolizumab/bevacizumab as preferred first-line drug therapy over the conventional sorafenib or atezolizumab monotherapies, which are used to improve survival outcomes and reduce disease progression in patients with unresectable HCC and non-decompensated liver disease.

METHODS
A comprehensive literature review was conducted using the PubMed, Scopus, ScienceDirect, clinicaltrials.gov, PubMed Central, Embase, EuropePMC, and CINAHL databases to identify studies that met the inclusion criteria using relevant MeSH terms. This systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines and risk of bias (RoB) were assessed using the Cochrane RoB 2 tool and Sevis.

RESULTS
In the atezolizumab/bevacizumab group, an improvement in overall tumor response, reduction of disease progression, and longer progression-free survival were observed compared to monotherapy with either sorafenib or atezolizumab. Hypertension and proteinuria were the most common adverse events, and the rates of adverse events were comparable to those with the monotherapy. Of the studies, there were two completed trials and two ongoing trials analyzed using high quality and low bias. A more thorough analysis was only performed on the completed trials.

CONCLUSION
Treatment of HCC with atezolizumab/bevacizumab combination therapy was confirmed to be an effective first-line treatment to improve survival in patients with unresectable HCC and non-decompensated liver disease compared to monotherapy with either sorafenib or atezolizumab.

Core Tip: Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is a leading cause of cancer-related deaths. Combination immunotherapy for the treatment of advanced HCC is attracting increasing attention because of the superiority of clinical results compared to sorafenib, the standard of care. Combination therapy with atezolizumab/bevacizumab has been compared to sorafenib and atezol-izumab monotherapies. Current findings indicate that combination therapy is as effective as first-line therapeutic options for improving survival rates in patients with unresectable HCC and non-decompensated liver disease.

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-85% of primary liver cancers, and is the sixth most common cancer and fourth leading cause of cancer-related deaths worldwide[1]. Surgical resection, thermal ablation, and liver transplantation represent the conventional approaches used for patients with early-stage HCC (Barcelona Clinic Liver Cancer [BCLC] stage A). Moreover, for patients who are not surgical candidates, systemic chemotherapy can be alternatively employed. Patients with early-stage HCC are usually suitable for curative treatments. However, the prognosis of patients with unresectable HCC is usually poor, with median survival times of 40 mo for intermediate HCC (BCLC stage B) and 6–8 mo for advanced HCC (BCLC stage C)[2]. Moreover, up to 70% of patients experience disease recurrence within 5 years, with no beneficial effects in the adjuvant setting[3].

Tumor cells can activate different immune checkpoint pathways that modify immunosuppressive functions. Specifically, in the last several decades, the emergence of immune checkpoint inhibitors (ICIs) that target the human programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway has led to the high potential to treat a wide spectrum of solid tumors including HCC[4].

Sorafenib (BAY 43-9006) is a multi-kinase inhibitor that blocks the activity of Raf serine/threonine kinase, as well as other receptor tyrosine kinases such as VEGFR-2 and VEGFR-3, platelet-derived growth factor receptor-β, c-KIT, fms-like tyrosine kinase 3, and RET. Its ability to block these pathways leads to the inhibition of tumor angiogenesis, tumor cell proliferation, and migration while increasing the rate of apoptosis[5,6].

In 2007, the United States Food and Drug Administration (FDA) approved sorafenib for the treatment of metastatic HCC due to its anti-angiogenic properties[6]. It has shown survival benefits by extending the median survival time of patients with unresectable HCC (10.7 mo compared to 7.9 mo in the placebo group)[2].

Atezolizumab, another ICI of interest, is a monoclonal antibody that targets PD-L1 [7] and prevents the interaction between the PD-1 and A7-1 receptors, resulting in the reversal of T-cell suppression[8]. Bevacizumab is an anti-angiogenic antibody that inhibits angiogenesis and neoplasm growth by targeting VEGF[9]. Anti-VEGF treatments can decrease VEGF-mediated immunosuppression and also improve anti-PD-1/PD-L1 functions[10,11].

Given the nature of these immunotherapies, it has been postulated that a combination of atezolizumab with bevacizumab should have safe and synergistic anti-tumor effects on HCC. The phase III IMbrave150 trial showed that bevacizumab combined with atezolizumab leads to better overall survival (OS) and progression-free survival (PFS) outcomes over sorafenib therapy in patients with unresectable HCC [12]. Moreover, atezolizumab/bevacizumab combination therapy was also demonstrated to be the first regimen to improve patients' quality of life, significantly delaying the median time to deterioration compared to sorafenib[12]. Given the better performance of atezolizumab/bevacizumab, FDA approved the combination drug therapy for patients with advanced HCC as first-line therapy on May 29, 2020[13].

The synergistic effects of atezolizumab-bevacizumab therapy compared to the sorafenib monotherapy are remarkable enough to warrant further study. Hence, this systematic review analyzed the documented evidence comparing the efficacy and safety of atezolizumab/bevacizumab combination therapy with monotherapy regimens, such as sorafenib or atezolizumab, in patients with unresectable HCC (Supplementary Figure 1).

MATERIALS AND METHODS

Criteria for considering studies

This study included a data collection of randomized controlled trials (RCTs), which evaluated adult patients (aged 18 and older) with unresectable HCC to receive

combination therapy of intravenous atezolizumab (1200 mg) plus bevacizumab (15 mg/kg) every 3 wk (or periodically). The study dataset was further divided into a control segment consisting of sorafenib monotherapy, atezolizumab monotherapy, or placebo.

The RCTs incorporated primary efficacy outcomes of mortality, measured as a median number of deaths and stratified hazard ratios (HRs). Moreover, the Response Evaluation Criteria in Solid Tumors (RECIST) criteria (Supplementary Table 1) measured secondary outcomes of OS, median OS, and median PFS as tumor response proportions or percentages. An example of the aforementioned was demonstrated in the overall confirmed objective response, confirmed complete and partial responses, stable disease, progressive disease, and disease control rate.

RCTs safety measurements evaluated included patients with adverse events (AEs) from causes that included serious treatment-related AEs and treatment-related mortality events. Additionally, AEs that resulted from drug dosing modifications and/or interruptions were evaluated along with drug withdrawal trials that included participants with Grade 3-5 AEs. Any unfavorable clinical or laboratory result associated with an investigational intervention during the clinical trial was considered an AE; hence, this included any unfavorable and life-threatening medical outcome that required inpatient hospitalization or prolongation of hospitalization.

All RCTs evaluated in the study had documented hepatitis virological status as well as a Child-Pugh classification of A or B (Supplementary Table 2). However, the data excluded trials involving patients who received treatments for medical conditions other than HCC, as well as participants with autoimmune liver disease or any autoimmune conditions and participants in Child-Pugh class C. Non-human studies, non-English and unpublished articles were also excluded.

Search methods for the identification of studies

A comprehensive and extensive literature review of published articles was conducted to identify RCTs that met the inclusion and exclusion criteria using appropriate MeSH terms. This systematic review was performed following the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses. The search was conducted using the Cochrane, Cochrane Central, PubMed Central, Scopus, Science-Direct, WHO trials, clinicaltrials.gov, Google Scholar, Embase, CINAHL, and MedLine databases. The following terms and Boolean operators were employed in MeSH and free-text searches to identify relevant articles: "Hepatocellular carcinoma," "liver tumor," "liver cancer," "atezolizumab and bevacizumab," and "sorafenib."

The data search was conducted until December 27, 2020. The search criteria were broadened by identifying additional studies from the reference lists of selected articles, as well as by the "related articles" function of PubMed. Additionally, the systematic review was registered in PROSPERO, the international prospective registry of systematic reviews of the National Institute for Health Research (CRD42021237736). For transparency, the study was pre-registered on the open science framework (URL: https://osf.io/esvk9), and in PROSPERO.

Data collection and analysis

Selection of studies: All articles used in this document were screened for eligibility *via* their titles and abstracts. Thereafter, the full-text of all chosen studies was examined in detail. Two independent reviewers were chosen to perform the screening and examination process according to predefined eligibility criteria for the qualitative review.

Data extraction and management

Two review authors (MKC and MZ) independently extracted the data and summarized the trial characteristics in each table. They were also involved in extracting the baseline characteristics of the participants, study design, geography, settings, methods, types of interventions (dosage, route of administration, regimen protocol), efficacy, and safety outcomes.

Assessment of risk of bias in included studies

This study assessed the risk of bias (RoB) in the included studies by using the revised Cochrane RoB 2 tool for randomized trials. This tool was used to assess the RoB across the following five domains: Bias arising from the randomization process, bias due to deviation from the intended intervention, bias due to missing outcome, bias in the measurement of outcome, and bias in the selection of the reported results. Moreover, the Robvis data software was used to create a RoB traffic light plot (Figure 1) and a
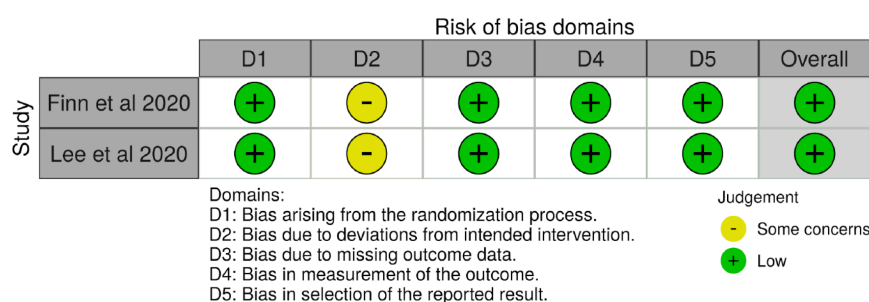
	D1	D2	D3	D4	D5	Overall
Finn et al 2020	+	−	+	+	+	+
Lee et al 2020	+	−	+	+	+	+

Domains:
D1: Bias arising from the randomization process.
D2: Bias due to deviations from intended intervention.
D3: Bias due to missing outcome data.
D4: Bias in measurement of the outcome.
D5: Bias in selection of the reported result.

Judgement
− Some concerns
+ Low

Figure 1 Traffic light plot showing the risk of bias of the two completed studies.

RoB summary plot (Figure 2).

Protocol for missing data

The studies that measured relevant objective data outcomes (*e.g.,* mean survival rates) according to their study protocols, but included non-retrievable online data, missing content or unclear data, were cleared up by reaching out to the original authors of the published reports. However, if no response was obtained from the original authors, the selected study was excluded from the analyses.

Data synthesis

A descriptive analysis of all study results was performed. All continuous variables such as mean and median were analyzed, and all dichotomous outcomes were investigated as proportions and percentages. Furthermore, epidemiological variables (*e.g.,* risk ratio, attributable risk, and numbers needed to treat) to measure certain effects of intervention such as mortality were estimated as deemed necessary.

RESULTS

The results of the literature search are summarized in Figure 3. Initially, 520 potentially eligible articles were considered. However, 326 full-text articles that were predominantly cohort studies and a few RCTs were evaluated after screening the abstracts. Subsequently, four RCTs were included in the literature search after excluding 516 articles according to the eligibility criteria. Of the four trials included, two were ongoing (La Roche[14] and Hack *et al*[15]), and two have been concluded (Finn *et al*[12], 2020, and Lee *et al*[16], 2020). Data from the ongoing clinical trials and completed studies are illustrated in Table 1. Hence, the two completed trials were included in the final analyses.

Study design and setting of included studies

This review included the two concluded trials in the present analyses (Finn *et al*[12] and Lee *et al*[16]) as well as the results of two currently ongoing trials of La Roche[14] and Hack *et al*[15] that will be used in future updates.

La Roche[14] is a Phase IIIb, single-component, multicenter study of atezolizumab/bevacizumab, which is currently ongoing. Hack *et al*[15] is also currently ongoing, and is evaluating randomized patients included in an intervention dataset (atezolizumab/bevacizumab) and patients assigned to the control portion of the dataset undergoing active surveillance. Patients included in the control were allowed to crossover to the intervention dataset (atezolizumab/bevacizumab) after confirmed recurrence.

Lee *et al*[16] is part of an open-label, multicenter, multi-segmental, phase 1b study also known as GO30140 study, which enrolled patients at 26 academic centers and community oncology practices in seven countries worldwide. The study included five cohorts, but only the results of the two HCC cohorts, Groups A (atezolizumab monotherapy) and F, are described within this review article. Finn *et al*[12] compared the intervention dataset (atezolizumab/bevacizumab) with the control dataset (sorafenib monotherapy) and compared patients from 111 sites in 17 countries. Hence, details of the trials and participants are shown in Table 1.

