Cancer stem cell-mediated therapeutic resistance in hepatocellular carcinoma

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Abstract
Hepatocellular carcinoma (HCC) is a highly heterogeneous malignancy. In the clinic, therapeutic resistance is largely attributed to tumor heterogeneity. Growing evidence indicates that cancer stem cells (CSCs) are the major source of tumor heterogeneity. Hence, uncovering the resistance mechanisms associated with CSC properties is essential for developing effective therapeutics. CSCs resemble embryonic stem cells. Embryonic development-related genes and signaling pathways are usually abnormally active and function as oncofetal drivers in HCC. Multiple strategies have been applied to identify oncofetal drivers. The mechanisms of CSC resistance could also provide reliable biomarkers to predict treatment failure. Precisely targeting these specific CSC properties may be effective in preventing or annihilating therapy resistance. This review provides an overview of drug resistance mechanisms associated with CSC traits and summarize therapeutic strategies against drug resistance.

Keywords: Hepatocellular carcinoma, heterogeneity, cancer stem cell, drug resistance, precision therapy
INTRODUCTION

Globally, hepatocellular carcinoma (HCC) is one of the most common and deadliest cancer types. Liver resection, ablation, and transplantation are potentially curative procedures but require an early diagnosis. Despite diligent surveillance, a large percentage of HCC patients present with intermediate or advanced stages of the disease, and treatments are often ineffective. To date, various molecular targeted drugs for advanced HCC have been developed. Both sorafenib and lenvatinib are inhibitors of multityrosine kinases that have been approved as a first-line treatment for advanced HCC management. Regorafenib has a molecular target spectrum similar to that of sorafenib and is applied as an alternative for sorafenib-resistant HCC patients. Cabozantinib and ramucirumab are novel tyrosine kinases used as second-line HCC treatments. Additionally, immune checkpoint inhibitors (ICIs) are also being considered as systemic HCC strategies. Some of the ICIs approved or in clinical research include agents (nivolumab, pembrolizumab, tislelizumab) against programmed cell death protein 1 (PD1), agents (atezolizumab, durvalumab, sintilimab) against PD-1 ligand (PD-L1), agents (ipilimumab, tremelimumab) against cytotoxic T-lymphocyte associated protein 4 (CTLA4) and other agents (cobolimab against T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3), and relatlimab against lymphocyte activation gene-3 (LAG-3))\(^{[1-4]}\). Due to these recently developed treatments, the cancer incidence rate and the cancer death rate have gradually declined. However, due to heterogeneity, many malignant HCC patients unavoidably acquire drug resistance. This treatment failure could be explained by the properties of cancer stem cells (CSCs).

CSCs are a profoundly heterogeneous subpopulation of “stem-like” cancer cells that behave like “tumor-initiating cells” or “sphere-forming cells”. Unlimited self-renewal, multilineage differentiation, and maintenance of pluripotency are key characteristics of CSCs, which are similar to those of embryonic stem cells (ESCs)\(^{[5]}\). Accumulating evidence suggests that drug resistance is largely attributed to CSCs, which account for much of the intratumor heterogeneity within each tumor population\(^{[6-9]}\). Integrating CSC properties into our understanding of drug resistance is crucial and may not only allow a better understanding of the mechanisms of chemoresistance but also facilitate the identification of potential biomarkers to predict treatment failure, as well as druggable targets for developing novel pharmacological strategies for improving the sensitivity of HCC to anticancer drugs. The present review summarizes recent studies on drug resistance mechanisms from the perspective of CSCs [Figure 1], provides an overview of therapeutic strategies for CSC-mediated drug resistance, and finally focuses on mono- or combination therapies against drug resistance [Table 1].

CSC AND HCC HETEROGENEITY

HCC is a highly heterogeneous disease with unique biological features and molecular backgrounds that may respond differently to different treatments. A well-documented study links heterogeneity to clinical outcomes. HCC heterogeneity consists of intertumor (within the tumor cell population) and intratumor (tumors from the same patient) heterogeneity. Convincing evidence shows that CSC heterogeneity is the major source of intratumor heterogeneity that induces chemoresistance and subsequent tumor relapse. Different CSC subpopulations function differently, exhibit different developmental states or express different gene expression profiles\(^{[10-11]}\). Currently, studies of liver CSCs have mainly focused on identifying liver CSC surface markers via cell-sorting and xenotransplantation analyses in immunodeficient mice. A number of the liver CSC markers in HCC appear to be oncofetal markers, as the development of HCC is similar to that of the development of fetal, normal, and regenerating livers. Classical stemness markers, including Nanog, SRY-box transcription factor 2 (SOX2), and Oct4, and liver CSC markers, including CD13, CD24, CD44, CD47, CD90, CD133, intercellular adhesion molecule 1 (ICAM1), epithelial cell adhesion molecule (EPCAM), Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), OV6,
Table 1. Targeted therapies for resistant HCC

<table>
<thead>
<tr>
<th>Clinical drug</th>
<th>Resistance mechanisms</th>
<th>Candidate biomarker</th>
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CTNNB1 mutation alters Wnt-β-catenin pathway
GDF1 induces ALK7-SMAD2/3 signalling cascade and suppresses LSD1 to boost CTA expression
SOX2, OCT4, HNRNPM, MBD2a, and FZD3 comprise a positive feedback loop
PKCa phosphorylates ZFP64 at S226 and induces its nuclear translocation, leading to transcriptional activation of macrophage CSF1
circMET activates miR-30-5p/Snail/DPP4/CXCL10 axis
PP2A: Protein phosphatase 2; DLK1: delta like non-canonical Notch ligand 1; DLL1: delta like canonical notch ligand 1; INSR: insulin receptor; IGFIR: insulin like growth factor 1 receptor; MBD2: methyl-CpG binding domain protein 2.

Figure 1. The mechanisms of heterogeneous CSCs that are responsible for the acquisition of therapeutic resistance. Selected mechanisms include CSC-associated potential biomarkers and developmental pathways.

CSCs exhibit features similar to those of ESCs due to their ability to retain the activity of vital and highly conserved developmental signaling pathways involved in managing the features of embryonic cells, normal organogenesis, and cell lineage differentiation, which may lead to the initiation or progression of poorly differentiated HCC[9]. Several important and well-characterized signaling pathways, including the protein kinase B (AKT), Hedgehog, Hippo, nuclear factor kappa-B (NF-κB), Notch, TGF-β and Wnt/β-catenin pathways, are involved in acquiring and maintaining stem-like traits, such as self-renewal, plasticity, and quiescence[8]. Interestingly, tumor cells expressing distinct CSC markers often exhibit activation of CSC pathways. For example, Notch and Jagged are highly expressed in CD133+ HCC CSCs[12]. Specifically, the molecular subtypes are usually indicative of tumor heterogeneity. The expression levels as well as the activation degree of CSC markers and signaling pathways vary among subtypes, which is a major challenge in preventing or abolishing CSC resistance[14].
MAJOR HINDRANCE OF RESISTANCE

Growing evidence shows that tumor cells harboring stem cell-like characteristics are more resistant to conventional therapeutic approaches than nonstem-like populations. The mechanisms of resistance occurrence and the acquisition of stem-like cell traits are tightly linked to the properties of CSCs, including plasticity, quiescence, CSC niches and the increased drug efflux activity of CSCs.

