Universidade de Lisboa

Faculdade de Farmácia

Cancer stem cells in liver cancer

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Monografia orientada pela Professora Doutora Marta Bento Afonso, Professora Auxiliar.

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Resumo

O cancro primário do fígado é o sexto tipo de cancro mais diagnosticado e a terceira principal causa de morte relacionada com cancro em todo o mundo, com cerca de 906 000 novos casos e 830 000 fatalidades reportadas anualmente. O carcinoma hepatocelular (HCC) e o colangiocarcinoma (CCA) são os dois cancros hepatobiliares mais comuns, representando 75%-85% e 10%-15% dos casos de cancro, respetivamente. Evidência acumulada sugere que uma pequena subpopulação especializada de células cancerígenas, conhecidas como células estaminais cancerígenas (CSCs), está implicada nos processos de iniciação, progressão, resistência à terapêutica e recorrência tumoral.

Este trabalho oferece uma revisão abrangente da literatura existente centrada em CSCs no fígado, visando elucidar as suas características moleculares únicas e mecanismos celulares que contribuem para o seu comportamento agressivo e resistência às terapêuticas convencionais.

Destacamos a heterogeneidade e a plasticidade das CSCs hepáticas, detalhando a sua capacidade de auto-renovação, diferenciação e de iniciação de tumores. As origens celulares dos cancros primários do fígado são descritas, abrangendo hepatócitos, colangiócitos e CSCs. Adicionalmente, o complexo papel do microambiente hepático no desenvolvimento e progressão do cancro do fígado é explorado, enfatizando a relação recíproca entre o microambiente tumoral e o nicho das CSCs. Discutimos, também, os mecanismos celulares reguladores das CSCs hepáticas, que podem servir como potenciais alvos terapêuticos e ferramentas de diagnóstico/prognóstico. Finalmente, esta tese realça os desafios e oportunidades associados à existência de CSCs, discutindo o seu papel na resistência ao tratamento, bem como possíveis estratégias para o desenvolvimento de terapêuticas personalizadas focadas nas CSCs hepáticas.

No geral, este trabalho fornece uma panorâmica ampla do papel das CSCs no desenvolvimento e progressão dos cancros primários do fígado, delineando as suas perspetivas terapêuticas e preparando o terreno para o desenvolvimento de tratamentos inovadores que possam melhorar os resultados dos doentes e revolucionar a gestão do cancro do fígado.

Palavras-chave: Cancro primário do fígado; carcinoma hepatocelular; colangiocarcinoma; células estaminais cancerígenas.

Abstract

Primary liver cancer ranks as the sixth most commonly diagnosed cancer and the third leading cause of cancer-related deaths worldwide, with approximately 906,000 new cases and 830,000 fatalities reported annually. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the two most common hepatobiliary cancers, accounting for 75%-85% and 10%-15% of liver cancer cases, respectively. Accumulating evidence implicates a specialized small subpopulation of cancer cells, known as cancer stem cells (CSCs), in driving tumour initiation, progression, therapy resistance and recurrence.

This work offers a comprehensive review of the existing literature focused on liver cancer stem cells, aiming to elucidate their unique molecular attributes and the mechanisms underlying their aggressive behaviour and resistance to conventional treatments.

We highlight the heterogeneity and plasticity of hepatic CSCs, elucidating their ability to self-renew, differentiate, and initiate tumour formation. The cellular origins of primary liver cancers are depicted, comprising hepatocytes, cholangiocytes and CSCs. Moreover, the intricate role of the liver microenvironment in the development and progression of liver cancer is explored, emphasizing the reciprocal influences between the tumoral microenvironment and the CSC niche. We also discuss the regulatory cellular mechanisms and hallmarks of liver CSCs, which can serve as potential therapeutic targets and diagnosis/prognostic tools. Finally, this thesis highlights the challenges and opportunities associated with CSCs, discussing their role in treatment resistance, and the potential strategies for developing personalized therapies aimed at targeting hepatic CSCs.

Overall, this work provides a broad overview of CSCs' role in the development and progression of primary liver cancers, outlining their therapeutic prospects and setting the stage for the development of innovative treatments that could improve patient outcomes and revolutionize the management of liver cancer.

Key-words: Primary liver cancer; hepatocellular carcinoma; cholangiocarcinoma; cancer stem cells.