As La Roche[14] and Hack *et al*[15] are currently ongoing, information for primary and secondary objectives are incomplete. It should be noted that while incomplete

Ongoing clinical trials	La Roche[14], 2020	Hack *et al*[15], 2020
Country of enrollment	Italy	170 sites in 25 countries (Asia)
Study design	Single-arm, multi-Center, randomized clinical control trial	Multi-center randomized open-label, clinical control trial
Study phase	IIIb	III
Study quality	NA (study is still ongoing)	NA (study is still ongoing)
Intervention	Atezolizumab plus bevacizumab	Atezolizumab plus bevacizumab
	Dose: atezolizumab 1200 mg IV infusion q3w + bevacizumab 15 mg/kg IV Q3W	Dose: atezolizumab 1200 mg every 3 wk + bevacizumab 15 mg/every 3 wk
Control	Standard of care	Active surveillance
	No specifications for control arm reported	
Number of patients	150	662
Intervention/control	Intervention not specified	Intervention 501
	Control: Not specified	Control: 119
Median age (range)	Not reported	Not reported
Intervention/control	Study included individuals > 18 yr	Study included individuals > 18 yr
-Duration of follow-up in mo	Not reported	Intervention: 8.6 mo
Intervention/control		Control: 6.5 mo
Types of outcomes reported	Overall survival	Overall survival
	Median progression-free survival	Median progression-free survival
	Grade 3-5 adverse events	Grade 3 or 4 adverse events
	Disease control	Disease control
	Objective response rate	
	Time to progression	
	Duration of response	
	Post-progression survival	
Data that could not be evaluated/data missing	NA (study is still ongoing)	NA (study is still ongoing)
Completed studies	Finn *et al*[12], 2020	Lee *et al*[16], 2020
Country/ies of Enrollment	111 sites in 17 countries, which include the United States, China, Japan, Germany, France, South Korea, Russia, Canada, and Taiwan	26 sites in 7 countries, which include the United States, Japan, South Korea, Taiwan, Australia, and New Zealand
Study design	Open-label, randomized clinical trial	Multi-arm study with five cohorts
		However, only the two cohorts focusing on hepatocellular carcinoma, Groups A and F, are described here in this study
Phase	III	Ib
Study Quality	Low risk of bias	Low risk of bias
Intervention	Atezolizumab plus bevacizumab	Atezolizumab plus bevacizumab
	Dose: 1200 mg atezolizumab + 15 mg/kg of bevacizumab IV q3w	Dose: 1200 mg atezolizumab + 15 mg/kg of bevacizumab IV q3w
Control	Sorafenib monotherapy	Atezolizumab monotherapy
	Dose: 400 mg sorafenib PO BID	Dose: 1200 mg atezolizumab
Number of patients	501	403
Intervention/control	Intervention: 336	Group A[1]: 104

Table 1 Characteristics of the two ongoing and two completed clinical trials

	Control: 165	**Group F+: 60**
		Control:
		59 included in efficacy analysis[1]
		58 included in safety analysis
		1 discontinued before receiving any treatment due to elevated alkaline phosphatase concentrations[1]
Median duration of follow-up (mo, [IQR])	Overall: 8.6 mo	Overall follow-up not given, see stratified data below
Intervention/control		Group A[1]: 12.4 (IQR 8.0-16.2)
	Intervention: 8.9	Group F+: 6.6 (IQR 5.5-8.5)
		Control: 6.7 (IQR 4.2-8.2)
	Control: 8.1	
Primary outcomes reported	Mortality rates	Mortality rates
	Hazard ratio for death	Hazard ratio for death
Secondary Outcomes reported	Overall survival	Overall survival
	Median progression free survival	Median progression free survival
	Grade 3-5 adverse events	Grade 3-4 adverse events
	Disease control	Disease control
	Objective response rate	Objective response rate
	Time to progression	Time to progression
	Duration of response	Duration of response
	Post-progression survival	Post-progression survival

[1]Group A: Patients with hepatitis B virus DNA of 500 IU/mL or less and ongoing anti-hepatitis B virus treatment for at least 3 mo before and at study entry; patients enrolled in Group F must have had hepatitis B virus DNA of 500 IU/mL or less measured up to 28 d before study entry and anti-hepatitis B virus treatment for at least 14 d before study entry. +: Group F: Patients in Child-Pugh class A, life expectancy of 3 mo and platelet count or > 75000/µL. NA: Not applicable.
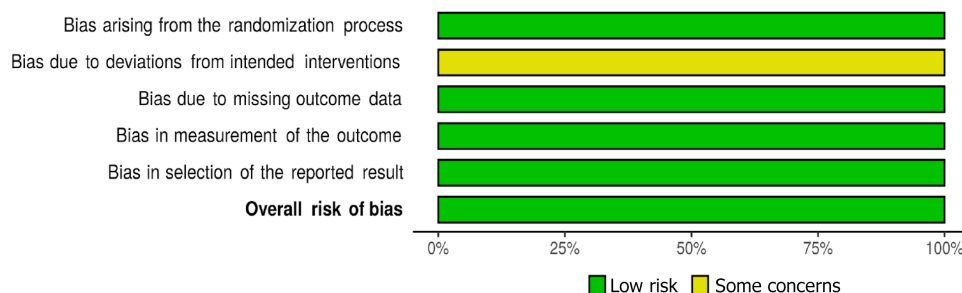

Figure 2 Summary plot of the risk of bias assessment of the two completed studies.

data were not assessed in this document, the authors of this manuscript fully intend to obtain updated data concerning related objectives in the future.

The Finn *et al*[12] and Lee *et al*[16] studies encompass a total of 724 patients and have been evaluated as follows. All clinical trials comprised a large sample of patients recruited from more than 310 sites across more than 20 countries. The countries included sites in North America (United States, Canada), Europe (United Kingdom, Germany, France, Italy, Poland, Spain, Russia, Czech Republic), and Asia-Pacific (China mainland, Japan, Republic of Korea, Taiwan, Australia, Hong Kong, Russia, Singapore, and New Zealand). The distribution of sites is shown in Figure 4. The specific patient profiles of La Roche[14] and Hack *et al*[15] have not yet been published (Table 1).
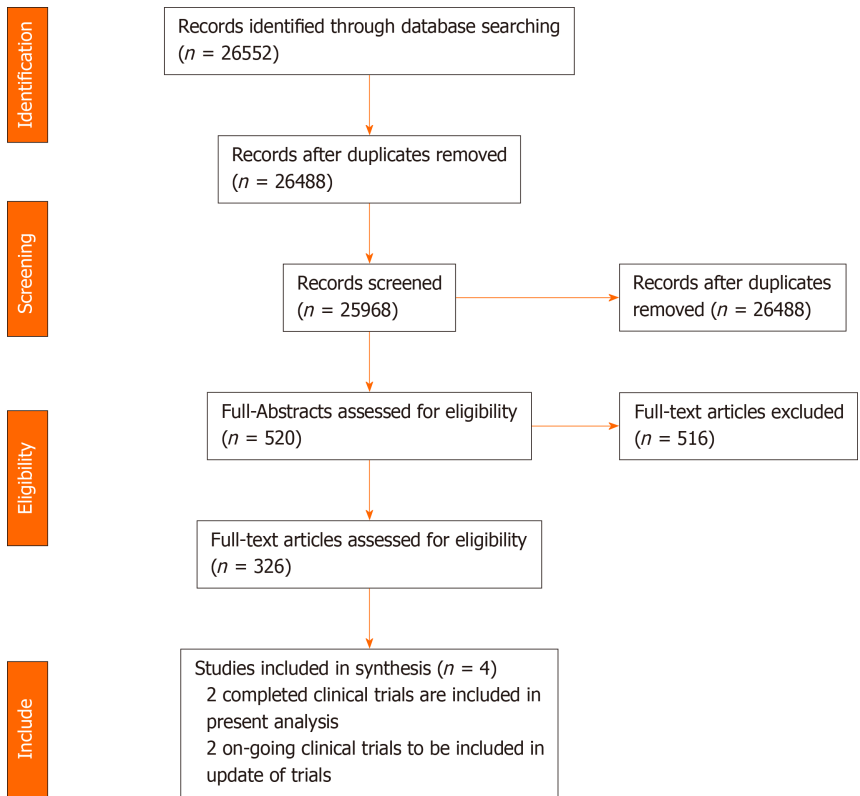
Figure 3 Preferred reporting items for systematic reviews and meta-analyses diagram.
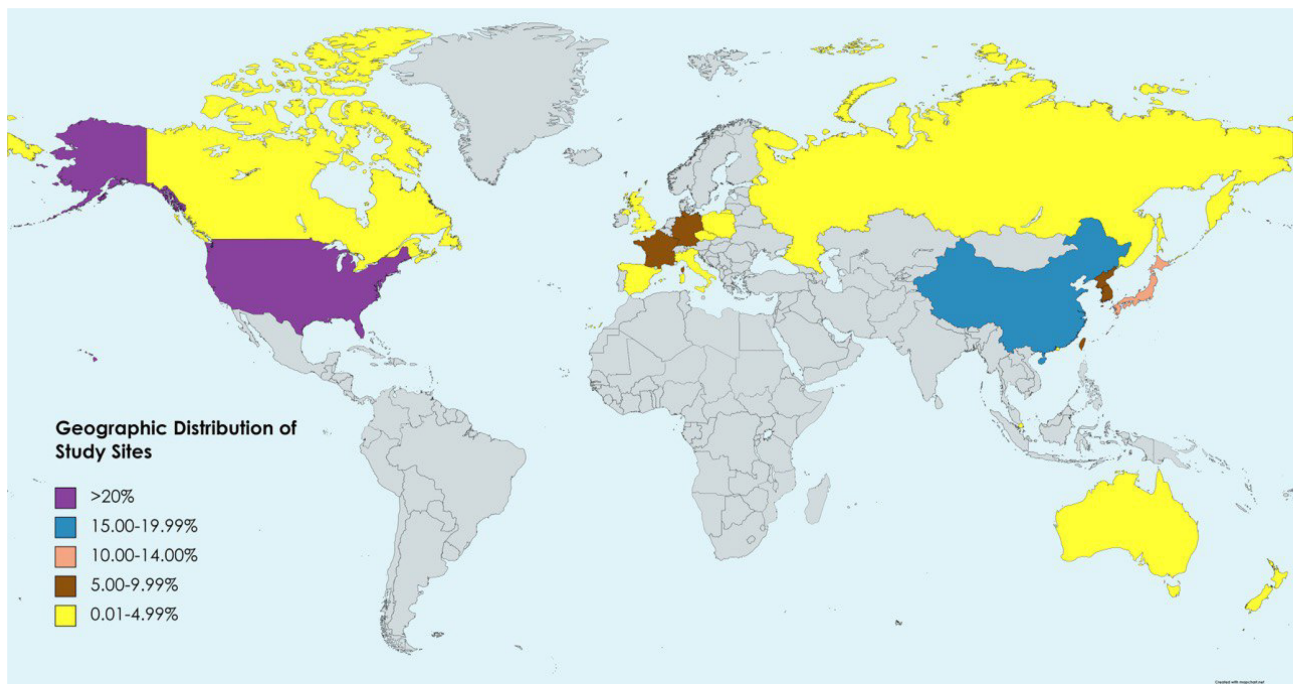
Figure 4 Distribution of study sites.

Participants

The clinical trials recruited adult volunteers of both genders, with locally advanced metastatic or unresectable HCC (or both). All trials used the American Association for the Study of Liver Disease criteria for histologic, cytologic, and clinical diagnostic confirmation. A documented hepatitis virological status was also required and a history of autoimmune disease was considered an exclusion criterion.

Eligible patients in the trials, who had not previously received systemic therapy for HCC, had an Eastern Cooperative Oncology Group (ECOG) performance score of either 0 or 1 and a Child-Pugh class of A or B (Supplementary Table 2). The studies attributed their exclusion of Child-Pugh class C to the increased risk of patient death due to related underlying cirrhosis. The patients with underlying cirrhosis were excluded from the study to avoid potentially confounding impact on the assessment of treatment-related antitumor efficacy. La Roche[14], Finn *et al*[12], and Lee *et al*[16] included patients who had HCC that was measurable as *per* the RECIST 1.1 (Supplementary Table 1). However, there was no specific mention of RECIST 1.1 in Hack *et al*[15].

Moreover, Hack *et al*[15], Finn *et al*[12], and Lee *et al*[16] employed BCLC staging (Supplementary Table 3). There was no specific mention of BCLC staging in La Roche [14].

Baseline characteristics of patients across the two completed trials and treatment modalities were adequately balanced (Table 2). Both studies had a median age range of about 63-years-old. Specifically, Finn *et al*[12] had a median age of 64 (interquartile range, [IQR], 56-71) and 66 (IQR, 59-71) years for its interventional (atezolizumab/bevacizumab) and control (sorafenib monotherapy), respectively. Whereas Lee *et al*[16] had a median age of 62 (IQR, 23-82) for Group A (atezolizumab/bevacizumab), 60 (IQR, 22-82) for Group F (atezolizumab/bevacizumab) and 63 (23-85) for the control (atezolizumab monotherapy), respectively. Both studies predominantly included the male sex (83%), Asian (62%) and Caucasian (30%) ethnicities, Child-Pugh class A (99%), and advanced BCLC (stage C) (84%) in the treatment and control groups. Both studies reported a higher prevalence of patients with extrahepatic spread, positivity for hepatitis B, and a history of alcohol use. Finn *et al*[12] included mostly ECOG 0 patients than ECOG 1, while the opposite was observed for Lee *et al*[16] Only about 35% of patients across the studies had alpha-fetoprotein > 400 ng *per* milliliter. Regarding PD-L1 status, more patients had tumor cell or immune cell ≥ 1% than any other classification, across treatment and control for both studies. Finn *et al*[12] showed the number of patients who previously experienced local therapy for HCC, and almost half of the patients had at least one treatment on both the atezolizumab/bevacizumab (48%, 161/336 patients) and sorafenib (52%, 85/165 patients) arms. Whereas Lee *et al*[16] did not show those patients who had prior local therapy for HCC.

There were no significant differences that were explicitly stated between interventional and control in the published manuscripts of Finn *et al*[12] and Lee *et al*[16]. The following baseline characteristics were evaluated for differences: Median age, sex, race, geographic region, Child-Pugh class, ECOG stage, BCLC stage, alpha-fetoprotein levels, macrovascular invasion, extrahepatic spread, hepatitis status, alcohol use, PD-L1 status, and prior local therapy for HCC. Moreover, regarding gastroesophageal varices (current/treated), there were no specific indications of statistically significant differences between the interventional and control groups in Finn *et al*[12] as well. On the other hand, Lee *et al*[16] did not report the prevalence of varices but stated that varices were managed when present, according to the standard of care.