Plasticity

Triggered by microenvironmental cues (e.g., oncogenic stress, inflammation, and senescence), cancer cells in a nontransformed differentiated state can shift to a tumorigenically transformed undifferentiated or CSC state; this process is referred to as “plasticity”\(^\text{[14]}\). Epithelial-to-mesenchymal transition (EMT) is the most typical example of plasticity. During tumor initiation, tumor cells with a differentiated epithelial phenotype lose apicobasal polarity and acquire a stem-like gene expression program and self-renewal capacity. In turn, the elevation of EMT not only enforces the tumor-initiating capacity but also exacerbates metastatic and therapeutic resistance potential\(^\text{[15,16]}\). CSC plasticity is usually a result of multilineage interconversion, transdifferentiation and dedifferentiation\(^\text{[17]}\). Aberrantly activated plasticity enables tumors to adapt to growth constraints and resist therapy\(^\text{[18]}\). In our recently published studies, we developed a hepatocyte differentiation model that mimics liver development by inducing human ESCs to differentiate into adult hepatocytes along hepatic lineages\(^\text{[19]}\). Inspired by this model, we found that chromodomain-helicase-DNA-binding protein 1-like gene (CHD1L)\(^\text{[20]}\), Claudin6 (CLDN6)\(^\text{[21]}\), poly (ADP-ribose) polymerase 1 (PARP1)\(^\text{[22]}\) and growth differentiation factor 1 (GDF1)\(^\text{[23]}\) are potential therapeutic targets linked to HCC lineage plasticity. These four oncofetal drivers are expressed in the embryonic stage, diminish during terminal differentiation, and reamplify abnormally in HCC. This dynamic change is accompanied by lineage plasticity as a shift from a hepatic lineage to a biliary or progenitor lineage. Notably, the phenotypic shift caused by a high abundance of CLDN6 and PARP1 confers sorafenib resistance. Further suppression of our candidate targets may adversely affect poorly differentiated HCC and sensitize patients to chemotherapeutic agents.

Quiescence

Quiescence or dormancy is another characteristic of CSCs that hinders standard therapy. Upon microenvironmental alterations, such as oxidative stress, hypoxia, nutrient deprivation or chemotherapeutic pressure, CSCs can temporarily transition into the Go phase of the cell cycle and remain dormant. Most standard cancer treatments are directed against proliferating cells, and quiescent or dormant CSCs can evade therapies and revert to the proliferative state when favorable conditions appear\(^\text{[24]}\). Quiescence may be triggered by altered CSC signaling pathways in response to unfavorable microenvironmental stimuli. For example, in HCC, the Smad-independent TGF-\(\beta\) pathway can activate the dormancy program. Restoration of miR122 expression can direct HCC stem-like cells toward cell differentiation and tumor dormancy\(^\text{[25]}\). TGF-\(\beta\) signaling participates in switching quiescent hepatic stellate cells (HSCs) to a myofibroblast (MFB) phenotype through the process of activation to transdifferentiation\(^\text{[16]}\). In addition, MIRLET7BH induces the activation of quiescent HSCs by provoking the SMO-involved Hedgehog pathway\(^\text{[27]}\).

CSC niche

The tumor microenvironment (TME) consists of multiple types of benign cells, extracellular matrix, and signaling molecules. By inducing inflammation, angiogenesis, hypoxia, and fibrosis, particularly in chronically damaged liver tissue, the TME contributes to tumor progression and response to therapy. HCC harbors CSCs in dedicated niches. The extracellular matrix (ECM) component residing in the TME is made up of glycosaminoglycans, fibronectins, collagens, proteoglycans, elastins, and other glycoproteins, which are required for the development of stemness-related CSC phenotypes. An ECM niche is a complex
network of macromolecules produced by cancer-associated fibroblasts (CAFs), macrophages, and endothelial cells (ECs) that regulates tissue architecture and intracellular signaling. The remodeling of the ECM in CSC niches allows CSCs and the ECM to interact abnormally, which is crucial for CSC senescence, quiescence, and phenotypic plasticity. CAFs display enhanced remodeling of the ECM and alter extracellular signaling, producing cytokines and growth factors that fuel cancer cell self-renewal. Activation of stem cell signals has been linked to CAF-mediated therapy resistance in some cases. By allowing tumor cells to disseminate, a soft matrix stiffness promotes tumor outgrowth. As HCC progresses, higher ECM stiffness favors proliferation and self-renewal of CSCs, whereas soft ECM may facilitate metastasis of CSCs\(^7\). 

Hypoxia is critical for the formation and maintenance of CSCs. In the hypoxic TME, HCC cells remain undifferentiated, clone faster, and express specific biomarkers for CSCs. Hypoxia can also enhance HCC stemness via hypoxia-inducible factor (HIF)-dependent mechanisms. Although hypoxic conditions enrich CSC subsets in HCC, biologically distinct liver CSC subpopulations with differing single-cell surface markers and hypoxia-responsive traits have been identified\(^1\). 

An increasing number of studies have shown a strong interplay between CSCs and angiogenesis. Angiogenesis nurtures CSCs by secreting a variety of instructive angiocrine factors to establish a vascular niche and promote the CSC phenotype and regulate CSC stemness. In turn, CSCs preferentially secrete exosomes or angiogenic factors to activate ECs. As CSCs differentiate, they become cancer vascular stem cells/progenitor cells and mimic ECs by forming blood vessels. This phenomenon is termed vasculogenic mimicry (VM), which directly affects tumor microcirculation. In addition, as a result of the vascular microenvironment, stem cells preserve undifferentiated dormancy and protect themselves from injury\(^7,24\). 

It is becoming increasingly apparent that CSCs and immune cells that infiltrate tumors communicate reciprocally, resulting in immune evasion and tumor progression, as well as having therapeutic implications. The polarization and activation of cells, including tumor-associated macrophages (TAMs), neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), B cells, and subsets of T lymphocytes, suppress adaptive immunity and signaling directly to cancer cells. By activating downstream stem cell pathways, growth factors and cytokines released from immune cells can boost self-renewal and increase treatment resistance. Clinical sensitivity to checkpoint blockade is variable, and many patients exhibit acquired or primary resistance. A patient's response to immunotherapy depends on the expression of cell surface molecules, antigenicity, and T-cell infiltration and function. CSCs can modulate these factors to determine a patient's sensitivity. As CSCs have been found to preferentially activate immune suppressive signaling, disrupting immune suppressive signaling could block self-renewal and improve therapeutic sensitivity\(^9,28\). 

**Increased drug efflux activity of CSCs**

ATP-binding cassette (ABC) transporters are capable of exporting a wide range of toxin-producing substances from cells and thus directly contribute to the acquisition of resistance. CSCs overexpress ABC transporters and dysregulate signaling pathway networks, leading to the acquisition of multidrug resistance and maintenance of self-renewal properties, respectively. Side population (SP) cells can be identified as CSCs. ABCG2 was the first ABC transporter to be identified as having phenotypic significance for SP cells. SP cells sorted from HCCLM3, MHCC97-H, MHCC97-L and Hep3B cells display CSC characteristics, remarkable levels of chemoresistance and ABCG2 expression\(^29,30\). TGF-β signaling contributes to drug resistance in liver cancer cells by inducing the expression of the xenobiotic nuclear receptor PXR\(^31\). The ATP-binding cassette transporter ABCF1 functions as a hepatic oncofetal protein and modulates
chemoresponse, migration, EMT and cancer stemness properties\textsuperscript{[32]}. SOX9 enhances sorafenib resistance by modulating ABCG2 expression. Thus, ABC transporter blockade may attenuate CSC-mediated chemoresistance\textsuperscript{[33]}.