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Abbreviations

AASLD - American Association for the Study of Liver Diseases
ABC - ATP-binding Cassette
AFP - Alpha-fetoprotein
AJCC - American Joint Committee on Cance
AML - Acute Myeloid Leukemia
APC - Adenomatous Polyposis coli
ARID1A - AT-rich Interaction Domain 1A
ARID2 - AT-rich Interaction Domain 2
BCLC - Barcelona Clinic Liver Cancer
BMP2 - Bone Morphogenetic Protein 2
BMP4 - Bone Morphogenetic Protein 4
CA 19-9 - Carbohydrate Antigen 19-9
CAF - Cancer-associated Fibroblasts
CCA - Cholangiocarcinoma
CCL17 - CC Chemokine Ligand 17
CD133 - Cluster of Differentiation 133
CD24 - Cluster of Differentiation 24
CD44 - Cluster of Differentiation 44
CD90 - Cluster of Differentiation 90
CD47- Cluster of Differentiation 47
CD34- Cluster of Differentiation 34
CD13- Cluster of Differentiation 13
CK1 - Casein Kinase 1
CLCF1 - Cardiotrophin-Like Cytokine Factor 1
CLIP - Cancer of the Liver Italian Program
COMP - Cartilage Oligomeric Matrix Protein
Co-Smad - co-mediator Smad
CSC – Cancer Stem Cell
CT - Computed Tomography
CTC - Circulating Tumour Cells
CXCL6 - chemokine (C-X-C motif) ligand 6
CXCL9 - chemokine (C-X-C motif) ligand 9
DC – Dendritic Cell
dCCA – Distal Cholangiocarcinoma
DLL1 - Delta-like Ligand 1

DLL3 - Delta-like Ligand 3

DLL4 - Delta-like Ligand 4

EASL - European Association for the Study of the Liver

EMT - Epithelial-Mesenchymal Transition

ESMO - European Society for Medical Oncology

FGFR - Fibroblast Growth Factor Receptor

FOLFOX - oxaliplatin/5-fluorouracil

FOXO - Forkhead box O

FSTL1 - Follistatin-like 1

FZD - Frizzled

GSK3 - Glycogen Synthase Kinase 3

HBV - Hepatitis B Virus

HCC - Hepatocellular Carcinoma

HCC-CCA – Mixed Hepatocellular-Cholangiocarcinoma

HCV - Hepatitis V Virus

HGF - Hepatocyte Growth Factor

HNF-4α - Hepatocyte Nuclear Factor-4α

iCCA - Intrahepatic Cholangiocarcinoma

IDH - Isocitrate Dehydrogenase

Ihh - Indian Hedgehog

IL-10 - Interleukin 10

IL-17A - Interleukin 17A

IL-17R - Interleukin 17R

IL-4 - Interleukin 4

IL-6 - Interleukin 6

IL-8 - Interleukin 8

I-Smad - Inhibitory Smad

JAG1 - Jagged Ligand 1

JAG2 - Jagged Ligand 2

JAK - Janus Kinase

K19 - Keratin 19

KEAP1 - Kelch Like ECH Associated Protein 1

LATS1/2 - Activate Large Tumour Suppressor 1 and 2

Lgr5 - Leucine-rich repeat-containing G-protein roupled receptor 5

LRP5/6 - Low-density Lipoprotein Receptor-related Proteins

LT – Liver Transplantation

MAPK - Mitogen-Activated Protein Kinase

MASLD - Metabolic Dysfunction-Associated Steatotic Liver Disease
MCP1 - Monocyte Chemoattractant Protein 1
MDSC - Myeloid-Derived Suppressor Cell
MRI - Magnetic Resonance Imaging
MST1/2 - Mammalian Ste20-like Kinases 1 and 2
mTOR - Mammalian Target of Rapamycin
NICD - Intracellular Domain of Notch
(NK) – Natural Killer
OSM – Oncostatin M
(OV)-6 - Oval Cell Marker
pCCA - Perihilar
PD-1 - Programmed Cell Death Protein 1
PET - Positron Emission Tomography
PI3K - Phosphoinositide 3-Kinase
PIP3 - Phosphatidylinositol (3,4,5)-Trisphosphate
PORCN - Porcupine
PPI - Protein-Protein Interaction
PS – Performance Status
ROS - Reactive Oxygen Species
R-Smad - Receptor-Regulated Smad
SAV1 - Salvador Family WW domain containing Protein 1
Shh - Sonic Hedgehog
SMO - Smoothened
STAT3 - Signal Transducer and Activator of Transcription 3
TACE - Transarterial chemoembolization
TAM - Tumour-associated Macrophages
TAN - Tumour-associated Neutrophils
TCF/LEF - T-cell Factor/Lymphoid Enhancer Factor
TERT - Telomerase Reverse Transcriptase
TGF-β - Transforming Growth Factor-Beta
TNM - Tumour-Node- Metastasis
TP53 – Tumour Protein 53
Treg - Regulatory T cells
VEGF - Vascular Endothelial Growth Factor
VEGFA - Vascular Endothelial Growth Factor A