Primary outcomes

Mortality rates: According to Finn *et al*[12], mortality occurred in 28.6% of patients (96/336) in the atezolizumab/bevacizumab group during a follow-up duration of 8.9 mo at the clinical data cut-off, and was significantly lower than that reported in the sorafenib group (39.4%; 65/165 patients) during a similar surveillance time of 8.1 mo ($P < 0.001$ by χ^2) (Table 3).

The overall mortality reported by Lee *et al*[16] in the atezolizumab/bevacizumab group was significantly different (higher in Group A, 27% [16/104 patients] and zero in Group F, [0/60 patients]; $P < 0.0001$ by χ^2), and was significantly lower than that in the atezolizumab group (31%; 18/59 patients) at a median follow-up of 12.4 mo ($P < 0.0001$ by χ^2). Lee *et al*[16] also showed a 7% mortality (7/10 patients) in Group A and did not report deaths related to AEs in Group F.

Using epidemiological analyses, this review estimated the relative risk (RR) of death from the combination therapy *vs* the monotherapy to be 0.72 (Finn *et al*[12]) and 0.87 (Lee *et al*[16]), respectively. The calculated RR reduction was 0.28 and 0.13, respectively, for both studies. The attributable risk was 0.108 (Finn *et al*[12]) and 0.04 (Lee *et al*[16]), and the number needed to treat (NNT) 9.2 for atezolizumab/bevacizumab *vs* sorafenib (Finn *et al*[12]), whereas the NNT was 25 for atezolizumab/bevacizumab *vs* atezolizumab alone (Lee *et al*[16]).

	Finn *et al*[12], 2020		Lee *et al*[16], 2020		
	Interventional arm	Control arm	Interventional arm		Control arm
	Atezolizumab-bevacizumab combination therapy	Sorafenib monotherapy	Atezolizumab-bevacizumab combination therapy given in both Arms A and F		Atezolizumab monotherapy
			Group A	Group F+	
	n = 336	*n* = 165	*n* = 104	*n* = 60	*n* = 59
Median age (IQR), yr	64 (56-71)	66 (59-71)	62 (23-82)	60 (22-82)	63 (23-85)
Gender, *n* (%)					
Male	277 (82)	137 (83)	84 (81)	54 (90)	49 (83)
Female	59 (18)	28 (17)	20 (19)	6 (10)	10 (17)
Race, *n* (%)					
White	123 (37)	52 (32)	20 (19)	14 (23)	9 (15)
Asian	188 (56)	96 (58)	75 (72)	45 (75)	47 (80)
Black or African American	6 (1.8)	4 (2.4)	7 (7)	1 (2)	2 (3)
Native Hawaiian or other Pacific Islander	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)
Unknown	19 (6)	12 (7)	2 (2)	0 (0)	0 (0)
Geographic region, *n* (%)					
Asian excluding Japan	133 (40)	68 (41)	59 (57)	39 (65)	39 (66)
Rest of the world (United States, Australia, New Zealand, and Japan)	203 (60)	97 (59)	45 (43)	21 (35)	20 (34)
Child Pugh, *n* (%)					
Child Pugh A5	239 (72)	121 (73)	77 (74)	43 (72)	42 (71)
Child Pugh A6	94 (28)	44 (27)	21 (20)	17 (28)	17 (29)
Child Pugh A7	0 (0)	0	6 (6)	17 (28)	17 (29)
Child Pugh B	1 (< 1)	0 (0)	NA	NA	NA
ECOG performance status, *n* (%)					
ECOG 0	209 (62)	103 (62)	52 (50)	27 (45)	25 (42)
ECOG 1	127 (38)	62 (38)	52 (50)	33 (55)	34 (58)
BCLC, *n* (%)					
BCLC stage A (early)	8 (2)	6 (4)	0 (0)	0 (0)	2 (3)
BCLC stage B (intermediate)	52 (15)	26 (16)	10 (10)	6 (10)	4 (7)
BCLC stage C (advanced)	276 (82)	133 (81)	94 (90)	54 (90)	53 (90)
Alpha-fetoprotein > 400 ng *per* milliliter	126 (38%)	61 (37%)	37 (36%)	18 (30%)	19 (32%)
Macrovascular invasion	129 (38%)	71 (43%)	55 (53%)	20 (33%)	25 (42%)
Extrahepatic spread	212 (63%)	93 (56%)	91 (88%)	47 (78%)	50 (85%)
Hepatitis B	164 (49%)	76 (46%)	51 (49%)	34 (57%)	32 (54%)
Hepatitis C	72 (21%)	36 (22%)	31 (30%)	11 (18%)	10 (17%)
Non-viral	100 (30%)	85 (52%)	22 (21%)	15 (25%)	17 (29%)
Alcohol use, *n* (%)					
Previous	166 (50)	79 (48)	58 (56)	39 (65)	32 (54)
Never	121 (36)	61 (37)	32 (31)	14 (23)	21 (36)

Current	48 (14)	25 (15)	14 (13)	7 (12)	6 (10)
Varices at baseline	88 (26%)	43 (26%)	NA	NA	NA
Varices treated at baseline	36 (11%)	23 (14%)	NA	NA	NA
PD-L1 status, *n* (%)	124	58	NA	NA	NA
TC and IC < 1%	45 (36)	25 (43)	25 (24)	15 (25)	18 (31)
TC or IC ≥ 1%	79 (64)	33 (57)	61 (59)	28 (47)	34 (58)
TC ≥ 5% or IC ≥ 5%	46 (37)	17 (29)	37 (36)	8 (13)	16 (27)
TC ≥ 10% or IC ≥ 10%	12 (10)	5 (9)	30 (29)	5 (8)	6 (10)
Data missing	NA	NA	18 (17)	17 (28)	8 (14)
Prior local therapy for HCC, *n* (%)					
At least one treatment	161 (48)	85 (52)	NA	NA	NA
Transarterial chemoembolization	130 (39)	70 (42)	NA	NA	NA
Radiofrequency ablation	47 (14)	24 (15)	NA	NA	NA
Prior radiotherapy	34 (10)	17 (10)	NA	NA	NA

BCLC: Barcelona Clinic Liver Cancer stage; ECOG: Eastern Cooperative Oncology Group; HCC: Hepatocellular carcinoma; IC: Immune cells; IQR: Interquartile range; NA: Not available; PD-L1: Programmed death-ligand 1; TC: Tumor cells.

Hazard ratio for deaths and PFS: According to Finn *et al*[12], the stratified hazard ratio (HR) for death was 0.58 (95% confidence interval [CI]: 0.42-0.79; *P* < 0.001), whereas Lee *et al*[16] did not report on HR for death but rather estimated the HR for PFS stratified HR 0.55 (80%CI: 0.40-0.74; *P* = 0.011) in Group F (Table 3).

Secondary outcomes

Overall/median survival: According to Finn *et al*[12], the estimated rates of OS at 6 and 12 mo were 84.8% (95%CI: 80.9-88.7) and 67.2% (95%CI: 61.3-73.1) in the atezolizumab/bevacizumab group, respectively. These results were significantly higher compared to 72.2% (95%CI: 65.1-79.4) and 54.6% (95%CI: 45.2-64.0) in the sorafenib group, respectively (*P* < 0.001) (Table 3). For Lee *et al*[16], median OS in Group A atezolizumab/bevacizumab was 17.1 mo (95%CI: 13.8 to not estimable), with only 55% (57 patients) still alive at the end of the surveillance. Median OS was not reached in atezolizumab/bevacizumab Group F. Additionally, neither Group F nor the atezolizumab group had estimable results as follows: (atezolizumab/bevacizumab: 95%CI: 8.3 mo to not estimable; atezolizumab group: 8.2 mo to not estimable).

Median PFS: Both studies reached significantly longer PFS in the atezolizumab/bevacizumab dataset *vs* their respective monotherapy data set. In detail, median PFS was 6.8 mo (95%CI: 5.7-8.3) for patients treated with atezolizumab/bevacizumab compared to 4.3 mo (95%CI: 4.0-5.6) for patients treated with sorafenib alone in Finn *et al*[12] study (*P* < 0.001). On the other hand, for Lee *et al*[16], median PFS was 7.3 mo (95%CI: 5.4-9.9) and 5.6 mo (95%CI: 3.6-7.4) in Group A and Group F (atezolizumab/bevacizumab), respectively, *vs* 3.4 mo (95%CI: 1.9-5.2) for atezolizumab monotherapy (*P* < 0.001) (Table 3).

Disease progression or death: At baseline, Finn *et al*[12] reported higher extrahepatic spread in 212/336 patients (63%) in the atezolizumab/bevacizumab group compared to 93/165 patients (56%) in the sorafenib group (*P* = 0.0005, by χ^2) (Table 2). These percentages were significantly lower than those observed by Lee *et al*[16], where extrahepatic spread was shown in 91/104 (88%), 47/50 (78%), and 50/69 (85%) patients, for Groups A, F (atezolizumab/bevacizumab), and atezolizumab monotherapy, respectively (*P* = 0.004 by χ^2). Moreover, in the study of Finn *et al*[12] atezolizumab/bevacizumab and sorafenib groups experienced similar disease progression (58.6% [97/336 patients] *vs* 66.1% [109/165 patients]; *P* = 0.10 by χ^2) (Table 3). The stratified HR for progression or death was estimated to be 0.58 (95%CI: 0.42-0.79; *P* < 0.001).

Tumor response rate: A better overall tumor-confirmed objective response with combination therapy compared to the respective monotherapies in the control groups

Table 3 Summary of the efficacy and safety findings

Ref.	Finn *et al*[12], 2020		Lee *et al*[16], 2020		
Schemes	Atezolizumab-bevacizumab combination therapy	Sorafenib monotherapy	Atezolizumab-bevacizumab combination therapy given in both Arm A and F		Atezolizumab monotherapy
Total patients			Group A	Group F+	
	n = 336	*n* = 165	*n* = 104	*n* = 60	*n* = 59
Primary efficacy outcomes					
Mortality					
n (%)	96 (28.6)	65 (39.4)	16 (27)	0 (0)	18 (31)
Two-tail *P* value	*P* = 0.0033	*P* = 0.0033	No *P* value reported	No *P* value reported	No *P* value reported
HR for disease progression, CI	0.59, 95%CI: 0.47-0.76	Not applicable	NA	NA	NA
Two-tail *P* value	*P* < 0.001				
HR for death, CI	0.58, 95%CI: 0.42-0.79	NA	NA	NA	NA
Two-tail *P* value	*P* < 0.001				
HR for progression-free survival, CI	NA	NA	0.55, 80%CI: 0.40-0.74		NA
Two-tail *P* value			*P* = 0.011		
Secondary efficacy outcomes tumor survival and progression of disease					
Overall/survival rate, *n* (%)	*n* not explicitly reported	*n* not explicitly reported	57 (55)	16 (27)	18 (31)
n (%)	-67.2	-54.6			
95%CI			CI not reported	CI not reported	CI not reported
	61.3-73.1	45.2-64			
Median overall survival in mo	Not estimable	13.2 mo	17.1 mo	Median overall survival was not reached	Median overall survival was not reached
95%CI					
		(10.4 to not estimable)	(13.8 to not estimable)	(8.3 to not estimable)	(8.2 to not estimable)
6 mo overall survival rates			NA	NA	NA
95%CI	84.80%	72.20%			
	80.9-88.7	80.9-88.7			
12 mo overall survival rates	67.20%	54.60%	NA	NA	NA
95%CI	61.3-73.1	45.2-64			
Median progression-free survival (mo), (95%CI)	6.8 mo	4.3 mo	7.3 mo	5.6 mo	3.4 mo
	(5.7-8.3)	(4.0-5.6)	(5.4-9.9)	(3.6-7.4)	(1.9-5.2)
Overall confirmed objective response				*n* not explicitly reported (20%)	*n* not explicitly reported (17%)
n (%) as *per* RECIST 1.1				(11-32)	(8-29)
95%CI	89 (27.3%)	19 (11.9%)	37 (36%)		
	(22.5-32.5)	(7.4-18)	(26-46)		
Confirmed objective response-complete response as *per* RECIST 1.1, *n* (%)	18 (5.5)	0 (0)	12 (12)	1 (2)	3 (5)