**THERAPEUTIC RESISTANCE IN HCC: FROM THE PERSPECTIVE OF CSC TRAITS**

Currently, molecular targeted therapies and immunotherapy are commonly used to treat HCC. Molecular targeted drugs consist of first-line sorafenib and lenvatinib and second-line regorafenib, cabozantinib and ramucirumab. Immunotherapy is a novel management option for HCC and principally includes ICIs against PD-1, PD-L1 and CTLA-4, such as nivolumab, pembrolizumab, MED14736, MPDL3280a, ipilimumab and tremelimumab\textsuperscript{[34]}. The indications, contraindications and interactions for the first- and second-line drugs cited in the review are listed in Table 2. Although these strategies have an increased survival benefit for advanced and metastatic HCC patients, anticancer efficacy remains unsatisfactory, as most patients will eventually acquire drug resistance due to the existence of CSCs; therefore, elucidation of the mechanisms of drug resistance from the CSC perspective is essential for developing effective therapies. Here, the review focuses on related articles published in the past five years concerning CSC-mediated therapeutic resistance in HCC.

**Sorafenib**

Sorafenib is a multikinase inhibitor that targets the tyrosine kinases vascular endothelial growth factor receptor (VEGFR)/platelet-derived growth factor receptor (PDGFR) and RAF serine/threonine kinases in the RAS/RAF/MEK/extracellular signal-regulated kinase (ERK) pathway\textsuperscript{[35]}. Recent studies have revealed that enriched CSCs contribute to sorafenib resistance. CD13 not only provokes HCC carcinogenesis but also induces sorafenib resistance by activating histone deacetylase 5 (HDAC5), lysine-specific demethylase 1 (LSD1)-NF-κB oncogenic signaling\textsuperscript{[36]}. CD24 regulates resistance to sorafenib by deactivating the mammalian target of rapamycin (mTOR)/AKT pathway\textsuperscript{[37]}. AKT/β-catenin HCC tumors harbor a subpopulation of cells that overexpress the cancer stem cell-like marker CD44, which may contribute to tumor sustenance and therapy resistance\textsuperscript{[38]}. The LGR5+ cell population, which harbors features of tumor-initiating cells (TICs), is resistant to sorafenib treatment\textsuperscript{[39]}. Tumor-infiltrating CCR4+ regulatory T cells (Tregs) display PD-1+TCF1+ stem-like properties and enhance immunosuppressive resources in the TME. CCR4+ Tregs promote sorafenib resistance by increasing IL-10 and IL-35 expression and suppressing CD8+ T cells\textsuperscript{[40]}.

In addition to liver CSC markers, CSC signaling pathways are another key factor involved in sorafenib-mediated resistance. Cyclin-dependent kinase 1 (CDK1), which is frequently overexpressed in HCC, reduces sorafenib efficacy via CDK1/PDK1/β-catenin signaling\textsuperscript{[41]}. The CD44-positive HCC subpopulation exhibits sorafenib resistance; however, Hedgehog signaling inhibition can sensitize this subpopulation to the drug\textsuperscript{[42]}. Nuclear factor (erythroid-derived 2)-like 2 (NRF2) is a transcription factor that is highly enriched in liver TICs and regulates HCC TIC properties and sorafenib resistance through regulation of the SHH (sonic hedgehog)/GLI (glioma-associated oncogene homolog) signaling cascade\textsuperscript{[43]}. Our published oncofetal driver CLDN6 is more refractory to sorafenib treatment because it enhances tumor lineage plasticity via the CLDN6/TJP2 (tight junction protein 2)/YAP1 (yeast aspartyl protease) interaction axis and further activates the Hippo signaling pathway\textsuperscript{[21]}. The Wnt target gene EPHB2 (EPH receptor B2) enhances CSC properties and drives sorafenib resistance via the TCF1/EPHB2/β-catenin positive feedback loop\textsuperscript{[44]}.

Increasing evidence suggests that noncoding RNAs (ncRNAs), mainly miRNAs and lncRNAs, are key factors in the development of drug resistance in HCC. miR-494 is associated with stem cell-like characteristics and decreases the sorafenib response by regulating the AKT/mTOR pathway and decreasing
Table 2. Indications, contraindications and interactions for the first- and second-line drugs cited in the review