1. The Liver: Anatomy, function and cellular composition

The liver is a pivotal organ located in the upper right portion of the abdomen, beneath the diaphragm. It plays a crucial role in numerous physiological processes, such as processing, distribution and metabolism of macronutrients, which provides the required energy for all the vital processes of the organisms.(1,2) Particularly, the liver manages glucose homeostasis through the storage, synthesis, metabolism, and release of glucose in response to the body energy demands.(3) It also contributes to lipid and cholesterol homeostasis, as it not only performs lipid oxidation, converting them into usable energy, but also stores surplus lipids in the form of triglycerides, which are then packaged with very low-density lipoprotein particles and secreted into the bloodstream for storage in various tissues, including adipose tissue.(4–6) Additionally, the liver produces bile, a substance that aids in the digestion and absorption of fats.(7) Finally, the liver plays a pivotal role in the metabolism of protein and amino acids.(8) It is responsible for producing most of the proteins secreted into the bloodstream, hence maintaining blood volume regulation, including those involved in blood clotting, immune system function, and nutrient transport. The liver is also involved in the conversion of amino acids into energy and the elimination of nitrogenous waste generated from protein breakdown through urea metabolism.(8,9)

Detoxification is another key function of the liver. The liver receives blood from the digestive tract through the portal vein, carrying nutrients, medications, and toxins absorbed from the intestines. The blood is filtered by the liver before it enters the systemic circulation, removing toxins and breaking down xenobiotic compounds, ensuring their elimination.(10)

The liver is constituted of several cell types of different embryological origin, namely hepatocytes, biliary epithelial cells or cholangiocytes, stellate cells, Kupffer cells, and liver sinusoidal endothelial cells, each with unique functions.(1,11)

These cells are neatly organized within the liver's functional structural unit, the lobule. The lobule comprises hepatocytes arranged in chords and organized around the central vein in a typically hexagonal pattern. The vertices of this hexagon contain portal triads, which encompass clustered branches of the hepatic artery, portal vein, and bile ducts. (1,11)

Hepatocytes, the predominant cell type in the liver, execute most of the liver's functions.(12) The second most prevalent cell population, the cholangiocytes, serve the typical epithelial role of lining the lumen of the bile ducts.(13)

Hepatic stellate cells, also known as Ito cells, can reside in the liver in either a quiescent or activated state. While they store vitamin A within lipid droplets in their quiescent state, hepatic stellate cells transform into myofibroblast-like cells upon liver damage. Activated stellate cells play a critical role in liver scaring by regulating collagen deposition and organization in the damaged liver, which can ultimately result in liver cirrhosis.(14)

Kupffer cells, a specialized type of macrophage residing in the liver, play a critical role in liver immune function and inflammation regulation.(15) Lastly, the liver sinusoidal endothelial form fenestrated sieve plates at the sinusoidal lumen, create pores essential for the exchange of proteins and particles between the liver and the plasma, while preserving certain barrier functions.(16)

2. Primary liver cancer

In 2020, primary liver cancer ranked as the sixth most frequently diagnosed cancer and the third leading cause of cancer-related deaths worldwide. Incidence and mortality rates are very close with ~ 906,000 new cases and ~ 830,000 fatalities reported per year. These consistently prove to be 2 to 3 times higher in men compared to women across most regions of the globe, ranking fifth in global incidence and second in mortality among men.(17)

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, accounting for 75% to 85% of liver cancer cases(17), and it typically develops from hepatocytes, the main functional cells of the liver.(18) In turn, cholangiocarcinoma (CCA) is the second most common type of hepatobiliary cancer, accounting for 10% to 15% of the cases. It originates from the bile ducts and is categorized into three main types: intrahepatic (iCCA), which originates from small bile ducts located inside the liver tissue; perihilar (pCCA), which occurs at the bile duct junction where the bile ducts exit the liver; and distal (dCCA), which affects the lower part of the bile ducts near the small intestine. Mixed hepatocellular-cholangiocarcinoma (HCC-iCCA) is a rare type of primary liver cancer, exhibiting features of both HCC and iCCA. Secondary liver cancers occur when

the primary tumour from another part of the body metastasizes to the liver through the blood or other routes.(18)

Several modifiable and non-modifiable risk factors have been identified for HCC and CCA. These include infectious, lifestyle, metabolic, and heritable factors, such as chronic infection with hepatitis B or C virus (HBV or HCV), chronic alcohol consumption, obesity, type 2 diabetes and smoking.(19) Despite shared risk factors, specific associations have been identified for each primary liver cancer type. While iCCA development has been linked mainly with primary sclerosing cholangitis, fibropolycystic liver disease, parasitic infection and intrahepatic biliary stones, HCC has been associated with exposure to aflatoxin, metabolic dysfunction-associated steatotic liver disease (MASLD), primary biliary cholangitis-driven cirrhosis, haemochromatosis and α1-antitrypsin deficiency.(20,21)

The exact molecular mechanisms through which these etiological factors lead to primary liver cancer remain not fully understood. However, it is well accepted that these risk factors induce chronic liver injury and inflammation, which, when sustained, subsequently contribute to the development of liver cancer.(22)