Confirmed objective response-Partial response as *per* RECIST 1.1, *n* (%)	71 (21.8)	19 (11.9)	25 (24)	11 (18)	7 (12)
Stable disease *n* (%) as *per* RECIST 1.1	151 (46.3)	69 (43.4)	37 (36)	28 (47)	19 (32)
Progressive disease					
n (%) as *per* RECIST 1.1	64 (19.6)	39 (24.5)	25 (24)	17 (28)	25 (42)
Disease control rate, *n* (%)	240 (73.6)	88 (55.3)	74 (71)	40 (67)	29 (49)
Ongoing objective response at data cut off, *n* (%)	77/89 (86.5)	13/19 (68.4)	NA	NA	NA
Safety outcomes (adverse events)					
Overall patients with an adverse event from any cause, *n* (%)	323 (98.2)	154 (98.7)	100 (96)	57 (95)	52 (90)
Treatment-related serious adverse events, *n* (%)	125 (38)	48 (30.8)	25 (24)	7 (12)	2 (3)
Treatment-related mortality	161 deaths (%) It was not explicitly stated how many deaths there were in relation to treatment in either intervention or control arm[1]		3 (3%)	0 (%)	NA
Adverse events leading to dose modifications, *n* (%)	163 (49.5)	95 (60.9)	50 (48)	9 (15)	5 (9)
Adverse events leading to withdrawal from any trial drug, *n* (%)	51 (15.5)	16 (10.3)	18 (17)	6 (10)	0 (0)
Number of participants with Grade 3 and above, *n* (%)	5-15 (4.6)	9 (5.8)	55 (53)	22 (37)	8 (14)
Types of Grade 3-4 adverse events					
Adverse events	Note: All stratified data reported below are Grade 3 or 4		Note: All stratified data reported below are Grade 3, except increased aspartate aminotransferase (note stratification)		
Hypertension, *n* (%)	50 (15.2)	19 (12.2)	15 (14)	3 (5)	1 (1)
Decreased appetite, *n* (%)	4 (1.2)	6 (3.8)	1 (1)	0 (0)	0 (0)
Fatigue, *n* (%)	8 (2.4)	5 (3.2)	1 (1)	0 (0)	0 (0)
Pyrexia, *n* (%)	4 (1.2)	2 (1.3)	2 (2)	0 (0)	0 (0)
Rash, *n* (%)	0 (0)	4 (2.6)	0 (0)	0 (0)	0 (0)
Diarrhea, *n* (%)	6 (1.8)	8 (5.1)	3 (3)	1 (2)	0 (0)
Abdominal pain, *n* (%)	4 (1.2)	4 (2.6)	4 (4)	0 (0)	0 (0)
Constipation, *n* (%)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
Cough, *n* (%)	0 (0)	1 (0.6)	0 (0)	0 (0)	0 (0)
Nausea, *n* (%)	1 (0.3)	1 (0.6)	NA	NA	NA
Weight decrease, *n* (%)	0 (0)	1 (0.6)	NA	NA	NA
Epistaxis, *n* (%)	0 (0)	1 (0.6)	NA	NA	NA
Asthenia, *n* (%)	1 (0.3)	4 (2.6)	NA	NA	NA
Infusion-related reaction, *n* (%)	8 (2.4)	0 (0)	NA	NA	NA
Palmar-Plantar	0 (0)	13 (8.3)	NA	NA	NA

erythrodysesthesia syndrome, *n* (%)					
Proteinuria, *n* (%)	10 (3)	1 (0.6)	7 (7)	3 (5)	0 (0)
Increased aspartate aminotransferase, *n* (%)	23 (7.0)	8 (5.1)	Grade 3: 3 (3)	2 (3)	2 (3)
			Grade 4: 2 (2)		
Increased alanine aminotransferase, *n* (%)	12 (3.6)	2 (1.3)	NA	NA	NA
Blood bilirubin increase, *n* (%)	8 (2.4)	10 (6.4)	NA	NA	NA
Decreased platelet count, *n* (%)	11 (3.3)	2 (1.3)	5 (5)	0 (0)	0 (0)
Pancreatitis, *n* (%)	1 (0.3)	2 (1.3)	NA	NA	NA
Hepatic Encephalopathy, *n* (%)	2 (0.6)	2 (1.3)	NA	NA	NA
Pulmonary Embolism, *n* (%)	3 (0.9)	2 (1.3)	NA	NA	NA
Cholangitis, *n* (%)	4 (1.2)	1 (0.6)	NA	NA	NA
Acute kidney failure, *n* (%)	1 (0.3)	3 (1.9)	NA	NA	NA
Gastrointestinal hemorrhage, *n* (%)	4 (1.2)	3 (1.9)	NA	NA	NA
Esophageal varices hemorrhage, *n* (%)	6 (1.8)	1 (0.6)	NA	NA	NA
Upper gastrointestinal hemorrhage, *n* (%)	2 (0.6)	2 (1.3)	NA	NA	NA
Asthenia, *n* (%)	1 (0.3)	4 (2.6)	NA	NA	NA
Types of Grade 5 adverse events					
Grade 5 adverse events, *n* (%)	15 (4.6)	9 (5.8)	0 (0)	0 (0)	0 (0)
	Not stratified[1]	Not stratified[1]			
Not evaluable/data missing					
Not evaluable, *n* (%)	8 (2.5)	14 (8.8)	NA	NA	NA
Data missing, *n* (%)	14 (4.3)	18 (11.3)	NA	NA	NA

[1]Group A: Patients with hepatitis B virus DNA of 500 IU/mL or less and ongoing anti-hepatitis B virus treatment for at least 3 mo before and at study entry. Patients enrolled in group F must have had hepatitis B virus DNA of 500 IU/mL or less measured up to 28 d before study entry and anti-hepatitis B virus treatment for at least 14 d before study entry. +: Group F: Patients in Child-Pugh class A, life expectancy of 3 mo and platelet count or > 75000/μL; CI: Confidence interval; HR: Hazard ratio; NA: Not available.

was reported. For Finn *et al*[12], a significantly higher overall tumor response was observed in 89/336 patients (27.3%; 95%CI: 22.5-32.5) with atezolizumab/ bevacizumab combination therapy than the one obtained with sorafenib in 19/165 patients (11.9%; 95%CI: 7.4-18.0) ($P < 0.001$ by χ^2). Whereas Lee *et al*[16] detected a better overall tumor response in Group A of the combination therapy compared to Group F and the atezolizumab group, which were similar (36% [95%CI: 26-46] *vs* 20% [95%CI: 11-32] *vs* 17% [95%CI: 8-29]; $P = 0.011$). Further details concerning the other indices of tumor response, including the confirmed partial/complete/ongoing objective responses are shown in Table 3.

Disease control rate: The disease control rate was significantly higher in both trials for atezolizumab/bevacizumab combination therapy than sorafenib or atezolizumab monotherapies (Table 3). The estimates were 73.6% (240/336 patients) *vs* 55.3% (88/165 patients) for atezolizumab/bevacizumab and sorafenib ($P < 0.001$ by χ^2), and 71% (74/104 patients), 67% (40/60 patients), and 49% (29/58 patients) for Group A, Group F and atezolizumab ($P = 0.016$ by χ^2), respectively.

Safety outcomes and AEs

Overall AEs: Finn *et al*[12] estimated similar AEs that were contributed from any cause in 98.2% (323/336 patients) and 98.7% (154/165 patients) for the atezolizumab/bevacizumab *vs* sorafenib monotherapy groups ($P = 0.17$ by χ^2), respectively. Likewise, according to Lee *et al*[16], 96% (100/104 patients), 95% (57/60 patients), and 90% (52/58 patients) for Groups A/F (atezolizumab/bevacizumab) and atezolizumab ($P = 0.13$ by χ^2), respectively (Table 3).

Treatment-related serious AEs: Finn *et al*[12] reported higher treatment-related AEs with atezolizumab/bevacizumab compared to sorafenib monotherapy (38% [125/336 patients] *vs* 30.8% [48/165 patients]; $P < 0.0001$ by χ^2). Lee *et al*[16] recorded 24% (25/104 patients), 12% (7/60 patients), and 3% (2/58 patients) for Groups A/F (atezolizumab/bevacizumab) and atezolizumab ($P < 0.001$ by Fisher's Exact Test), respectively (Table 3).

Grade 3-5 AEs: Details of the treatment-related deaths and severe AEs as well as other indices of safety are shown in Table 3. Finn *et al*[12] and Lee *et al*[16] reported Grade 3-5 AEs in 10% and 15% of participants, respectively. In the Finn *et al*[12] study, hypertension occurred in 15.2% and 12.2% for the combination therapy and sorafenib monotherapy groups, and 14%, 5%, and 1% in Groups A/F (atezolizumab/bevacizumab) and atezolizumab, respectively (Lee *et al*[16]). Furthermore, Proteinuria occurred in 3% and 0.6% in the combination therapy and sorafenib monotherapy groups, respectively, whereas in 7%, 5%, and 0% for Groups A/F (atezolizumab/bevacizumab) and atezolizumab groups, respectively. Other Grade 3-5 AEs that were reported included abdominal pain, fatigue, rashes, pyrexia, and palmar-plantar erythrodysesthesia syndrome (Table 3). Finn *et al*[12] registered fewer Grade 5 AEs in the combination therapy compared to sorafenib monotherapy (4.6% *vs* 5.8%), whereas Lee *et al*[16] study registered 0% of such events.

RoB of included studies

The RoB assessment for Finn *et al*[12] and Lee *et al*[16] trials resulted in high quality with a low RoB. However, in the two ongoing studies (La Roche[14] and Hack *et al*[15]), the RoB was not evaluated given that incomplete information existed (Figures 1 and 2).

Randomization and allocation concealment

Both completed trials (Finn *et al*[12] and Lee *et al*[16]) showed adequate randomization with a low RoB arising from the randomization process that was performed through an interactive voice-response or Web-response system in permuted blocks. There was also fair allocation concealment. For Lee *et al*[16], an independent statistician was responsible for generating the randomization sequence, which was subsequently stored within the interactive voice systems.

Blinding and bias arising from deviations from intended interventions

In both completed studies (Finn *et al*[12] and Lee *et al*[16]), open-label trials were implemented, and consequently had neither blinding nor masking of interventions. Lee *et al*[16] described 26 participants who deviated from the initially assigned atezolizumab/bevacizumab to atezolizumab monotherapy without describing post-crossover efficacy and safety results. Moreover, Finn *et al*[12] found it less cumbersome to not administer intravenous placebo infusions. Hence, due to the lack of blinding or masking and because of deviations from intended interventions, the two completed studies were estimated to have some RoB concerns.

Bias arising from incomplete or missing outcome data, measurement of the outcome, and selection of the reported results

All RCT results showed a low risk of attrition bias from the missing outcome data. Both studies also had appropriate measurements of survival outcomes (Finn *et al*[12]). For example, both used PFS and objective tumor response with the RECIST 1.1 (Supplementary Table 1) as well as the HCC-specific mRECIST by investigator assessment and independent faculty review. Thus, they displayed a low risk of measurement bias as well as lower selective reporting bias.

DISCUSSION

In the past, early-stage HCC has been treated with surgical treatment and/or thermal ablation. These procedures have been associated with high recurrence rates and therefore considered to have poor prognosis. The development of immunotherapy has led to new alternatives in treating HCC patients with advanced stages of the disease, who are considered unresectable with the standard surgery. Different treatments have been used for unresectable cases of HCC, including ICIs and VEGF inhibitors such as sorafenib, atezolizumab, and bevacizumab (Supplementary Figure 2).

In this systematic review, the results of Finn *et al*[12] and Lee *et al*[16] studies were summarized. The purpose was to combine with their studies the ongoing results of the two additional trials of La Roche[14] and Hack *et al*[15] to determine which of the therapeutic regimens could support a stellar efficacy and safety profile. Two clinical trials included 724 participants in about 137 sites in over 19 different countries were identified (Figure 4). The completed trials of Finn *et al*[12] and Lee *et al*[16] recruited participants mostly from the United States, Canada, the United Kingdom, Germany, France, Italy, Poland, Spain, Russia, Czech Republic, China mainland, Japan, Republic of South Korea, Taiwan, Australia, Hong Kong, Russia, Singapore, and New Zealand, which are mostly high and middle-income countries[17]. Both trials were assessed as having an overall low RoB outcome.

The results of this review demonstrate that the combination of atezolizumab/bevacizumab had a beneficial effect on improving the overall efficacy and safety in treating patients with early-stage HCC compared to sorafenib or atezolizumab monotherapy. The combination therapy resulted in the prevention of mortality, increased OS and PFS rates, as well as better disease control and response rate. However, the proportion of participants with AEs from any cause were similar in both trials (Table 3).

Specifically, Finn *et al*[12] showed a higher OS rate with atezolizumab/bevacizumab than sorafenib groups along with a 42% reduced hazard of death at 6 and 12 mo of surveillance[12]. PFS rate and time were significantly higher in the atezolizumab/bevacizumab group when compared to sorafenib (54.5% *vs* 37.2% and 6.8 mo *vs* 4.3 mo, respectively)[12]. Finn *et al*[12] reported a significantly lower mortality rate in the atezolizumab/bevacizumab than the sorafenib group at a median follow-up of 8.6 mo (28.6% *vs* 39.4%). The overall confirmed objective response, disease control rate, and median time to deterioration of quality of life were better in the atezolizumab/bevacizumab group compared to the sorafenib group[12]. The objective response in the Finn *et al*[12] and Lee *et al*[16] studies were more than two times higher using the combination therapy than the monotherapy (27.3% *vs* 11.9% and 36% *vs* 17%, respectively).

In the Lee *et al*[16] study, the atezolizumab/bevacizumab combination was given in both Groups A and F as specified in the study protocol. Median OS was estimated to be 17.1 mo in the atezolizumab/bevacizumab group but was not estimated for the atezolizumab monotherapy group. In both Groups A and F, objective response was confirmed with the primary endpoint according to RECIST 1.1. Secondary efficacy outcomes were also achieved that included objective response (based on RECIST 1.1 assessment) and independent review assessment (HCC specific mRECIST) showing PFS, duration of response, and time to radiographic progression. Irrespective of PD-L1 status, there was a progressive survival benefit with atezolizumab/bevacizumab compared to the atezolizumab monotherapy. This progressive survival benefit was observed despite the difficulty experienced comparing efficacy within Groups A and F due to the varied follow-up periods[16]. Moreover, Lee *et al*[16] reported that responses with long surveillance were expected to change in Group F. Lee *et al*[16] also recorded a 27% and 0% mortality in the combination therapy Groups A and F, which was significantly lower compared to the 31% in the monotherapy group at the median 12.4 mo follow-up duration. The overall response rate was statistically significant in the combination therapy group (atezolizumab/bevacizumab) compared to the monotherapy group, especially with sorafenib[16]. Thus, in this study[16] the primary endpoint was PFS as *per* RECIST 1.1 and OS. IMbrave 150 results demonstrated a significantly better PFS, OS, and response rate with atezolizumab/bevacizumab combination therapy than with sorafenib[18].