<table>
<thead>
<tr>
<th>First/second-line treatment</th>
<th>Drugs</th>
<th>Indications</th>
<th>Contraindications</th>
<th>Interactions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-line</td>
<td>Sorafenib</td>
<td>• Advanced renal cell carcinoma (RCC)</td>
<td>• Patients who are hypersensitive to sorafenib or any other component of this drug</td>
<td>• Decrease efficacy: strong CYP3A4 inducers (e.g. carbamazepine, dexamethasone,</td>
<td>[95,96]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unresectable HCC</td>
<td></td>
<td>phenobarbital, phenytoin, rifampin, rifabutin, St. John’s wort; Neomycin</td>
<td></td>
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<td></td>
<td></td>
<td>• Locally recurrent or metastatic, progressive,</td>
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<tr>
<td></td>
<td></td>
<td>• Differentiated thyroid carcinoma refractory to radioactive iodine treatment</td>
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<tr>
<td></td>
<td>Lenvatinib</td>
<td>• Radioactive iodine-refractory differentiated thyroid cancer (DTC)</td>
<td>None</td>
<td>• Increase toxicity: CYP3A, P-gp, and BCRP inhibitors (e.g. Ketoconazole); P-gp inhibitors: [e.g. Rifampicin (600 mg as a single dose);]</td>
<td>[97,98]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Unresectable or advanced HCC</td>
<td></td>
<td>• Decrease efficacy: CYP3A4 inhibitors [e.g. Rifampicin (600 mg daily for 21 days);]</td>
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<tr>
<td></td>
<td></td>
<td>• Advanced RCC</td>
<td></td>
<td>• Prolong the QT/QTc interval: Drugs known to prolong QT/QTc intervals (e.g. Everolimus)</td>
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<tr>
<td></td>
<td></td>
<td>• Endometrial carcinoma</td>
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<tr>
<td></td>
<td>Atezolizumab and</td>
<td>• Treatment of advanced or unresectable HCC not amenable to curative or</td>
<td>None</td>
<td>• There are no known drug interactions with atezolizumab bevacizumab</td>
<td>[99,100]</td>
</tr>
<tr>
<td></td>
<td>bevacizumab</td>
<td>locoregional therapies, who have not received prior systemic therapy</td>
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<tr>
<td></td>
<td>combination</td>
<td>• Barcelona clinic liver cancer (BCLC) stage B or C</td>
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<td></td>
<td></td>
<td>• Child-Pugh A classification of liver function</td>
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<tr>
<td></td>
<td></td>
<td>• Oesophagogastroduodenoscopy for varices within the last 6 months</td>
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<tr>
<td></td>
<td></td>
<td>• ECOG performance status 0 or 1.</td>
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<tr>
<td>Second-line</td>
<td>Regorafenib</td>
<td>• Colorectal cancer</td>
<td>None</td>
<td>• Decrease efficacy: strong CYP3A4 inducers (e.g. rifampin, phenytoin, carbamazepine,</td>
<td>[101]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Gastrintestinal stromal tumors</td>
<td></td>
<td>phenobarbital, and St. John’s Wort)</td>
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<td></td>
<td></td>
<td>• HCC</td>
<td></td>
<td>• Increase toxicity: strong CYP3A4 inhibitors (e.g. clarithromycin, grapefruit juice,</td>
<td></td>
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<tr>
<td></td>
<td>Cabozantinib</td>
<td>• Advanced RCC</td>
<td>None</td>
<td>• Increase breast cancer resistance protein (BCRP) substrates toxicity: BCRP substrates</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• HCC</td>
<td></td>
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<tr>
<td></td>
<td>Ramucirumab</td>
<td>• Gastric cancer</td>
<td>None</td>
<td>• Increase the risk of exposure-related adverse reactions: strong CYP3A4 inhibitors</td>
<td>[103]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Non-small cell lung cancer (NSCLC)</td>
<td></td>
<td>• Reduce efficacy: strong CYP3A inducers</td>
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<tr>
<td></td>
<td></td>
<td>• Colorectal cancer</td>
<td></td>
<td>No information provided</td>
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<tr>
<td></td>
<td></td>
<td>• HCC</td>
<td></td>
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<tr>
<td></td>
<td>Pembrolizumab</td>
<td>• Unresectable or metastatic melanoma</td>
<td>Hypersensitivity to pembrolizum or any component of the formulation.</td>
<td>Thalidomide analogues: Pembrolizum may enhance the adverse/toxic effect of thalidomide analogues.</td>
<td>[104,105]</td>
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<tr>
<td></td>
<td></td>
<td>• NSCLC</td>
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<tr>
<td></td>
<td></td>
<td>• Head and neck squamous cell cancer</td>
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<td></td>
<td></td>
<td>• Relapsed or refractory hodgkin lymphoma</td>
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<tr>
<td></td>
<td></td>
<td>• Primary mediastial large B-cell lymphoma</td>
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</tr>
</tbody>
</table>
• Urothelial carcinoma
• Microsatellite instability-high or mismatch repair deficient cancer
• Gastric cancer
• Esophageal cancer
• Recurrent or metastatic cervical cancer
• HCC
• RCC
• Merkel cell carcinoma
• Endometrial carcinoma
• Tumor mutational burden-high cancer
• Cutaneous squamous cell carcinoma
• Triple-negative breast cancer

**Nivolumab**

- Hodgkin lymphoma, classical
- HCC
- Colorectal cancer, metastatic (microsatellite instability-high or mismatch repair deficient cancer)
- Head and neck squamous cell cancer (recurrent or metastatic)
- Melanoma
- NSCLC, metastatic, progressive
- Small cell lung cancer, metastatic
- Advanced RCC

- Hypersensitivity to nivolumab or any component of the formulation

**Ipilimumab**

- Metastatic colorectal cancer
- Unresectable or metastatic melanoma
- Advanced RCC

- Hypersensitivity to ipilimumab or any component of the formulation; active life-threatening autoimmune disease, or with organ transplantation graft where further immune activation is potentially imminently life-threatening

- Belimumab: Monoclonal antibodies may enhance the adverse/toxic effect of Belimumab
- Immunosuppressants: May diminish the therapeutic effect of nivolumab

**ECOG:** Eastern Cooperative Oncology Group; **CYP3A4:** cytochrome P450 3A4.

PARP levels\(^{[45]}\). N6-methyladenosine-modified circRNA-SORE functions as a miRNA sponge to sequester miR-103a-2-5p and miR-660-3p and maintains sorafenib resistance by competitively activating the Wnt/β-catenin pathway\(^{[46]}\). Overexpression of IncRNA SNHG1 (small nucleolar RNA host gene 1) supports sorafenib resistance by activating the AKT pathway via upregulation of SLC3A2 (solute carrier family 3 member 2), and its increased nuclear expression is promoted by miR-21 in HCC cells\(^{[47]}\). Translation regulatory IncRNA 1 (TRERNA1) acts as a miR-22-3p sponge to positively regulate NRAS expression at the posttranscriptional level. TRERNA1 enhances HCC sorafenib resistance by activating the RAS/Raf/MEK/ERK signaling pathway\(^{[48]}\). Under long-term sorafenib exposure, HCC cells enrich a fraction of quiescent stem-like cells. Single-cell RNA analysis has demonstrated the vital role of stemness and EMT in causing sorafenib resistance. ZNFX1 antisense RNA 1 (ZFAS1) is a novel regulator IncRNA found to be overexpressed in higher stages (III/IV) of HCC and in poorly (G3)/undifferentiated HCC (G4) compared with levels at early stages (I/II) and in highly (G1)/moderately (G2) differentiated HCC. Overexpression of ZFAS1 increases the expression of stemness genes (e.g., EpCAM, CD24, CD90, CD133, DLK1, and CK18/19) and EMT markers (e.g., S100A4/A6, Twist, and
Vimentin). Thus, ZFAS1 is regarded as a critical mediator of sorafenib resistance via the induction of stemness and EMT phenotypes\[49\].

**Lenvatinib**

Lenvatinib is another oral multikinase inhibitor that selectively inhibits the tyrosine kinases VEGFR1-3, fibroblast growth factor receptor (FGFR)-1-4, PDGFR-α, c-Kit and RET and is recommended as a first-line systematic therapy in patients with advanced HCC\[50\]. CD73 is a novel biomarker and poor prognosis indicator of HCC, and overexpression of CD73 is essential for the sustainment of CSC features, thereby promoting HCC progression and metastasis. CD73 boosts lenvatinib resistance by upregulating SOX9 (sex determining region Y-box 9), and the increase in SOX9 expression induced by CD73 is achieved through reactivation of AKT signaling, followed by enhancement of SOX9 transcription via the downstream AKT signaling target c-Myc. Moreover, CD73 hinders SOX9 ubiquitination and proteasome degradation by abolishing GSK3β\[51,52\]. By applying a kinome-centered clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (CRISPR-Cas9) genetic screen, EGFR was found to be negatively correlated with HCC sensitivity to lenvatinib. Notably, EGFR is an essential receptor tyrosine kinase regulator of CSCs\[53\]. In terms of mechanism, lenvatinib inhibits FGFR and then triggers EGFR-PAK2-ERK5 signaling activation\[54\]. In addition, IGF1R is responsible for the acquisition of further lenvatinib resistance in HCC cells\[55\]. Another genome-scale CRISPR-Cas9 knockout screening identified two important resistance genes, neurofibromin 1 (NF1) and dual specificity phosphatase 9 (DUSP9). Removal of NF1 and DUSP9 limits the response of HCC to lenvatinib. The absence of NF1 activates both the PI3K/AKT and mitogen-activated protein kinase (MAPK)/ERK signaling pathways, while DUSP9 loss activates the MAPK/ERK but not the PI3K/AKT pathway, thereby downregulating FOXO3 activity, followed by FOXO3 degradation\[56\]. Integrin subunit beta 8 (ITGB8) is a novel contributor to lenvatinib resistance. Elevated expression of ITGB8 may be regulated via the transcription factor NF-xB. ITGB8-mediated resistance to lenvatinib occurs through an induced increase in the expression of HSP90, which inhibits AKT ubiquitination and promotes AKT stabilization, thereby activating the AKT signaling pathway\[57\]. ST6 beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) is a tumor-derived secreted protein that is positively regulated upstream of fibroblast growth factor (FGF)-19 in HCC cells. FGF19 is a critical oncogenic driver gene that facilitates CSC-like properties in liver CSCs\[58\]. Intriguingly, low levels of FGF19 eliminated lenvatinib susceptibility. However, FGF19 was re-expressed in lenvatinib-resistant HCC cells; therefore, FGF19 is a potential biomarker of lenvatinib-susceptible HCC. Proteome and secretome analyses revealed that FGF19 transcriptionally activates ST6GAL1 via STAT3 phosphorylation, and serum ST6GAL may be a useful biomarker for the identification of lenvatinib-susceptible FGF19-driven HCC\[59\].