2.1. Pathogenesis

Although the specific molecular mechanisms underlying the aetiology of HCC and iCCA may differ, the chronic inflammatory state and subsequent fibrosis play a critical role in initiating and advancing the progression of both primary liver cancers.(23) Since the liver has an exceptional regenerative capacity, chronic inflammation-induced damage in hepatocytes and cholangiocytes leads to a marked increase in cell proliferation and regeneration. This continuous cycle of cellular damage and regeneration escalates the risk of genetic mutations, thereby contributing to liver carcinogenesis.(23)

Some of the most common mutations associated with HCC development as a result of chronic inflammation include those in the promoter region of telomerase reverse transcriptase (*TERT*), tumour protein p53 (*TP53*), and β-catenin (*CTNNB1*). Other frequent genetic mutations reported are AT-rich interaction domain 1A (*ARID1A*), AT-rich interaction domain 2 (*ARID2*), axin 1 (*AXIN1*), kelch like ECH associated protein 1 (*KEAP1*), and vascular endothelial growth factor A (*VEGFA*).(24–26)

In turn, two main molecular classifications of iCCA have been proposed based on transcriptomic profiling, namely the inflammation and proliferation classes, accounting for 38% and 62% of iCCA cases, respectively. Some of the most relevant genetic alterations in the inflammatory class are in the expression of pro-inflammatory cytokines interleukin (IL)-6, IL-4, IL-10, IL-17R and IL-17A, as well as signal transducer and activator of transcription 3 (STAT3).(27) In the proliferation class, genetic alterations involve mainly oncogenic signalling, such as *KRAS, BRAF MAPK,* and *MET*, deletion of *TP53* and activation of β-catenin (*CTNNB1* and *AXIN1*).(27,28)

The chronic inflammation process is also associated with alterations of the liver immune defence mechanisms, facilitating the evasion of cancer cells from immune surveillance, such as a diminished pro-inflammatory/anti-inflammatory tumour-associated macrophage ratio, the infiltration of myeloid-derived suppressor cells (MDSCs), the generation of pro-tumorigenic cytokines, the dysregulation of the senescence-associated secretome, and the leakage of metabolites and pathogens from the gut to the liver. These immune-related changes collectively foster an environment that supports tumour growth and progression in the liver.(23)

Hepatic chronic inflammation has also been implicated in the selection, maintenance and regulation of liver cancer stem cells (CSCs), a small subset of cells within the tumour that possess self-renewal capacity, tumour-initiating potential, and resistance to conventional cancer therapies. Although the precise mechanisms remain obscure, augmented levels of inflammatory cytokines and reactive oxygen species (ROS), hypoxia, and the activation of developmental signalling pathways, play crucial roles in impairing normal stem cell function, generation and regulation of liver CSCs.(29–31) A more detailed discussion on the role of liver CSCs in primary liver cancer will be provided in subsequent topics.

Still, liver chronic inflammation is not a *sine qua non* requisite for primary liver cancer pathogenesis. Liver cancer can also develop as a result of direct changes in hepatocellular DNA, as in the cases of exposure to aflatoxin or HBV infection. Aflatoxin is known to induce genomic instability, chromosomal aberrations, and DNA strand breaks, leading to the activation of oncogenes and inactivation of tumour-suppressor genes, such as the *TP53*. Concurrently, HBV infection can also directly affect the genome via viral integration.(32)

Overall, the understanding of the intricate interplay between chronic inflammation and direct or indirect hepatic genetic mutations can shed light on the diverse pathways

through which primary liver cancers develop, paving the way for novel effective targeted therapeutic interventions.

2.2. Diagnosis of liver cancer

Early and accurate diagnosis, correct disease staging, and tailored treatment approaches are crucial for improving patient outcomes and overall survival rates in HCC and iCCA.(33,34)

Diagnosis is based on a multidisciplinary approach, relying on patient's medical history, clinical examination, imaging studies and laboratory analysis with or without biopsy confirmation, contingent upon the specific circumstances of each case.(33,34)

The exhaustive review of the patient's medical history aims to identify risk factors, such as chronic HBV or HCV infection, MASLD, cirrhosis, and alcohol abuse, while physical examination may reveal hepatomegaly, jaundice, or other signs suggestive of liver disease.(33,34)

Imaging techniques, such as ultrasonography, computed tomography (CT) scan, magnetic resonance imaging (MRI), and positron emission tomography (PET) scan, are instrumental in diagnosing HCC and iCCA. Ultrasound serves as the first-line imaging technique for surveillance and detection of liver lesions. CT scans offer detailed cross-sectional images, while MRI provides excellent contrast for soft tissue and vascular evaluation. PET scans, combined with CT or MRI, may detect distant metastasis and assess tumour metabolism.(33,34)

Tumour markers, such as alpha-fetoprotein (AFP) for HCC and carbohydrate antigen 19-9 (CA 19-9) for iCCA, complement imaging findings and support disease diagnosis and progression. However, these tumour markers lack optimal sensitivity and specificity, being typically used alongside imaging techniques.(33,34)

When imaging results and tumour marker levels yield inconclusive outcomes, a percutaneous liver biopsy may be conducted to obtain tissue samples for histological analysis and definitive diagnosis.(33,34)

2.3. Staging of liver cancer

Staging of HCC and iCCA is essential in determining prognosis and guiding treatment decisions.