Ongoing trials by La Roche[14] and Hack *et al*[15] are phase III randomized trials with atezolizumab/bevacizumab. Although the trials have not yet been finalized, the results to date are considered meaningful and support the objective of their study. In Finn *et al*[12], and Lee *et al*[16] studies, the profile of safety outcomes were rather comparable with the exception concerning the AEs related to dose modifications which were lower in the combination (atezolizumab/bevacizumab) therapy group

than monotherapy (sorafenib) group (Finn *et al*[12]). Moreover, Finn *et al*[12] also showed a slightly higher rate of AEs with Grade 3 and above, especially regarding objective response in the atezolizumab/bevacizumab group compared to the sorafenib group (86.5% *vs* 68.4%, respectively). The rate of AEs leading to withdrawal from any drug trials in the atezolizumab/bevacizumab therapy group was significantly greater compared to the sorafenib group (15.5% *vs* 10.3%, respectively). The rate of AEs more than Grade 3 was less in the atezolizumab/bevacizumab group than the sorafenib group (4.6% *vs* 5.8%, respectively).

Overall, combination therapy demonstrated a better safety profile in both studies. There was a difference in the types of Grade 3-5 AEs reported in Finn *et al*[12] such as palmar-plantar erythrodysesthesia syndrome and increased bilirubin as well as infusion-related reactions, which were not reported in the study by Lee *et al*[16]. In Finn *et al*[12], hypertension occurred at a slightly higher rate in the atezolizumab/bevacizumab group than in the sorafenib monotherapy group (15% *vs* 12%, respectively). Infusion-related reaction only occurred in the atezolizumab/bevacizumab group (2.4% of cases) and additionally, palmar-plantar erythrodysesthesia syndrome was detected in the sorafenib group (8.3%). Furthermore, the occurrence of abdominal pain and asthenia was low in the atezolizumab/bevacizumab group (1.2% and 0.3%, respectively) compared to the sorafenib group (2.6% and 2.6%, respectively). There was also a mild increase in proteinuria (3%), aspartate aminotransferase (7%), alanine aminotransferase (3.6%) in those treated with atezolizumab/bevacizumab combination therapy compared to those treated with sorafenib alone (proteinuria [0.6%], aspartate aminotransferase [5.1%], alanine aminotransferase [1.3%]). There was also a slight decrease in platelet count in the atezolizumab/bevacizumab group (3.3%) compared to sorafenib monotherapy (1.3%)[12].

In Lee *et al*[16], AEs leading to withdrawal from the trial were reported only in Groups A and F (17% and 10%, respectively). The rate of AEs Grade 3 and above was greater in combination therapy (53% for Group A and 37% for Group F) than atezolizumab monotherapy (14%). Some differences in the Grade 3-5 types of AEs especially were identified with Group A when compared to atezolizumab alone. For instance, when considering the prevalence of hypertension as one of the most commonly occurring AEs, it was present in 14% of Group A and 5% of patients in Group F. Thus, these figures were slightly higher than the atezolizumab group (1%). Additionally, fatigue, abdominal pain, and asthenia occurred only in 1% and 4% of patients in Group A, when compared to Group F or atezolizumab monotherapy. Proteinuria in combination therapy was similar in Groups A and F (7% *vs* 5%)[16].

A major limitation of this review was that supportive evidence was based on a limited number of completed clinical trials used in the treatment of HCC. Moreover, there was inadequate applicability in low-income countries or developing countries where these novel immune therapies may not be available. Despite the limitations, the analysis of the studies reviewed in this document was considered overall satisfactory.

Additional study limitations included that both Lee *et al*[16] and Finn *et al*[12] studies were open-label trials that held a higher risk for bias. Although independent faculty reviewers were used to reduce the potential biases, there were no blinding or masking of the investigators and participants, thus further sustaining the potential for bias. In Lee *et al*[16], it was challenging to compare the efficacy among Groups A and F due to their different follow-up periods. Also, Lee *et al*[16] reported crossover participants from monotherapy to combination therapy; however, post-crossover results were not mentioned. The aforementioned could have further created some kind of reporting bias, decreasing the quality of the study. Although both RCTs were satisfactory in terms of quality, they did not present robust evidence. Additionally, in-depth cost-effectiveness analysis, which could have provided further support, was not performed.

It is of great importance to consider cost-effectiveness for combination therapies to be effectively administered worldwide. A study (Hou and Wu[19], 2020) conducted in China, stated that in the base-case analysis, atezolizumab/bevacizumab gained a marginal 0.811 quality-adjusted life-year (QALY) and 1.297 overall life-years with an augmented cost of $49994 as compared with sorafenib, which led to an incremental cost-effectiveness ratio (ICER) of $61613/QALY[16]. The study by Wang *et al*[20] (2021), conducted using the IMIbrave 150 trial evaluation, reported that atezolizumab/bevacizumab treatment resulted in an increase of 0.623 Life-years, 0.484 QALYs, and $158781 *per* patient at the base case analysis[20]. The ICER was $322500 *per* QALY (95%CI: 136275-801509 *per* QALY)[20]. The negative incremental net benefit of -0.810 QALY reported by Hou *et al*[19] (2020) as well as the ICER of $322500 *per* QALY reported by Wang *et al*[20] (2021) was considered to be rather expensive for combination therapy implementation.

Conversely, both completed trials[12,16] demonstrated promising results in terms of better combination therapy efficacy with atezolizumab/bevacizumab compared to monotherapy (either sorafenib or atezolizumab). PD-1 and PD-L1 play key roles by escaping tumor immune surveillance in tumor progression and survival[15,21]. The PD-1 and PD-L1 antibody inhibitors act against PD-1 or PD-L1, and stimulate T-cell mediated immunity. Although PD-1 is mostly expressed on T cells, they also activate PD-L1 on cancer cells and antigen-presenting cells[15,21]. Therefore, PD-L1 inhibitors cause the resurrection of T-cell mediated anti-tumor immune effects unless other T-cell regulatory proteins are blocked such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), thus resulting in improved cancer immunotherapy[15,21].

The CTLA-4 antibody inhibitor (ipilimumab) and PD-1 inhibitors (pembrolizumab, nivolumab) have both been approved for treating various solid tumors including lung cancer, renal cell cancer, and ovarian cancer. Other anti-tumor agents such as kinase inhibitors, checkpoint inhibitors, and targeting agents are used in combination with PD-1 antibody inhibitors[11,22,23]. Even though clinical data show monotherapy as a successful immunotherapy regimen when focusing on safety and efficacy the clinical data shows that novel combination therapies are superior to monotherapy[7].

CONCLUSION

In this review, findings confirm that atezolizumab/bevacizumab combination therapy can be an effective first-line treatment option to either sorafenib or atezolizumab monotherapy in patients with advanced HCC and non-decompensated liver disease. However, due to the small number of RCTs included, this systematic review may be considered insufficiently robust to provide strong recommendations. Consequently, further research and larger RCTs with cost-effectiveness analysis are necessary to validate our observations and identify the most efficacious and safe therapeutic regimen.

ARTICLE HIGHLIGHTS

Research background

Despite the use of the current standard therapy, the prognosis of unresectable hepato-cellular carcinoma (HCC) patients is poor, with median survival times of 40 mo in intermediate HCC (Barcelona Clinic Liver Cancer [BCLC] stage B) and 6–8 mo in advanced HCC (BCLC stage C). Although patients with early-stage HCC are usually suitable for therapies with curative intention, up to 70% of patients may manifest disease relapses at 5-year surveillance. Moreover, no treatment has been demonstrated to be useful in the adjuvant setting.

Research motivation

This systematic review described the evidence for atezolizumab/bevacizumab combination therapy *vs* sorafenib or atezolizumab monotherapies in improving survival outcomes and reducing disease progression in patients with unresectable HCC.

Research objectives

To evaluate the efficacy and safety of atezolizumab/bevacizumab *vs* sorafenib or atezolizumab alone, in patients with unresectable HCC with non-decompensated liver disease.

Research methods

A comprehensive literature review of published articles was conducted to identify studies that met our inclusion criteria using relevant mesh terms. This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines and we assessed for risk of bias using the Cochrane ROB tool and Sevis tool to create the traffic light plots and summary plots.

Research results

There was an improvement in overall tumor response, reduction of disease progression, and longer progression-free survival in the atezolizumab/bevacizumab

group compared to the monotherapy of either sorafenib or atezolizumab.

Research conclusions

This study confirms that combination treatment of atezolizumab plus bevacizumab could be a promising alternative to the standard of care sorafenib as a first-line treatment in patients with unresectable HCC and non-decompensated liver disease.

Research perspectives

Given the scarcity of randomized controlled trials specifically focusing on this therapeutic strategy, further research is needed to strengthen the current evidence. Two completed clinical trials were analyzed in this research; however, this review will be updated upon the completion of two ongoing trials. Moreover, further evaluation regarding the cost-effectiveness of combination therapy *vs* monotherapy is still needed valuable information.

ACKNOWLEDGEMENTS

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Anatomical *vs* nonanatomical liver resection for solitary hepatocellular carcinoma: A systematic review and meta-analysis

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Abstract

BACKGROUND

The long-term survival of patients with solitary hepatocellular carcinoma (HCC) following anatomical resection (AR) *vs* non-anatomical resection (NAR) is still controversial. It is necessary to investigate which approach is better for patients with solitary HCC.

AIM

To compare perioperative and long-term survival outcomes of AR and NAR for solitary HCC.

METHODS

We performed a comprehensive literature search of PubMed, Medline (Ovid), Embase (Ovid), and Cochrane Library. Participants of any age and sex, who underwent liver resection, were considered following the following criteria: (1) Studies reporting AR *vs* NAR liver resection; (2) Studies focused on primary HCC with a solitary tumor; (3) Studies reporting the long-term survival outcomes (> 5 years); and (4) Studies including patients without history of preoperative treatment. The main results were overall survival (OS) and disease-free survival (DFS). Perioperative outcomes were also compared.

RESULTS

A total of 14 studies, published between 2001 and 2020, were included in our meta-analysis, including 9444 patients who were mainly from China, Japan, and Korea. AR was performed on 4260 (44.8%) patients. The synthetic results showed that the 5-year OS [odds ratio (OR): 1.19; *P* < 0.001] and DFS (OR: 1.26; *P* < 0.001) were significantly better in the AR group than in the NAR group. AR was

associated with longer operating time [mean difference (MD): 47.08; $P < 0.001$], more blood loss (MD: 169.29; $P = 0.001$), and wider surgical margin (MD = 1.35; $P = 0.04$) compared to NAR. There was no obvious difference in blood transfusion ratio (OR: 1.16; $P = 0.65$) or postoperative complications (OR: 1.24, $P = 0.18$).

CONCLUSION
AR is superior to NAR in terms of long-term outcomes. Thus, AR can be recommended as a reasonable surgical option in patients with solitary HCC.

Key Words: Hepatocellular carcinoma; Anatomical resection; Non-anatomical resection; Meta-analysis; Systematic review; Solitary tumor

Core Tip: Anatomical hepatectomy is considered an effective way to treat hepatocellular carcinoma (HCC) in theory. However, there is still no consensus about which surgical technique between anatomical and non-anatomical hepatectomy is more suitable for patients with solitary HCC. This study aimed to compare the long-term survival outcomes between anatomical and non-anatomical hepatectomy in HCC patients undergoing curative resection. Patients with a solitary tumor undergoing AR were associated with a better overall survival.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed cancer and the fourth most common cause of cancer-related death worldwide[1]. It is estimated that there are about 841000 new cases and 782000 deaths annually[2], causing a heavy economic burden on society and government. The main risk factors for HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, aflatoxin, obesity, and type 2 diabetes[3]. China and Eastern Africa are the most high-risk HCC areas globally with a high prevalence of HBV and exposure to aflatoxin. Surgical resection is still considered the first-line treatment for HCC in patients with preserved liver function[4,5], especially for patients who have a solitary HCC. The ideal candidates for surgical resection are patients with a single tumor at an early stage, Child–Pugh class A, no clinically significant portal hypertension, and good performance status[6]. However, the high incidence of postoperative recurrence of HCC remains an unresolved challenge.

Anatomical resection (AR), which was first proposed in the 1980s, was defined as complete removal of one Couinaud's segment (*i.e.*, segments I-VIII) or a combination of contiguous territories of the third-order subsegmental portal venous branches smaller than one Couinaud's segment[7]. In theory, AR can produce a better survival outcome by systematic removal of the tumor-bearing portal territories. However, as reported recently, some studies have found that non-anatomical resection (NAR) could achieve a more satisfactory outcome compared with AR[8-10]. Others have concluded that AR can significantly improve the long-term survival results[11,12]. Thus, the superiority of AR for solitary HCC is not clear.

The aim of the present study was to compare the long-term outcomes of AR and NAR for solitary HCC.

MATERIALS AND METHODS

Protocol and guidance

This systematic review and meta-analysis is reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement. The protocol for this review was registered with PROSPERO (number: CRD42020213382).

Search strategy

The electronic databases PubMed, Medline (Ovid), Embase (Ovid), and Cochrane Library were searched for eligible studies from the inception of each database to September 30, 2020. Only studies published in English were included. The following algorithm was applied: (anatomic resection OR anatomical resection OR non-anatomic resection OR non-anatomical resection OR nonanatomic resection OR non-anatomical resection OR limited resection OR systematic resection OR partial resection OR wedged resection) AND (single hepatocellular carcinoma OR solitary hepatocellular carcinoma). Two reviewers (Liu H and Hu FJ) performed the initial literature screening independently. The titles and abstracts were reviewed to identify all potential articles.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) Studies reporting AR *vs* NAR liver resection; (2) Studies focused on primary HCC with a solitary tumor; (3) Studies reporting the long-term survival outcomes (> 5 years); and (4) Studies including patients without history of preoperative treatment.

The exclusion criteria were as follows: (1) Noncomparative studies; (2) Conference abstracts and case reports; (3) Review articles and editorials; and (4) Studies without data of interest. Duplicated studies by the same authors or centers would be distinguished carefully. The largest patient cohorts were included in this analysis. However, if the patient samples were enrolled at different times, both were included.