The expression of the ncRNA circMED27 is elevated in HCC serum and is positively associated with a poor prognosis for HCC patients. circMED27 induces insensitivity to lenvatinib by sponging miR-655-3p, which acts as a tumor suppressor by inhibiting the β-catenin pathway\[60\].
Regorafenib

Regorafenib is an oral multikinase inhibitor approved as a second-line treatment for unresectable HCC. Regorafenib targets a broad range of relevant protein kinases, including VEGFR1-3, TIE2, FGFR1-2, PDGFR, KIT, RAF, and RET. Although regorafenib brings clinical benefits, drug resistance is inevitable [63].

Gankyrin augments β-catenin mRNA levels by increasing the expression of the RNA-binding protein HuR. The upregulation of β-catenin promotes the expression of c-Myc, which contributes to the tolerance to sorafenib and regorafenib mediated by Gankyrin in HCC [64]. Pin1 is a high-abundance gene in regorafenib-resistant HCC cells. Pin1 contributes to regorafenib resistance by affecting EMT traits. Inhibition of Pin1 suppresses EMT and metastasis by reducing the levels of the EMT regulators E-cadherin and Snail; additionally, Pin1 interacts with another EMT regulator, Gli1 [65]. Through CRISPR/Cas9 screening, the Hippo signaling pathway, which is responsible for regorafenib resistance, was identified. Inactivation of large-tumor suppressor 2 (LATS2) leads to YAP dephosphorylation, and YAP inhibition resensitizes regorafenib-insensitive HCC cells to regorafenib [66]. RAB27A is a Rab GTPase that controls the release of exosomes. Exosomes secreted by liver CSCs transfer regorafenib resistance to differentiated HCC cells when RAB27A is activated. Moreover, RAB27A-derived exosomes promote Nanog expression to maintain CSC self-renewal ability. Downregulating RAB27A sensitizes HCC cells to regorafenib [67]. Interestingly, acute regorafenib administration enhances Wnt/β-catenin signaling in hepatoblast-like HCC cells, along with the activation of hepatic stem/progenitor markers. In turn, the activation of Wnt/β-catenin signaling caused by Wnt3a-R-Spo1 intervention avoids apoptosis from regorafenib stimuli. In addition, long-term regorafenib tolerance leads to enhanced TGF-β signaling activity and increased expression of the CSC markers CD24 and CD133 [69]. Interleukin-6 receptor alpha (IL-6Rα) is induced in response to sorafenib and is essential for IL-6-mediated sorafenib resistance in HCC. Transcription factor 3 (ATF3) binds to the IL-6Rα promoter and induces IL-6Rα expression. The ATF3-IL-6Rα cascade is also activated in regorafenib-resistant HCC cells. Blockade of IL-6Rα sensitizes HCC to sorafenib and regorafenib both in vitro and in vivo [69].

Immune checkpoint inhibitors

Despite the promising outcomes of ICIs for HCC treatment, not all patients are sensitive to ICIs and inevitably acquire resistance to ICI therapy. A large number of studies have shown that CSCs are necessary for immunosuppressive microenvironment formation, resulting in immune evasion. The molecular mechanisms mainly include elevated expression of immunosuppressive factors and activation of CSC signaling pathways correlated with ICI resistance. The Wnt/β-catenin signaling pathway and TGF-β signaling pathway are closely connected with immune evasion, exclusion and resistance [70].

An HCC immune-excluded class (cold tumors), defined by Wnt/CTNNB1 mutations, is refractory to ICIs in patients treated with anti-PD-1/PD-L1 monoclonal antibodies (81%), anti-CTLA-4 monoclonal antibodies or combinations of anti-PD-1/PD-L1 antibodies with anti-CTLA-4, anti-KIR or anti-LAG3 antibodies (19%) [71]. MYC; p53-/- HCC tumors show highly active β-catenin signaling, which directly promotes immune escape. In addition, the β-catenin-driven MYC-lucOS;CTNNB1 or MYC-luc;CTNNB1 models are insensitive to nivolumab and pembrolizumab. Of particular interest, CTNNB1 mutation may be used as a biomarker for HCC patient exclusion [72]. In our recent studies, the secreted protein GDF1, which belongs to the TGF-β superfamily, was found to be highly expressed in embryonic stem cells and is gradually downregulated in the endoderm, liver progenitor cells, premature hepatocytes, and hepatocytes but reactivated in HCC. Overexpression of GDF1 leads to tumor dissemination and metastasis. Ectopic expression of GDF1 can induce tumor-lineage plasticity through the activin receptor-like kinase 7 (ALK7)-mothers against decapentaplegic homolog 2/3 (SMAD2/3) signaling cascade. Intriguingly, GDF1-mediated lineage plasticity might be an Achilles heel for HCC immunotherapy. A wide panel of cancer-testis antigens (CTAs) are activated in HCC by GDF1 via suppression of the epigenetic regulator lysine-specific demethylase 1 (LSD1). The inhibition of LSD1 is mediated by SMAD2/3 binding to its promoter region in a
GDF1-dependent manner. HNRNPM (heterogeneous nuclear ribonucleoprotein M) is an elevated oncofetal splicing factor that has the same expression pattern as GDF1 in HCC. HNRNPM is essential for maintaining the stem cell-like properties and tumorigenesis of HCC cells. Moreover, HNRNPM mediates the immunosuppressive tumor environment in HCC. Mechanistically, HNRNPM binds to the flanking introns of MBD2 to promote its alternative splicing. MBD2a, one of the isoforms of MBD2, enhances FZD3 expression and activates the Wnt/β-catenin signaling pathway. FZD3 and β-catenin further promote the expression of SOX2 and OCT4. Interestingly, ectopic expression of SOX2 and OCT4, in turn, upregulates HNRNPM expression via direct promoter binding. Patients with anti-PD1-resistant HCC are frequently positive for zinc finger protein 64 (ZFP64). ZFP64 induces anti-PD1 resistance by shifting macrophage polarization to an abnormal activation phenotype (M2) and inhibiting the TME. Mechanistically, protein kinase C alpha (PKCα) phosphorylates ZFP64 at S226 and induces its nuclear translocation, leading to transcriptional activation of macrophage colony-stimulating factor (CSF1). This results in a shift of macrophages to the M2 phenotype. Clinically, anti-PD1 resistance is frequently observed in individuals with an active PKC/ZFP64/CSF1 axis.

circMET (hsa_circ_0082002) is an onco-circRNA that is upregulated in HCC, and overexpression of circMET promotes EMT and the formation of an immunosuppressive tumor microenvironment. Generally, the effect of immune tolerance triggered by circMET occurs through the Snail/dipeptidyl peptidase 4 (DPP4)/CXCL10 axis. In detail, circMET works as a sponge for miR-30-5p; Snail is a novel identified target of miR-30-5p, and the upregulation of Snail significantly promotes DPP4 expression. The miR-30-5p/Snail/DPP4 axis further degrades CXCL10 to trigger immunosuppression.