Multiple staging systems have been proposed for HCC, of which the Tumour-Node-Metastasis (TNM), Okuda, Barcelona Clinic Liver Cancer (BCLC) systems, and Cancer of the Liver Italian Program (CLIP) score are the most commonly used.(35) Each of these systems has its advantages and drawbacks, and, although none of these staging systems universally accommodate all tumour variables, some scoring systems are optimal for certain populations. Still, the most recent guidelines by the American Association for the Study of Liver Diseases (AASLD)(36), the European Society for Medical Oncology (ESMO) and the European Association for the Study of the Liver (EASL)(37) recommend the BCLC staging system for prognosis prediction and treatment stratification.

The BCLC staging system comprises 5 prognostic stages, 0 and A through D, based on the following variables: tumour stage, functional status of the liver, patient's physical status and cancer-related symptoms. It also links each stage to a first-line treatment recommendation. Briefly, patients with early HCC who may benefit from ablative treatment are classified under stages 0 or A, while those with intermediate HCC fall under stage B. Patients with advanced stage HCC are included in stage C and may benefit from intra-arterial or systemic treatments. Finally, patients with end-stage disease are categorized under stage D and are recommended to receive symptomatic treatment.(36,37) The different first-line treatments assigned to the different stages will be discussed below.

Regarding iCCA, several staging systems have been also proposed to classify the cancer based on tumour characteristics and extent of disease. Among these, the American Joint Committee on Cancer (AJCC) TNM staging system is the most widely used and accepted. The AJCC TNM staging system categorizes iCCA patients into different stages based on the tumour size (T), extent of regional lymph node involvement (N), and presence of distant metastasis (M), and each category is followed by a number (0 to 4). This system provides a comprehensive and standardized approach for assessing tumour progression and prognosis, thereby assisting clinicians in treatment planning and predicting patient outcomes. In brief, stage 0 [Tis (*in situ*) N0 M0] includes non-invasive carcinoma, limited to the innermost layer of the bile duct lining. Stage I (T1 N0 M0) refers to a localized tumour confined to the bile duct without invasion of blood

vessels or distant sites. Stage II (T2 N0 M0) refers to tumours that have extended into nearby blood vessels but have not reached major structures or distant sites. Stage III (T3 N0 M0 or T1-3 N1 M0) indicates further expansion into surrounding structures and regional lymph nodes, but without distant metastasis. Finally, stage IV (T4 N0-1 M0 or any T N2 M0 or any T any N M1), suggests extensive invasion, lymph node involvement, and possible distant metastasis.(38)

2.4. Treatment of liver cancer

The treatment approach for HCC depends on the stage of the disease. For early-stage HCC (BCLC 0/A), surgical resection, potential curative options include surgical resection, liver transplantation, and local ablation therapies such as radiofrequency or microwave ablation. Transarterial chemoembolization (TACE) and radioembolization are employed for intermediate-stage HCC (BCLC B) to restrict tumour blood supply and deliver localized therapy. In advanced-stage HCC (BCLC C), systemic therapies such as targeted therapies aim to slow tumour growth and enhance the immune response. The combination of an immune checkpoint inhibitor (atezolizumab), and an antiangiogenic agent (bevacizumab) is currently the first-line treatment for advanced HCC.(34) (Figure 1)

Briefly, programmed cell death protein 1 (PD-1) is an immunosuppressive protein commonly expressed on the surface of T cells. Its ligand, PD-L1, is frequently overexpressed on the surface of HCC tumour cells. The binding of PD-L1 from hepatic cancer cells to the PD-1 receptor results T cell inactivation and, therefore facilitate evasion of tumour cells from the immune system.(39) Atezolizumab is a monoclonal antibody against PD-L1, blocking its interaction with PD-1 and consequently reversing T cell suppression.(40) On the other hand, vascular endothelial growth factor (VEGF) is know to play a significant role in hepatic tumour growth and progression by facilitating tumour angiogenesis. It also mediates immunosuppression by promoting the recruitment and proliferation of immunosuppressive cells such as regulatory T cells (Treg), MDSCs, and tumour-associated macrophages (TAMs), together with the suppression of antigen-presenting dendritic cells (DCs).(41) Bevacizumab is a monoclonal antibody that targets the VEGF protein, inhibiting its cancer promoting effects. Therefore, the combination of these two immunotherapies provides a potent dual approach against tumour growth by boosting the immune response against cancer cells while also inhibiting tumour blood supply.(40)