Data extraction and quality assessment

Essential information and continuous or dichotomous data for special outcomes of each eligible article were extracted by two independent investigators (Liu H and Hu FJ), using the customized data extraction form that included the following items: Study ID; year of publication; country; sample size; age of participants; number of male patients; HBV and HCV infection; cirrhosis; hepatic function (Child–Pugh class A/B); α-fetoprotein (AFP); des-γ-carboxy prothrombin (DCP); indocyanine green retention rate at 15 min (ICGR-15); tumor characteristics (size and microvascular invasion); perioperative characteristics (operating time, amount of blood loss, blood transfusion, and surgical margin); postoperative complications; duration of hospital stay; duration of follow-up; and long-term outcomes [overall survival (OS) and disease-free survival (DFS)]. If OS and DFS were not summarized in tables or texts directly, they were calculated from the Kaplan–Meier graph using Engauge Digitizer (version 7.2). Disagreements were settled through discussion until reaching a consensus.

Two authors independently assessed the quality of the included studies using the modified Newcastle-Ottawa Scale, which included three broad perspectives: Selection of study groups, comparability of the groups, and ascertainment of exposure or outcome of interest[13,14]. Total score ranged from 0 to 9. Scores > 6 were regarded as high quality[15].

Statistical analysis

The meta-analysis was performed using Cochrane Collaboration's Review Manager 5.3 software. The intervention effect was expressed as odds ratios (ORs) for dichotomous outcomes and mean differences (MDs) for continuous outcome measures, with 95% confidence intervals (CIs). Heterogeneity was assessed by χ^2 and I^2 tests. A random effects model was used routinely only if there was no obvious heterogeneity among the included studies ($I^2 < 40\%$)[16].

Sensitivity analysis and publication bias

Sensitivity analysis was conducted by deleting the included studies in sequence to recognize the stability of the total effect. Funnel plot was used to assess the publication bias. Begg's test and Egger's test were used to evaluate the symmetry of the funnel plot.

RESULTS

Eligible studies and characteristics

A total of 853 records were retrieved, and 799 records were excluded by reading titles and abstracts because of irrelevance to our theme. By assessing full-text articles of the remaining studies, 14 (with data for 9444 participants) that compared the outcomes between AR and NAR for patients with solitary HCC were included in this meta-analysis[10,12,17-28]. They were published between 2001 and 2020. Eight studies using propensity score matching aimed to reduce the bias and confounding variables[10,12, 18,20,21,23,24,26]. All the included studies were from Asia (Table 1), including two from China[18,20], three from Korea[10,19,22], and nine from Japan[11,12,17,21,23-38]. Most studies were marked 7 or 8 stars (Supplementary Table 1). All studies were deemed of high quality. Detailed search steps were described using the PRISMA 2009 flow diagram (Figure 1).

Pooled outcomes showed that the patients in the AR group were characterized by a lower proportion of cirrhosis, smaller tumor size, lower ICG-R15, longer surgical time, and more intraoperative blood loss in comparison with those in the NAR group. The data and the forest plots are displayed in Supplementary Table 2 and Figure 2.

Long-term outcomes

For OS of the two groups, the postoperative 5-year survival rates were 69.8% and 63.7%, respectively (Table 2). All included studies reported 5-year OS, and the pooled outcome showed that the AR group was associated with a better survival (OR: 1.19, fixed model, I^2 = 32%, 95%CI: 1.08-1.30, Z = 3.69, P < 0.001)[10,12,17-28]. Concerning 5-year DFS rates, there were 11 studies including 7655 patients. Patients who underwent AR tended to have a better 5-year DFS in comparison with the NAR group (OR: 1.26, fixed model, I^2 = 37%, 95%CI: 1.15-1.39, Z = 4.82, P < 0.001)[10,12,17,19-24,26,27]. Ten studies analyzed 1-year DFS of 2110 patients undergoing liver resection, and the pooled result displayed that there was no difference in 1-year DFS (OR: 1.21, random model, I^2 = 47%, 95%CI: 0.85-1.72, Z = 1.05, P = 0.29)[10,12,19-26] or 1-year OS (OR: 1.19, fixed model, I^2 = 0%, 95%CI: 0.79-1.78, Z = 0.83, P = 0.41)[10,12,19-26]. Details of the data and forest plot are shown in Supplementary Table 3 and Figure 3 respectively.

Sensitivity analysis and publication bias

A sensitivity analysis was performed by omitting the included studies in turn to recognize the stability of synthesized 5-year OS. OS was steady as pooled ORs did not alter significantly after eliminating the enrolled studies in sequence (Figure 4A). No evidence of bias was observed in the selected funnel plot (Figure 4B), and other clinical outcomes were also displayed (Figure 5). Similarly, the Begg's test (Z = 0.22, P = 0.827) and Egger's test (bias coefficient = 0.026, SE = 0.471, t = 0.05, P = 0.957) were conducted to evaluate funnel plot symmetry. These results demonstrated no obvious evidence of publication bias.

DISCUSSION

In the management of HCC, the attainment of long-term survival is compromised by the choice of therapeutic method. Although there are various alternative treatment choices, liver resection is still considered the most ideal curative option for HCC, especially for patients with a solitary tumor[6,39]. Whether to perform AR or NAR is a sophisticated decision based on considering the balance between radical resection and avoiding postoperative liver failure from removing too much liver parenchyma, especially in patients with cirrhosis. AR is always related to major liver resection, which may induce a high risk of postoperative liver dysfunction. On the contrary, NAR aims to decrease the incidence of postoperative complications including liver failure. The oncological and long-term benefit of AR is always a debate, and has been studied for many years[40-43]. Due to the high heterogeneity of HCC at both the molecular and clinical levels[44], it is difficult to conduct a high-quality randomized controlled trial (RCT) comparing AR and NAR. A recent meta-analysis using propensity score matching has shown that AR can yield better local control of the disease[45]. Previous studies have suggested that AR provides better long-term outcomes[27,38,46]. Comparable findings have been found by other studies between AR and NAR[9,10,12,19,22-24,31,32,36]. Thus, it remains unclear whether AR has oncological and prognostic superiority as an effective treatment for HCC.

Table 1 Characteristics of the studies included in the meta-analysis

Ref.	Country	Enrollment of period	Design/center	Patients		Quality score
				AR	NAR	
Cho *et al*[10], 2019	Korea	Jan 2008-Sep 2014	R/Single	59	59	8
Eguchi *et al*[27], 2008	Japan	1994-2001	R/Multiple	2267	3514	**7**
Hirokawa *et al*[23], 2015	Japan	Jan 2001-Dec 2005	R/Multiple	72	72	8
Hokuto *et al*[12], 2018	Japan	Jan 2007-Dec 2015	R/Single	20	20	8
Ishii *et al*[26], 2014	Japan	Jan 2002-Dec 2010	R/Single	44	44	8
Jung *et al*[18], 2019	Korea	Jan 2006-Dec 2014	R/Single	936	388	8
Kaibori *et al*[21], 2017	Japan	2003-2007	R/Multiple	355	355	7
Kim *et al*[22], 2016	Korea	Jan 2003-Dec 2009	R/Single	27	72	7
Kudo *et al*[25], 2014	Japan	Apr 2000-Mar 2012	R/Single	121	112	7
Okamura *et al*[24], 2014	Japan	Sep 2002-May 2013	R/Single	64	64	8
Shin *et al*[19], 2018	Korea	Jan 2006-Dec 2015	R/Single	53	63	7
Shindoh *et al*[52], 2020	Japan	Jan 2011-Oct 2017	R/Single	38	165	7
Yamamoto *et al*[28], 2001	Japan	1990-1994	R/Single	90	114	7
Zhao *et al*[20], 2017	China	Jan 2004-Dec 2013	R/Multiple	114	114	8

R: Retrospective; AR: Anatomical resection; NAR: Non-anatomical resection.

Table 2 Results of meta-analysis comparison of anatomical resection and non-anatomical resection

	Studies	Patients		MD/OR (95%CI)	P value	Study heterogeneity			
		AR	NAR			χ^2	df	I^2, %	P value
Operating time (min)	9	782	954	47.08 (26.30-67.86)	< 0.001	60.82	8	87	< 0.001
Blood loss (mL)	8	749	921	169.29 (65.88-272.70)	0.001	110.72	7	94	< 0.001
Blood transfusion	8	749	921	1.16 (0.84-1.60)	0.36	8.75	6	31	0.19
Surgical margin (mm)	6	322	494	1.35 (0.06-2.64)	0.04	7.68	5	35	0.17
Complication	5	512	684	1.24 (0.91-1.70)	0.18	4.14	4	3	0.39
1-yr OS	10	929	975	1.08 (0.69-1.68)	0.73	3.17	8	0	0.92
1-yr DFS	10	929	975	1.21 (0.85-1.72)	0.29	16.92	9	47	0.05
5-yr OS	14	4260	5184	1.19 (1.08-1.30)	< 0.001	19.04	13	32	0.12
5-yr DFS	11	3113	4542	1.26 (1.15-1.39)	< 0.001	15.76	10	37	0.11

AR: Anatomical resection; NAR: Non-anatomical resection; OS: Overall survival; DFS: Disease-free survival; MD: Mean difference; OR: Odds ratio; CI: Confidence interval; df: Degrees of freedom.

The pooled outcomes showed that, compared with NAR, complete removal of the tumor-bearing third-order portal territories was associated with significantly improved long-term outcomes, including 5-year OS and DFS, with no increase in postoperative complications and transfusion. Our results thus contribute to current knowledge by providing evidence that AR is a satisfactory treatment strategy that can achieve the ideal long-term outcomes in solitary HCC. Several included studies showed that AR is not superior to NAR in terms of long-term outcomes, which disagrees with our pooled outcomes. Shin *et al*[19] reported that the outcomes of NAR are comparable with those of AR in single HCC < 3 cm. Kim *et al*[22] found that the long-term survival of NAR for solitary HCC < 5 cm is comparable to that achieved with AR. The reason for this is the different tumor characteristics in that study. Specifically, the tumor size and the proportion of microvascular invasion in the AR
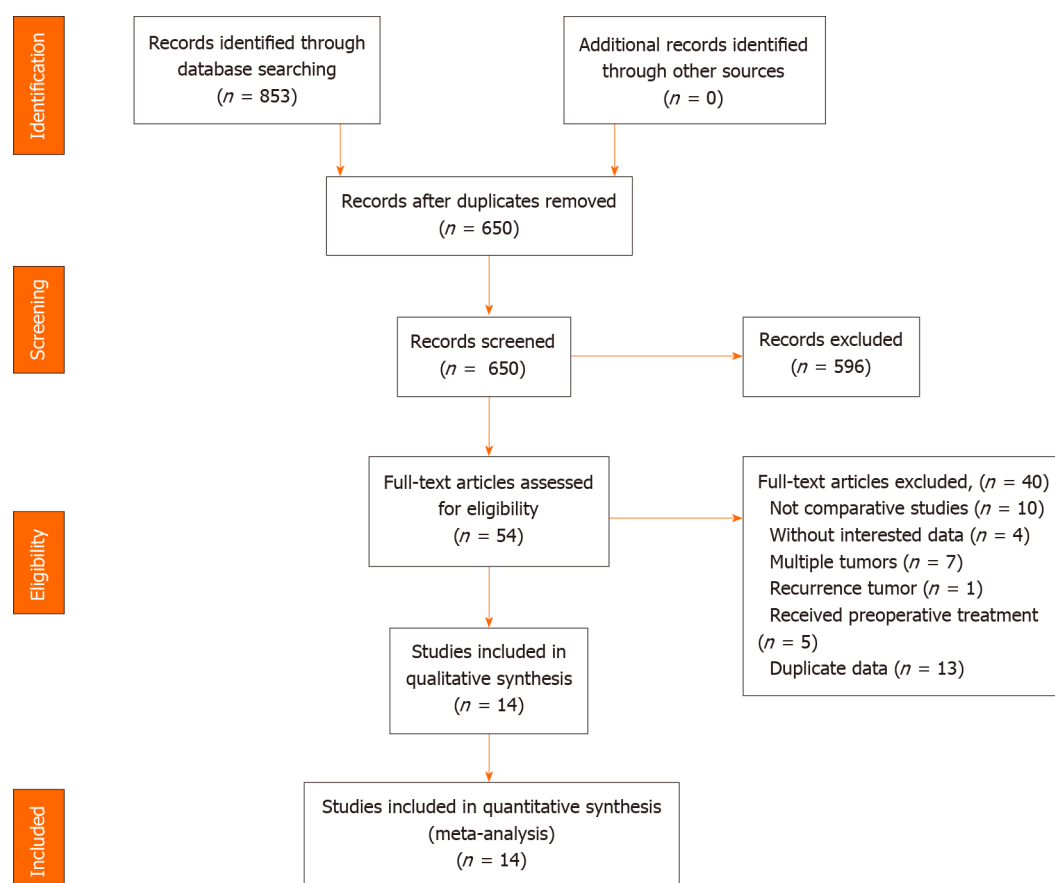
Figure 1 Flow chart of selection process in this meta-analysis.

group were larger than those in the NAR group. Hirokawa *et al*[23] also presented similar outcomes by using propensity score matching. This might be because the included patients had no macroscopic vascular invasion, which decreased the advantage of AR. Limited by the reported data, we did not conduct a subgroup analysis in term of tumor size. Further studies are needed to determine the optimal choice for the application of AR for different tumor sizes.