**THERAPEUTIC STRATEGIES FOR CSC-MEDIATED DRUG RESISTANCE**

**Drug resistance-related CSC identification strategies**

The identification of drug resistance-related CSCs can be achieved by multi-omics (e.g., transcriptomics, proteomics, metabolomics, lipidomics, glycomics) in publicly accessible repositories (e.g., gene expression omnibus (GEO), CancerDR, canSAR, Genomics of Drug Sensitivity in Cancer data, and Platinum) or institutional sources. However, due to intratumor heterogeneity, tissue biopsy sampling errors may occur. Strikingly, liquid biopsy has recently gained popularity as a noninvasive and repeatable means of detecting drug resistance indicators in body fluids. Liquid biopsy can alleviate the difficulties in collecting tissue biopsies, provide insight into spatial and/or temporal heterogeneity, and facilitate dynamic therapeutic response monitoring (detection of resistance mechanisms).

Multiple techniques have been applied for analysis and post-analysis after sample collection and processing. To date, drug-resistant drivers have been measured via real-time quantitative polymerase chain reaction (qPCR), immunoblotting, immunohistochemistry, immunofluorescence, flow cytometry, mass spectrometry and next-generation sequencing (NGS). Recent achievements in NGS, such as bulk-cell RNA sequencing, genome-wide CRISPR sequencing, long-read RNA sequencing, circular RNA sequencing and single-cell RNA sequencing (scRNA-seq), have provided extraordinary insights into tumor heterogeneity, treatment resistance, tumor relapse, and metastasis. Ho et al. adopted single-cell genomics to unveil the landscape of intratumoral heterogeneity and identify a rare cell subpopulation of CD24+/CD44+ cells within the EPCAM+ population in HCC. Zhou et al. applied lineage tracing and scRNA-seq to reveal the increasing tumorigenicity and the ability to form cancers of differential lineages of Prom1+ HCC cells, highlighting the heterogeneity and dynamics of Prom1+ HCC cells, which confer a dedifferentiated status and stem cell traits. CD24, CD44, and PROM1 have already been verified to be responsible for drug resistance in HCC.
**Preclinical models for evaluating CSC-mediated drug resistance**

Tumor-derived cell lines and patient-derived xenografts (PDXs) are the two most common models used for drug resistance studies because they are able to retain the majority of the molecular characteristics of primary tumors. A large variety of tumor-derived cell lines are acceptable for studies on the molecular prediction of drug response and biomarker discovery. Caruso et al. performed whole-exome RNA and microRNA sequencing to screen the efficacy of 31 anticancer agents in 34 liver cancer cell lines and distinguished genetic alterations and gene expression patterns correlated with drug response\[^{81}\]. Large-scale functional screening using RNAi or CRISPR/Cas9 is another promising technique for studying CSC-mediated drug resistance. For instance, Wei et al. utilized CRISPR/Cas9 library screening and identified phosphoglycerate dehydrogenase (PHGDH) as an essential gene for sorafenib resistance\[^{82}\]. Overexpression of PHGDH leads to regorafenib and lenvatinib resistance in HCC. In particular, PHGDH is required for cancer stem cell maintenance\[^{83}\]. Despite the ease of manipulation and suitability for high-throughput screening of tumor-derived cell lines, they are unable to fully reflect the native 3D environment of tumor cells. Instead, PDXs more closely mimic parental tumors. He et al. developed the first public liver cancer PDX model database, which contains 116 PDTXs generated from HCC\[^{84}\]. The molecular and drug response data, as well as the clinical annotation, are available from: http://www.picb.ac.cn/PDXliver/. Despite this, long engraftment periods and low engraftment efficiency are still the major drawbacks hindering the application of the PDX model in large-scale drug response testing. To overcome the shortcomings of 2D culture and PDX, a 3D preclinical organoid model was developed. Coexisting CSC subclones display different sensitivities to drug therapies, but HCC organoids retain the histological architecture, genetic background and heterogeneity of the parent tumor; therefore, organoid models allow the personalized management of targeted therapies. Broutier et al. first successfully developed human primary liver cancer organoid lines to identify patient-specific drug sensitivities\[^{85}\]. Nuciforo et al. generated long-term organoid cultures to test sorafenib sensitivity\[^{86}\]. Wang et al. established patient-derived organoid models to examine how CD44 and Hedgehog signaling contribute to sorafenib tolerance and determine whether combination therapy with sorafenib and a compound targeting Hedgehog signaling is effective in overcoming resistance\[^{42}\]. In addition to the above models, immunocompetent mice, including spontaneous, chemically induced and genetically engineered models, are also widely used in the study of drug resistance\[^{87}\]. Overall, the development of novel preclinical models and the combined application of multiple preclinical models will undoubtedly improve the accuracy of drug sensitivity and/or resistance predictions in a patient-specific manner [Figure 2].

**MONO- OR COMBINATION THERAPIES TO AGAINST DRUG RESISTANCE**

**Therapeutic strategies targeting CSCs in HCC**

Recent advances in CSC biology have accelerated research on CSC eradication. Multiple therapeutic strategies are being developed to target CSCs. CSCs can be effectively targeted by targeting core signaling cascades. AKT, Hedgehog, Notch, TGF-β, and Wnt pathway inhibitors suppress CSC characteristics in HCC. Considering the crosstalk between CSCs and HCC cells, intricate CSC niches contribute to both tumor growth and resistance to therapy. Single or combined treatments that target the CSC niches are effective at treating CSCs. Targeting CSC surface markers provides a straightforward approach for addressing CSCs. It has been suggested that some ABC transporters are abundantly expressed in SP cells and positively associated with the acquisition of drug resistance; thus, targeting critical molecules in SP cells may be an effective strategy to eradicate CSCs. Epigenetic modifications, including chromatin remodeling, DNA methylation and histone modification, are involved in the regulation of CSC properties. However, epigenetic modulation is rarely targeted to specific genes, resulting in an alteration of gene function that is not specific. In light of the roles of miRNAs and IncRNAs in regulating the CSC phenotype and HCC progression, miRNA/IncRNA-targeted therapies are expected to have great therapeutic potential\[^{88,89}\].
**Sorafenib**