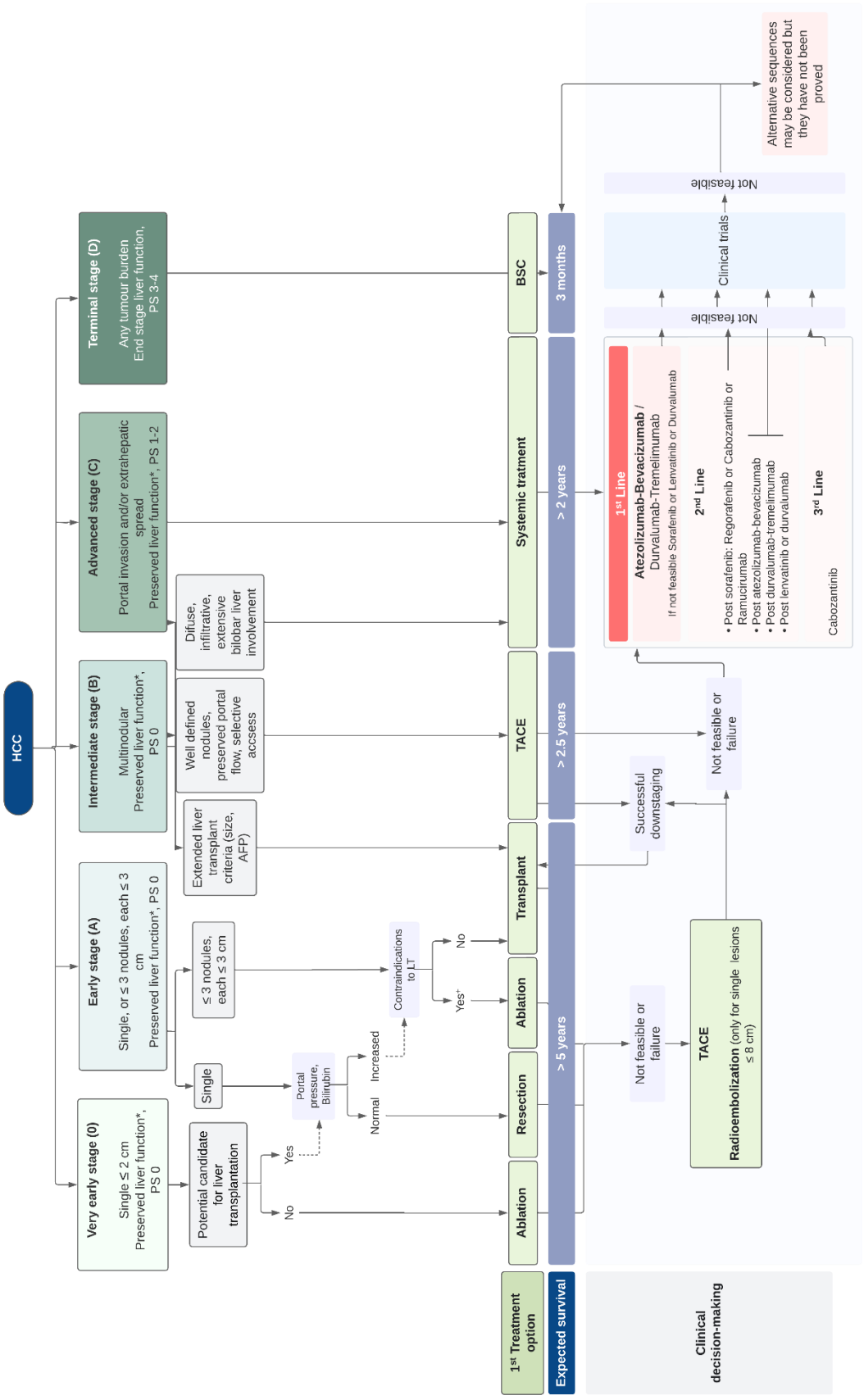

Figure 1 - BCLC staging and treatment strategy in 2022. Adapted from (34)

LT, liver transplantation; PS, performance status.

*Except those with tumour burden acceptable for transplant.

+Research may be considered for single peripheral HCC with adequate remnant liver volume.