Perioperative outcomes showed that AR was associated with longer operating time, more blood loss, and wider surgical margins when compared to NAR. To our knowledge, AR is always related to major liver resection, and is generally regarded as a more technically demanding operation. Unlike other tumors, underlying liver function plays an important role in patients' prognosis after initial liver resection[47, 48]. As is known to us, impaired liver function is associated with a worse prognosis. Because of the superiority of AR and the preference of surgeons, AR is always conducted in patients with better liver function compared to NAR, and our synthetic results proved this. Although part of included studies used propensity score matching to decrease confounders as much as possible, liver function is still a potential confounder which cannot be bypassed, and we need take it into consideration when interpreting the result. Less remnant liver volume, more intraoperative loss, and longer operating time were related to AR, which theoretically increased the risk of postoperative complications such as liver failure. Although AR is a more challenging procedure than NAR, we did not observe differences in the blood transfusion ratio or postoperative complications. Thus, our results offer powerful support for surgeons to choose AR.

It is estimated that close to 70% of patients with HCC will relapse within 5 years following surgery[49]. HCC has a unique pattern of metastasis *via* the portal vein. The mechanisms of recurrence can be either intrahepatic metastasis from the initial tumor or a *de novo* multicentric tumor[50]. Intrahepatic metastasis may be due to residual micrometastases from the HCC spreading through the portal venous system[7,51]. AR can theoretically prevent the progression of HCC by eradicating the primary tumor and microvascular metastasis. Several studies[12,17,18] have demonstrated that OS was significantly better after AR than NAR. The outcomes were in accordance with the outcomes of our meta-analysis. Hence, our finding of a better 5-year DFS after AR than
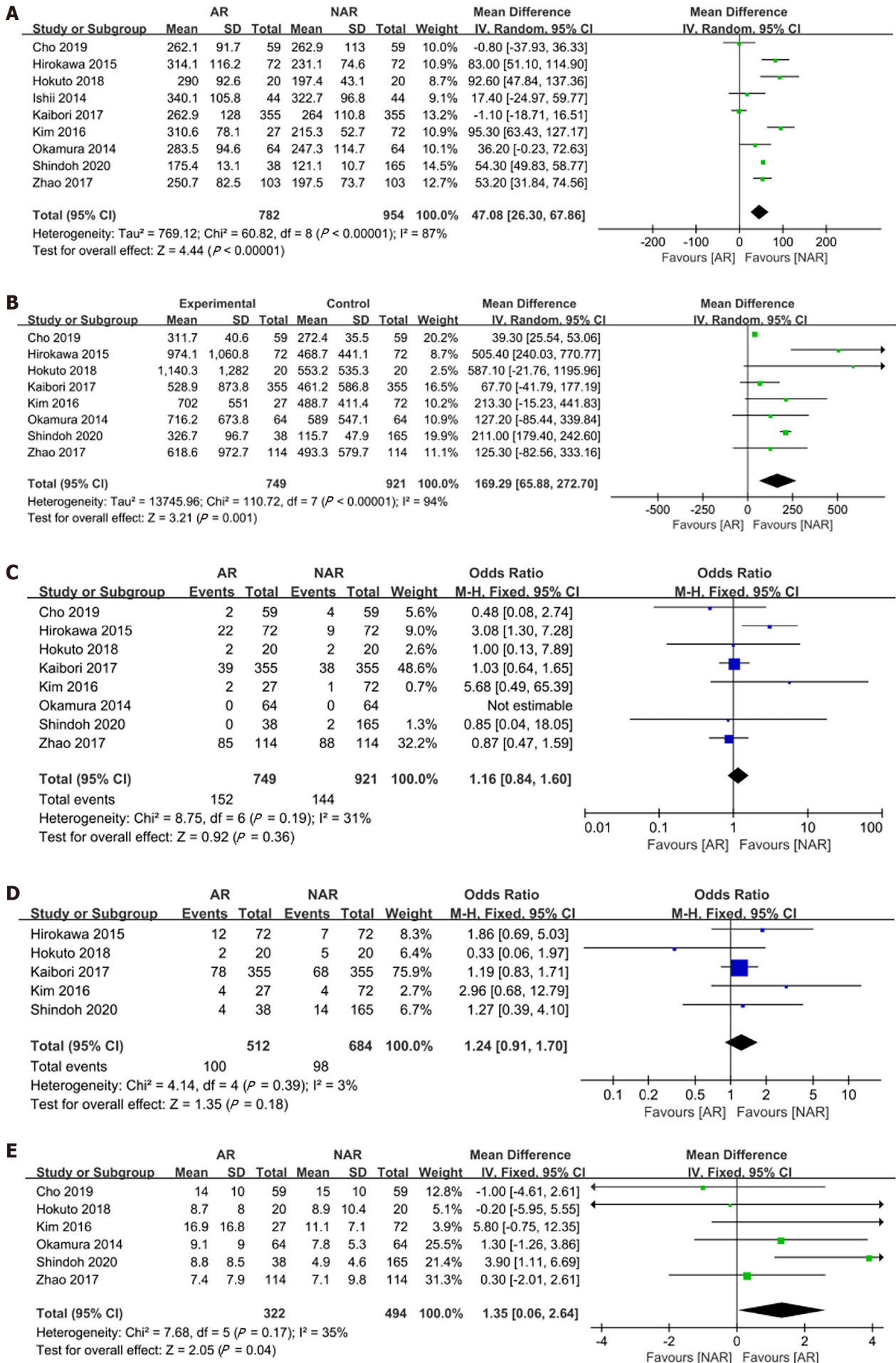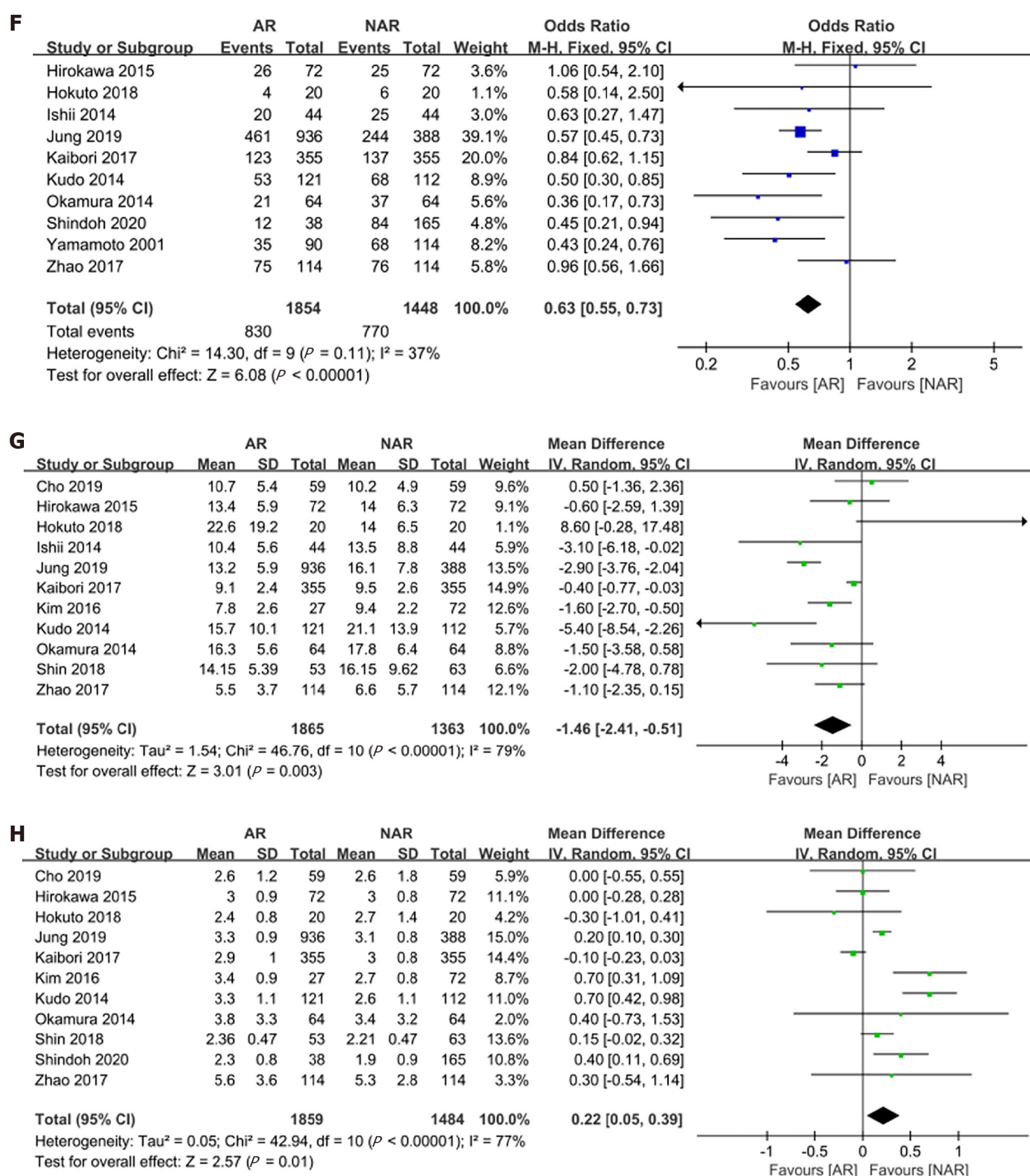
Figure 2 Forest plots of perioperative outcomes. A: Operating time; B: Blood loss; C: Blood transfusion; D: Postoperative complications; E: Surgical margin; F: Cirrhosis; G: Indocyanine green retention at 15 min; H: Tumor size. CI: Confidence interval.

NAR indicated that this procedure is advantageous for improvement of long-term survival.

Our study had several limitations. First, there were no RCTs and most were retrospective. Included samples mainly consisted of Japanese cohorts. Selection bias of enrolled studies might not have been completely negligible, even after the adjustment of propensity scoring. Second, among different medical centers, a standard surgical procedure was not available, and the experience of surgeons may have had an impact on perioperative outcomes, especially operating time, blood loss, and morbidity. Third, the sample size of several included studies was small. Prognosis of HCC is highly dependent on the selection and quality of repeat treatment for recurrence[52], which is another crucial factor that deserves to be further analyzed. There is still a need for a well-designed RCT that is characterized by larger samples and multiple centers to verify the advantage of AR over NAR for patients with solitary HCC.
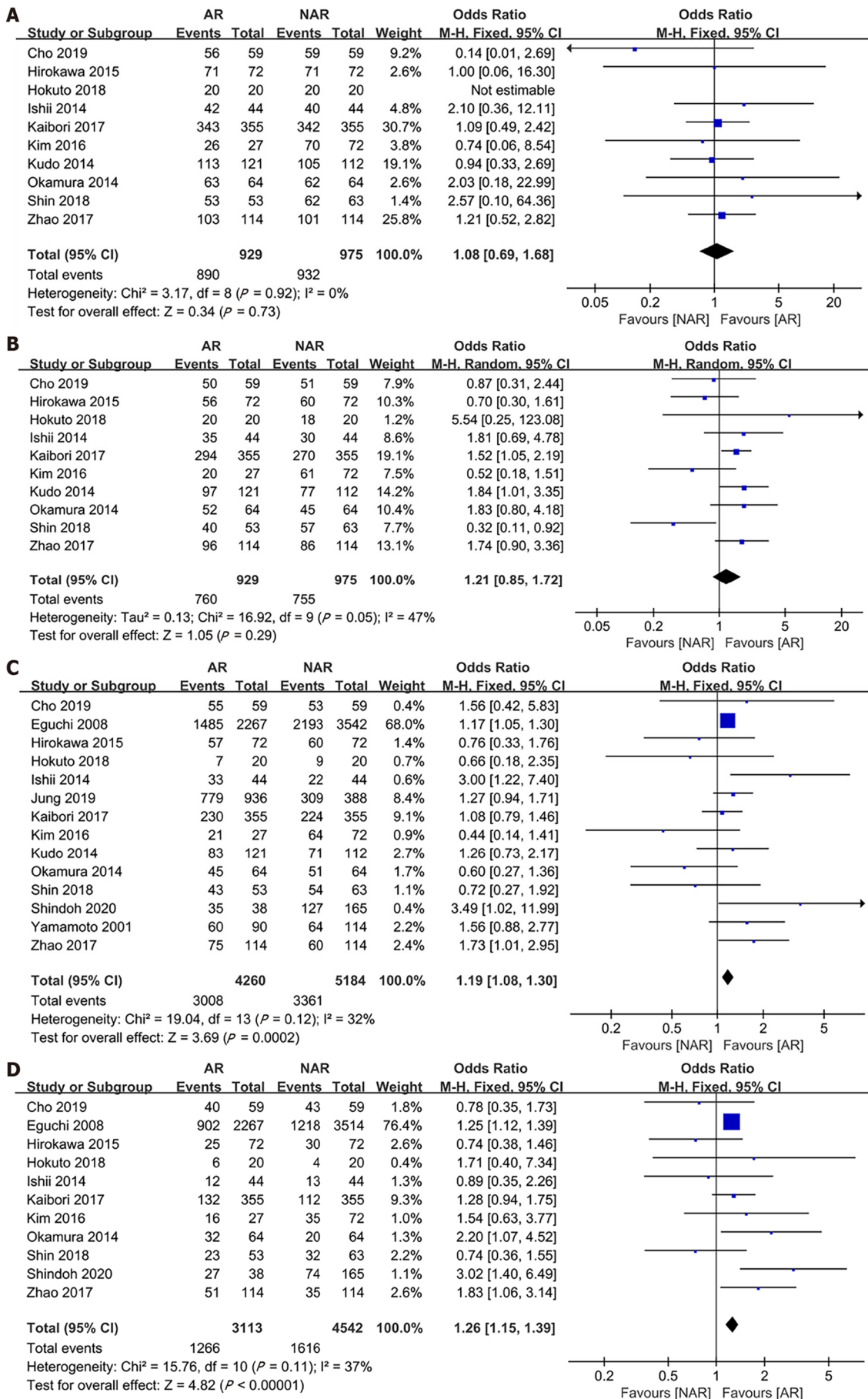
Figure 3 Forest plots of primary outcomes. A: 1-year overall survival (OS); B: 1-year disease-free survival (DFS); C: 5-year OS; D: 5-year DFS. CI:
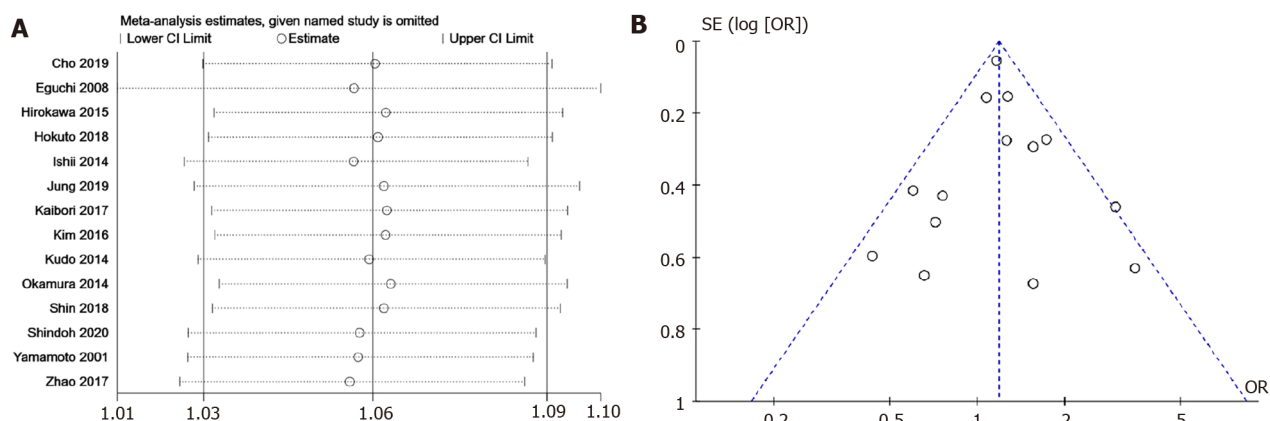
Confidence interval.