Our published oncofetal driver CLDN6 strikingly enhances tumor lineage plasticity, resulting in activation of the Hippo signaling pathway via the CLDN6/TJP2/YAP1 axis. A de novo anti-CLDN6 monoclonal antibody conjugated with a cytotoxic agent (mertansine, DM1) (CLDN6-DM1) was established. Cotreatment with sorafenib and CLDN6-DM1 exhibited an even more dramatic synergistic effect in inhibiting tumor formation than treatment with sorafenib alone. Another oncofetal driver, PARP1, is prone to induce stem cell pluripotency and sorafenib resistance in HCC. Sorafenib treatment stimulates DNA damage repair. The PARP inhibitor olaparib profoundly inhibits key pluripotent transcription factors and DNA damage repair signaling, potentially through CHD1L-mediated condensation of the chromatin structure at promoter regions. A synergistic combination of olaparib and sorafenib is more effective than sorafenib alone in eliminating HCC residual tumors. CD13 is necessary for sorafenib-induced resistance, and the blockade of CD13 with its inhibitor ubenimex can suppress tumor growth and restore sorafenib sensitivity in HCC. Multiple lines of evidence suggest that Jak/Stat signaling is activated in HCC CSCs. Jak/Stat inhibitors (AZ960 and TG101209) remarkably suppress CSC properties and delay the formation of AKT/β-catenin-driven HCC tumors, which may be an ideal method to overcome drug resistance. LGR5 is a well-characterized CSC marker in HCC. The LGR5+ cell compartment can be ablated through diphtheria toxin (DT). Simultaneous administration of 5-FU and DT obviously improves the efficacy of 5-FU in combatting HCC, but this is not the case with the combination of sorafenib and DT. Studies have shown that the CDK1/PDK1/β-catenin signaling pathway is activated in HCC. Abrogation of CDK1 with its inhibitor RO3306 confers sensitivity to sorafenib in HCC cells; a 50% tumor incidence was reported in the sorafenib-treated group, while only 17% incidence was observed in the combinatorial treatment group. CD44+ populations in HCC are more refractory to sorafenib. For CD44-positive HCC patient-derived organoids, restricting Hedgehog signaling improves sensitivity to sorafenib. Cotreatment with sorafenib and GANT61 (a Hedgehog signaling inhibitor) has a highly synergistic effect on inhibiting malignant properties in CD44-positive HCC, both in vitro and in vivo. The Wnt target gene EPHB2 has been identified to form an EPHB2/β-catenin/TCF1 positive feedback loop that elevates HCC stemness and sorafenib resistance. Currently, there are no available EPHB2 kinase inhibitors. Intravenous injection of rAAV-8-EPHB2 in an immunocompetent mouse model enhanced the efficacy of sorafenib treatment and further blocked...
tumorigenicity\[44\]. The lncRNA SNHG1 participates in sorafenib resistance by activating the AKT pathway via regulation of SL3A2. SNHG1 depletion boosts sorafenib antitumor responses. Compared with control tumors, anti-SNHG1 treatment reduced tumor size by 54.5%, while combinational therapy reduced tumor size by 76.4%\[47\]. ZFAS1 is a novel regulatory IncRNA that mediates sorafenib resistance via the induction of stemness and EMT phenotypes, and silencing ZFAS1 was found to kill quiescent HCC stem-like cells (EpCAM+CD133+) to overcome sorafenib resistance\[50\]. CCR4+ TIL-Tregs display enhanced immunosuppressive PD1+TCF1+ stem-like specificity; thus, targeting CCR4+ regulatory T cells using N-CCR4-Fc, a neutralizing pseudoreceptor, can eliminate sorafenib resistance and increase the tumor response to immune checkpoint blockade, which has been shown to suppress tumor growth, reduce tumor weight and prolong the survival of mice with liver cancer\[40\].

**Lenvatinib**

Treatment of 12 patients with EGFR\textsuperscript{high} HCC who were unresponsive to lenvatinib with the widely used EGFR inhibitor erlotinib plus lenvatinib resulted in beneficial clinical outcomes (trial identifier NCT04642547). Lenvatinib plus gefitinib reduced the total tumor burden by 76.5% after four weeks. Erlotinib effectively reverses lenvatinib resistance\[54,55\]. Loss of NF1 and DUSP9 activates the PI3K/AKT and MAPK/ERK signaling pathways that confer lenvatinib resistance. In addition, despite NF1 knockout, trametinib (a MEK inhibitor) is still capable of halting HCC growth, which provides evidence that trametinib may mediate the susceptibility of HCC cells to lenvatinib\[56\]. The ITGB8/HSP90/AKT axis offers an attractive strategy for treating lenvatinib-unresponsive HCC, and the AKT inhibitor MK-2206 or the HSP90 inhibitor 17-AAG could elevate the efficacy of lenvatinib treatment. Lenvatinib treatment completely retards tumor growth in mice raised from ITGB8 knockdown cells, while control small hairpin RNA (shRNA) cells grew steadily\[57\].

**Regorafenib**

Gankyrin has been reported to promote HCC tumorigenesis, metastasis and resistance to sorafenib or regorafenib through β-catenin/c-Myc signaling. c-Myc pathway inhibition by shRNA or 10058-F4 largely abolishes c-Myc-dependent metabolic reprogramming, and 10058-F4 in combination with sorafenib or regorafenib has more significant effects on the inhibition of tumor cell growth\[44\]. Pin1 is insensitive to regorafenib treatment and EMT properties via the Gli1/Snail/E-cadherin pathway in HCC. All-trans retinoic acid (ATRA) has recently been identified as a Pin1 inhibitor. ATRA reduces the number of metastatic nodules in lung tissue by downregulating Pin1 and Snail and upregulating E-cadherin, which indicates that ATRA alleviates regorafenib resistance-induced metastasis by reversing EMT\[65\]. The Hippo signaling pathway could act as a mediator for the efficacy of regorafenib in HCC. The YAP inhibitor verteporfin significantly augments the cytotoxicity of regorafenib in regorafenib-tolerant HCC cells, which offers an attractive strategy for treating regorafenib-insensitive HCC\[66\]. RAB27A-dependent exosome secretion from CSCs supports regorafenib resistance by inducing Nanog expression. RAB27A depletion via CRISPR/Cas9 alleviates Nanog expression and strongly increases the efficacy of regorafenib, significantly restraining the growth of xenografts\[67\]. Long-term regorafenib tolerance contributes to enhanced Transforming growth factor (TGF-β) signaling activity, and TGF-β type 1 receptor (TGFβ-R1) inhibition leads to a decrease in colony formation and an increase in cell death in resistant spheroids. In addition, in the zebrafish model, HuH7 cells migrated in 21.6% of zebrafish, regorafenib-resistant cells migrated in 43% of zebrafish, and TGFβ-R1 inhibitor-treated regorafenib-resistant cells only migrated in 12.9% of zebrafish. The combination of a TGFβ-R1 inhibitor and regorafenib diminishes pSTAT3, pSMAD2 and pERK (44/42) expression and increases regorafenib sensitization\[44\]. The ATF4-ATF3-IL-6Rα cascade is responsible for sorafenib- and regorafenib-induced resistance. IL-6Rα can be blocked with sarilumab, an FDA-approved antibody drug, and the combination of sorafenib or regorafenib and sarilumab is even more effective, with a
significant and pronounced reduction in tumor growth and tumor weight in HCC PDX models compared with sorafenib or regorafenib alone. Blockade of IL-6Ra might be a novel therapeutic approach to improve the efficacy of sorafenib/regorafenib in advanced HCC patients[69].