For iCCA, surgical resection can manage early-stage disease if tolerated by the patient. In advanced-stage iCCA, gemcitabine/cisplatin/durvalumab is the first-line systemic therapy.(33) (Figure *2*)

The first-line standard of care for advanced iCCA had remained unchanged for the past decade, consisting of gemcitabine and cisplatin chemotherapy.(42) Gemcitabine is a nucleoside analog that exerts its antitumoral properties by causing chain termination during DNA synthesis.(43) The disruption in DNA replication leads to cell cycle arrest and triggers apoptosis in cancer cells. Cisplatin is a platinum-based drug that forms cross-links with the DNA strands in cancer cells. These cross-links also hinder DNA replication and transcription, leading to DNA damage and cancer cell apoptosis.(44)

However, the newly released 2023 EASL-ILCA *Clinical Practice Guidelines on the management of intrahepatic cholangiocarcinoma* recommend that patients with unresectable, advanced iCCA should be treated with gemcitabine/cisplatin, as first-line chemotherapy, together with the monoclonal antibody against PD-L1, durvalumab.(33)

Moreover, other chemotherapy regimens like oxaliplatin/5-fluorouracil (FOLFOX), and targeted therapies, including the fibroblast growth factor receptor (FGFR) inhibitors pemigatinib and infigratinib, are regarded as second-line systemic therapy.(33) (Figure ***2***)

FGFRs play a role in cell proliferation, differentiation and angiogenesis, being *FGFR2* fusion one of the genetic mutations implicated in the development and progression of CCA. This mutation is involved in regulating various cellular mechanisms, including tumour cell migration, anchorage, and aggregation, contributing to tumour mass formation. Pemigatinib and infigratinib target these specific FGFR alterations, inhibiting cancer cell growth and survival, leading to tumour shrinkage.(45,46)

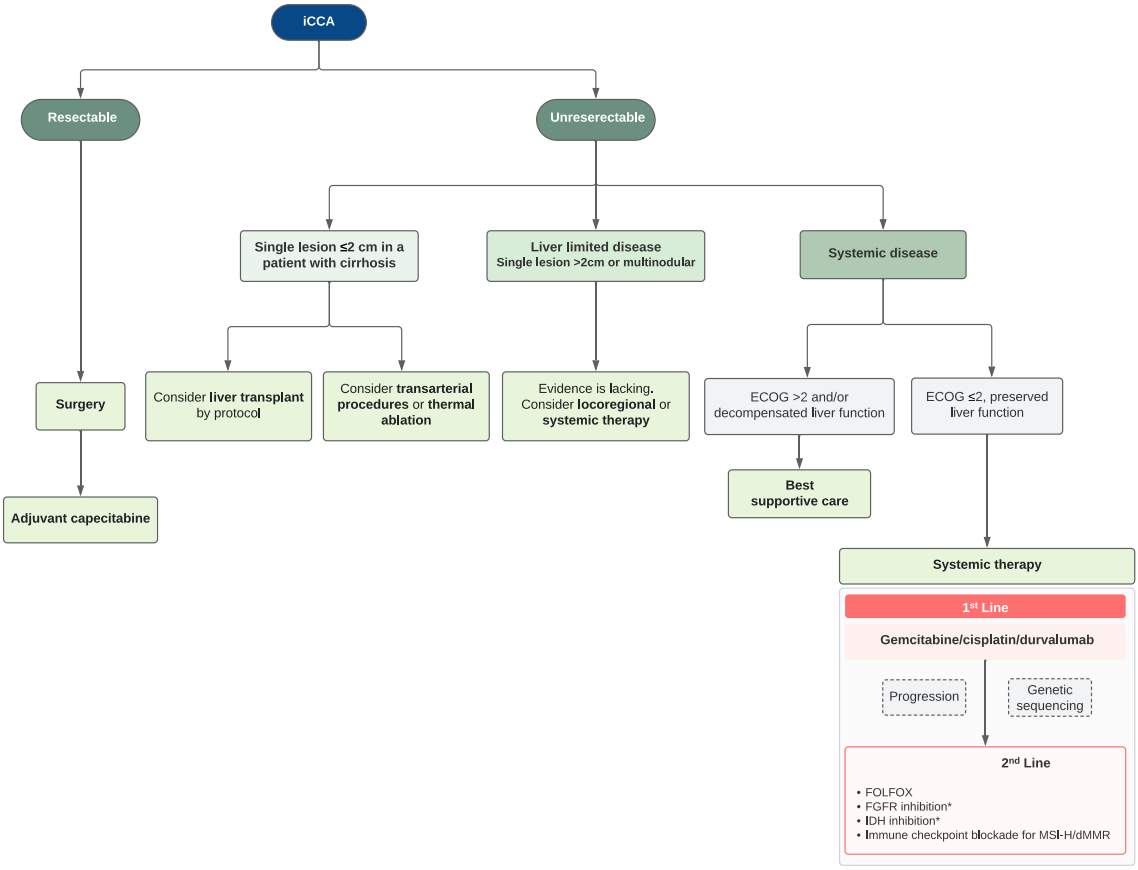

Figure 2 - Diagnosis and management of intrahepatic cholangiocarcinoma. Adapted from (33)

IDH, isocitrate dehydrogenase; PET positron emission tomography.