Figure 4 Sensitivity analysis and funnel plot of 5-year overall survival for subjects with hepatectomy using anatomical resection *vs* non-anatomical liver resection. A: Sensitivity analysis; B: Funnel plot.
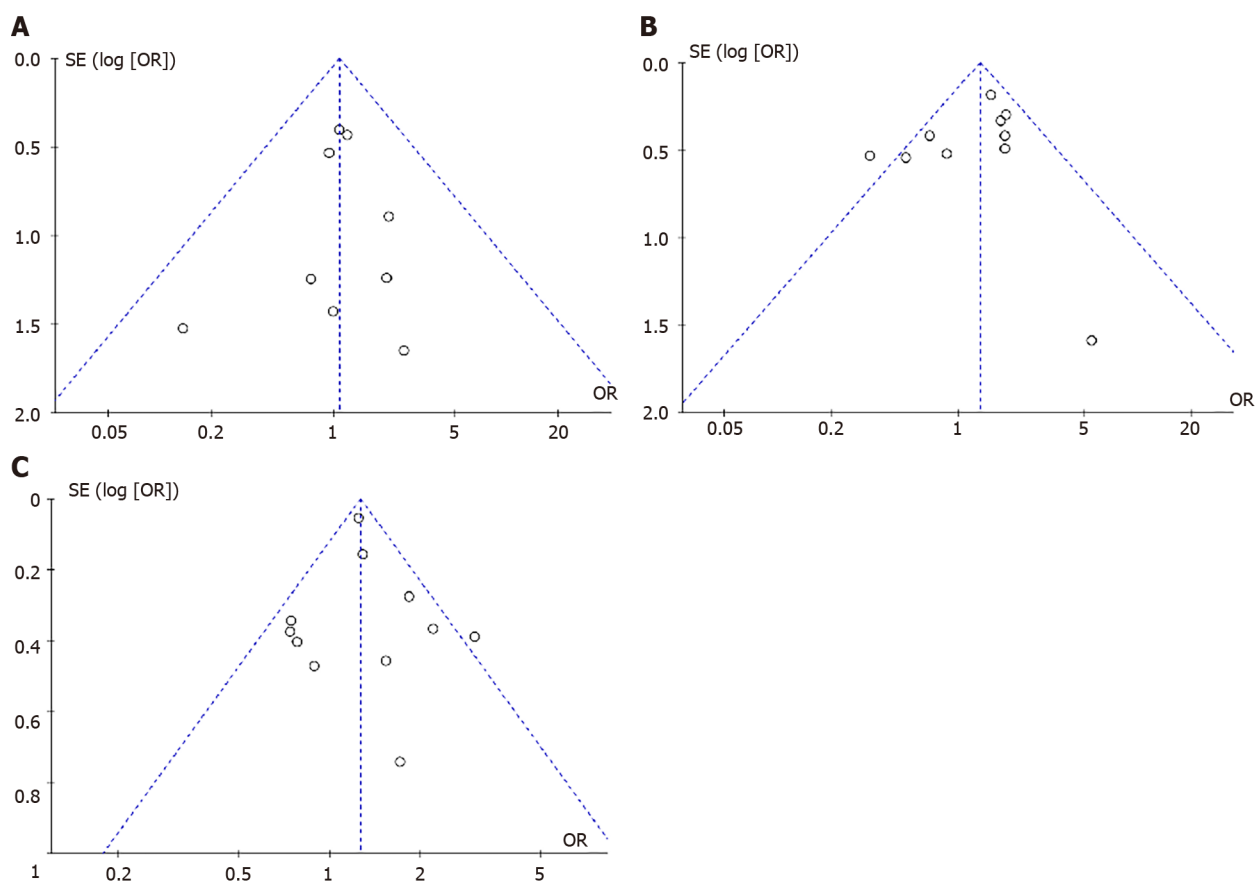

Figure 5 Funnel plots of primary outcomes. A: 1-year overall survival; B: 1-year disease-free survival (DFS); C: 5-year DFS. OR: Odds ratio.

CONCLUSION

In conclusion, this meta-analysis indicated that AR improves the 5-year DFS and OS in patients with solitary HCC. Thus, AR should be recommended as the primary option as long as such a surgical maneuver is feasible.

ARTICLE HIGHLIGHTS

Research background

Patients diagnosed with solitary hepatocellular carcinoma (HCC) always receive liver resection. More and more patients are undergoing anatomical hepatectomy which aims to eradicate tumor. Accumulating studies had been performed to compare these two kinds of surgical technique. However, it is still not yet whether anatomical hepatectomy is superior to non-anatomical hepatectomy.

Research motivation

Clarifying the survival benefits of anatomical and non-anatomical hepatectomy is of vital importance for patients with solitary HCC. Furthermore, it will be instructive for doctors to choose better surgical method.

Research objectives

To perform a systematic review and meta-analysis on short- and long-term results of anatomical and non-anatomical hepatectomy in patients with solitary HCC.

Research methods

PubMed, Medline (Ovid), Embase (Ovid), and Cochrane Library were searched for articles from the inception of each database to 2020 according to the designed extraction scheme, and statistical analysis was performed using Cochrane Collaboration's Review Manager 5.3 software. The quality of included papers was assessed with the modified Newcastle-Ottawa Scale. The main results of this study included overall survival (OS) and disease-free survival (DFS).

Research results

Fourteen studies (9444 patients) comparing anatomical and non-anatomical hepatectomy were included for final analysis with 4260 cases of anatomical resection (AR) and 5184 cases of non-anatomical resection (NAR). Anatomical hepatectomy was associated with a higher 5-year OS [odds ratio (OR): 1.10, 95% confidence interval (CI): 1.08-1.30] and DFS (OR: 1.26, 95%CI: 1.15-1.39). AR was associated with longer operating time [mean difference (MD): 47.08; $P < 0.001$], more blood loss (MD: 169.29; $P = 0.001$), and wider surgical margin (MD = 1.35; $P = 0.04$) compared to NAR. There was no obvious difference in blood transfusion ratio (OR: 1.16; $P = 0.65$) or postoperative complications between the two groups (OR: 1.24, $P = 0.18$).

Research conclusions

This meta-analysis confirmed that AR is superior to NAR in terms of long-term outcomes. Thus, AR can be recommended as a reasonable surgical approach in patients with solitary HCC.

Research perspectives

There are some limitations that should be taken into consideration when interpreting the results. The most vital limitation is that the included studies are non-randomized controlled trial and retrospective. Future studies with large-scale and well-designed randomized controlled trial are needed to further verify the benefits of anatomical hepatectomy for patients with solitary HCC.

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LETTER TO THE EDITOR

Hepatocellular carcinoma biomarkers, an imminent need

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Abstract

Hepatocellular carcinoma (HCC) is the most common malignant neoplasm of the liver and one of the deadliest cancers worldwide. The identification of novel, highly specific and more sensitive biomarkers for HCC is crucial because existing ones are deficient and non-confirmatory without histological biopsy or imaging techniques.

Key Words: Hepatocellular carcinoma; Biomarker; Blood; Urine; Feces; Gut microbiota

Core Tip: The identification of specific, sensitive and validated biomarkers for hepatocellular carcinoma (HCC) is complex because of the variability in genetic profiles, but their requirement is urgent to achieve earlier detection of HCC. Body fluids and feces for biomarker detection constitute feasible and low cost screening tools for early diagnosis, prognosis and treatment of HCC.

TO THE EDITOR

I read the review by Guan *et al*[1], "Biomarkers for hepatocellular carcinoma based on body fluids and feces", published in the April 2021 issue of the *World Journal of Gastrointestinal Oncology*, with profound interest.

Hepatocellular carcinoma (HCC) is the leading malignant neoplasm of the liver and one of the most common lethal cancers worldwide. For this reason, an early detection is crucial to decrease mortality, since symptomatology appears at later stages of the disease. The identification of novel, highly specific and sensitive biomarkers, or a combination of them, is of special concern because the existing ones are deficient and non-confirmatory without histological biopsy or imaging techniques. The utilization of body fluids and feces for biomarker detection constitutes a feasible minimally- or non-invasive and low-cost screening method that facilitates studies for early diagnosis, prognosis and treatment of HCC.

Since HCC can arise from a variety of etiological factors, such as metabolic disorders, virus infections or toxin exposure, the genetic profiles are considerably variable, resulting in diverse hepatic immune microenvironments. This implies that the metabolomic, proteomic and glycomic profiles should be better clarified for the various HCCs in order to improve overall understanding of the disease and allow for identification of appropriate and validated biomarkers[2].

Alpha-fetoprotein (AFP) is the most common biomarker utilized for HCC diagnosis, but its sensitivity is only 39%-65% and its performance at early stages of the disease is suboptimal. Therefore, to improve detection of the malignancy, AFP has to be combined with imaging findings as well as other parameters, such as age and sex, which increase the sensitivity. In addition, AFP-L3, the highest glycoform of AFP, has exhibited much higher sensitivity, and the AFP-L3/AFP ratio can be considered as a risk factor for the development of HCC[3].

Several metabolites have displayed higher accuracy than AFP, showing aberrant levels that can be detected at earlier stages of HCC[4]. Circulating tumor (ct)DNA also has great potential to became a biomarker, since it contains several tumor-specific mutations or epimutations, constituting a good approach for HCC detection and prognosis, and to serve as a tool for monitoring therapeutic response. Additionally, several different micro (mi)RNAs and other non-coding (nc)RNAs have been shown to be deregulated in HCC, implying that their aberrant expression should be evaluated and validated as potential prognostic biomarkers. Unfortunately, miRNA variabilities have been detected depending on whether they are measured in serum or plasma, thereby complicating interpretation[5-8].

Moreover, circulating tumor cells (CTCs) have shown partial sensitivity but high specificity and are considered to have great potential in prediction of recurrence and prognostic evaluation of HCC. On the other hand, extracellular vesicles (EVs), such as exosomes and microvesicles, the contents of which are very heterogeneous, do not present better diagnostic performances than CTCs or circulating cell-free DNA, but they do have good potential as future therapeutic agents[5,8,9].

The above-mentioned biomarkers, ctDNA, miRNAs, CTCs and EVs, are tumor-specific, which is of great advantage, because they exhibit the heterogeneity of the tumor and its evolution. These features cannot be detected with other plasma biomarkers.

Additionally, several urine molecules have the potential to be classified as biomarkers for prevention, detection, progression monitoring, and recurrence prediction of HCC[10]. It is possible that they can be used as auxiliary diagnostic tools in combination with AFP. Moreover, feces-based biomarkers, which reflect the gut microbiota — which is itself known to vary with different pathological stages, are under evaluation for their potential utility in early diagnosis, prognosis and progression monitoring of HCC. In addition, the use of antibiotics to modulate gut microbiota appears to be a favorable strategy to influence the progression of HCC. Promising results have also been obtained with probiotics in mouse HCC models, which have shown a reduction in the development of this malignant neoplasm, opening avenues of possible application as a therapy in humans in the future[11-13].

The identification of more specific and sensitive biomarkers for HCC, and their variability over time, is an urgent requirement due to their critical role for early detection and prognosis, for choosing appropriate therapy, or for use as a tool to follow-up the patient's treatment response. Ideally, biomarkers should detect HCC months before the tumor is visible, to improve surveillance and facilitate initiation of an earlier therapy. Clearly, the identification of new biomarkers for prompt HCC detection is complex, nonetheless because of the diverse type of tumors (genetic heterogeneity). However, efforts must be made to combat this devastating tumor malignancy. Moreover, the performance of new biomarkers will have to be clinically validated to optimize the current therapeutical strategies.

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