**Immune checkpoint inhibitors**

Our recent study showed that GDF1 suppresses LSD1 to enhance CTA expression. The combination of an LSD1 inhibitor and an anti-PD1 antibody is more effective than a single treatment in restraining tumor growth and metastasis and prolonging overall survival. Blockade of LSD1 may boost the immune system and broaden the therapeutic window for ICIs in HCC patients[23]. Studies have established links between HNRNPM and immunotherapeutic resistance. HNRNPM-specific antisense oligonucleotides (ASOs) can inhibit cancer stemness, recruit more CD8+ T cells and suppress the WNT/β-catenin signaling pathway; hence, HNRNPM inhibition can block immune evasion and elevate the efficacy of ICI therapy[73]. A recent study suggested that the PKCa/ZFP64/CSF1 axis is critical for triggering immune evasion and anti-PD1 resistance in HCC patients. Gö6976, an inhibitor of PKCa, inhibits activation of the PKCa/ZFP64/CSF1 axis in a dose- and time-dependent manner. The Gö6976-treated group had significantly lower tumor weights than the vehicle group. Gö6976 combined with anti-PD1 therapy significantly inhibits tumor growth and evidently improves the tumor immune microenvironment, thus overcoming anti-PD1 resistance[74]. The circMET/miR-30-5p/Snail/DPP4 axis has been reported to be involved in the immune tolerance of HCC. Sitagliptin is a selective DPP4 inhibitor that has been approved for diabetes treatment, and sitagliptin plus anti-PD1 therapy resulted in 100% tumor regression. The combination therapy with sitagliptin and anti-PD1 antibody increased CD8+ T lymphocyte trafficking and sensitized tumors to PD1 blockade in the HCC clinical setting[75].

For unbiased and easily accessed information on the management of liver injury attributable to prescription and nonprescription medications, the potential hepatotoxicity of indicated drugs for HCC treatment is shown in Table 3. The RUCAM (Roussel Uclaf Causality Assessment Method) causality assessment score yields total scores that can range from -9 to 14; drug causality can be “highly probable” (score > 8), “probable” (score 6-8), “possible” (score 3-5), “unlikely” (score 1-2) or “excluded” (score ≤ 0). For “highly probable”, RUCAM sometimes uses other terms, including “highly likely” and “definite”[90].

**CONCLUSIONS**

For many years, pharmaceutical therapies have largely relied on sorafenib, which has enabled some improvements in survival but not in the quality of life. Recently, a phase 3 randomized, open-label study, the IMbrave150 study (NCT03434379), showed that atezolizumab (anti-PD-L1 antibody) plus bevacizumab (anti-VEGF antibody) significantly improved overall survival (OS) and progression-free survival (PFS) versus sorafenib and had an acceptable safety profile in patients with unresectable HCC after longer follow-up, which confirmed atezolizumab plus bevacizumab as the first-line standard of care for advanced HCC[91,92]. Another phase III randomized trial, the HIALAYA trial (NCT03298451), compared a single priming dose of tremelimumab (anti-CTLA-4 antibody) with once-monthly durvalumab (anti-PDL1 antibody) to sorafenib. Tremelimumab plus durvalumab led to better OS than sorafenib as a first-line treatment for patients with unresectable HCC[93]. Other clinical trials are underway to improve the efficacy of combination therapy in advanced HCC. For example, a phase III randomized trial is examining apatinib plus camrelizumab (anti-PD1 antibody) versus sorafenib (NCT03764293), a phase II study is exploring nivolumab and regorafenib (NCT04310709), and a phase II study is investigating regorafenib plus tislelizumab (anti-PD1 antibody) (NCT04183088)[94].
Table 3. Hepatotoxicity of indicated drugs in case reports

<table>
<thead>
<tr>
<th>Drugs</th>
<th>RUCAM score</th>
<th>Causality assessment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorafenib</td>
<td>7</td>
<td>Probable</td>
<td>[108]</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Highly probable</td>
<td>[109]</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>Probable</td>
<td>[110]</td>
</tr>
<tr>
<td>5-FU</td>
<td>6</td>
<td>Probable</td>
<td>[111]</td>
</tr>
<tr>
<td>Crizotinib</td>
<td>8</td>
<td>Probable</td>
<td>[112]</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Highly probable</td>
<td>[113]</td>
</tr>
<tr>
<td>Atezolizumab</td>
<td>7</td>
<td>Probable</td>
<td>[114]</td>
</tr>
<tr>
<td>Ipilimumab</td>
<td>9</td>
<td>Highly probable</td>
<td>[115]</td>
</tr>
<tr>
<td>Pembrolizumab</td>
<td>9</td>
<td>Highly probable</td>
<td>[116]</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Probable</td>
<td>[117]</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>NA</td>
<td>Probable</td>
<td>[110]</td>
</tr>
<tr>
<td>Paclitaxel+Seggio+Sintilimab</td>
<td>8</td>
<td>Highly likely</td>
<td>[118]</td>
</tr>
</tbody>
</table>

NA: Not available.

There has been a dramatic improvement in our understanding of HCC biology. Biological features and clinical properties differ among HCC patients, which is likely due to heterogeneity. Clinically, heterogeneity is largely responsible for tumor progression, metastasis, relapse, and resistance to treatment. Because CSCs are the major source of tumor heterogeneity, CSC characteristics are undoubtedly tightly associated with drug resistance. Intriguingly, CSCs have features similar to those of ESCs, which suggests the importance of developmental signals in resistance to therapeutics. Hence, deep exploration of stemness properties will largely facilitate further insights into treatment resistance.

Although studies on CSCs are broadening our understanding, the implementation of effective precision medicine remains a challenge. Reliable biomarkers to predict treatment failure are still lacking. Precise targeting of specific CSC properties might serve as a novel strategy to eliminate resistant CSC subpopulations. In addition, combined application of multiple techniques and models or development of new effective approaches will allow detailed characterization of heterogeneity and elucidation of the mechanisms underlying therapeutic resistance and thus result in more favorable outcomes.

DECLARATIONS
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Authors’ contributions
Initiated the study and finalized the manuscript: Liu M
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Read and approved the final manuscript: All authors

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REFERENCES
et al. Hepatoma Res


immunotherapy makes strides against HCC. *Cancer Discov* 2022;12:OF1. DOI PubMed


Highlights of prescribing information for lenvima® (lenvatinib) capsules, for oral use. Available from: https://www.lenvima.com/-/media/Project/EISAI/Lenvima/PDF/prescribing-information.pdf [Last accessed on 22 Sep 2022].


Tzadok R, Levy S, Aouizerate J, Shibolet O. Acute liver failure following a single dose of atezolizumab, as assessed for causality using the updated RUCAM. *Case Rep Gastrointest Med* 2022;2022:5090200. DOI PubMed PMC


Al-Nattah S, Lata Sharma K, Caldis M, Spengler E, Nicholas Rose W. Plasmapheresis for pembrolizumab-induced hepatitis in a patient with squamous cell carcinoma and prior orthotopic liver transplantation. *Case Reports Hepatol* 2022;2022:5908411. DOI PubMed PMC