*For patients harbouring these targetable mutations. FGFR, fibroblast growth factor receptor.

Finally, palliative care is an essential component of the treatment plan for patients with advanced liver cancer, with a focus on symptom management, supportive care, and enhancing quality of life.(33)

The diagnosis, staging, and treatment of HCC and iCCA require a multidisciplinary approach and personalized patient care. Early detection, precise staging, and timely interventions are crucial in improving patient outcomes and propelling advancements in liver cancer management. Ongoing research and advancements in diagnostic tools and treatment modalities offer hope for better outcomes and a higher quality of life for patients diagnosed with HCC and CCA.(33)

3. Cancer stem cells

Liver cancer is characterized by the uncontrolled proliferation of cells with morphological and functional heterogeneity. Tumoral heterogeneity can be divided into inter-tumour and intra-tumour heterogeneity.(47)

Inter-tumour heterogeneity refers to the differences between tumours in different patients diagnosed with the same type of histological tumour by showing different patterns of epigenetic regulation, mutations and gene expression. This is a critical factor in personalized therapy.(47,48)

On the other hand, intra-tumoral heterogeneity refers to the complexity within a single tumour, characterized by the presence of distinct cell clones, each bearing different genetic mutations. Intra-tumoral heterogeneity can highly impact the effectiveness of therapies at an individual level.(47,48)

Two main models have been proposed to explain tumour growth and heterogeneity - the stochastic model or clonal evolution model, and the hierarchy or CSC model.(49)

The clonal evolution model postulates that initially all tumour cells are biologically equivalent but, over time, they could accumulate different genetic and epigenetic alterations, leading to the formation of distinct cell clones within the tumour. These clones carry diverse traits influencing tumour aggressiveness, invasiveness, and treatment resistance, among other cancer features. This model suggests that all cancer cells display equal tumour-initiating capacity, implying that tumour-initiating cells cannot be isolated based on the intrinsic characteristics of a subpopulation of tumour cells.(50)

Conversely, the hierarchy model argues that the tumour-initiating activity is restricted to a subpopulation of cells within the tumour, the CSCs. These cells possess stem/progenitor cell-like abilities such as self-renewal, differentiation and proliferation, contributing to tumorigenicity and chemoresistance. As a result of CSCs proliferation and differentiation, the tumour comprises a hierarchy of cells with highly tumorigenic CSCs at the apex, giving rise to intermediate progenitors, which then yield differentiated progeny at the bottom of the hierarchy. In contrast with the clonal evolution model, the hierarchy model infers that CSCs can be identified and isolated based on their intrinsic traits.(49)

Despite their contrasting views and differing emphasis on CSCs and their microenvironments, the clonal evolution and hierarchy models are not mutually

exclusive. Indeed, a hybrid model known as the cellular plasticity model postulates that cancer cells can transition between stem and differentiated states.(51) This interconversion could be stimulated by intrinsic tumour cell processes or microenvironmental cues, leading either to the reacquisition of stem cell properties by differentiated tumour cells or the differentiation of CSCs into non-stem cancer cells.(52)

CSCs were first discovered 29 years ago in a pioneering study in human acute myeloid leukaemia (AML) in which an AML-initiating cell population was isolated.(53) However, it wasn't until 9 years later, in 2003, that these cells were successfully isolated from a solid tumour in a groundbreaking study related to breast cancer.(54) Through the years, CSCs have been identified in various hematologic and solid tumours, including those of the colon(55), pancreas(56), brain(57) and liver(58). Notably, the presence of CSCs has been consistently associated with a poor prognosis in cancer patients, underscoring their clinical significance.(58)

3.1. Cellular origin of liver cancer

Initially, it was believed that primary liver cancers only arose from the malignant transformation of mature epithelial liver cells. Given the distinct histopathological and morphological features of HCC and CCA, it was assumed that hepatocytes exclusively drove the development of HCC, while cholangiocytes were the sole responsible for the emergence of CCA. However, HCC and CCA also share some similarities, not only with respect to several risk factors, but also concerning molecular features.(19)

Molecular alterations, such as mutations in potentially tumour-supressing genes are frequently found in both HCC and iCCA and alter several cellular processes. Mutations in genes involved in chromatin remodelling (*ARID1a*), protein deubiquitination (*BAP1*), cell cycle regulation (*CDKN2A* and *KRAS*) and phosphoinositide 3-kinase (PI3K) signalling (*PIK3CA* and *PTEN*) have been found in both liver cancer types.(59–61) In accordance, the rare, mixed HCC-iCCA cancer presents cells with a phenotype that is intermediate between hepatocytes and cholangiocytes, bearing the same genetic mutations.(19)

Overall, these similarities in the molecular and phenotypical features of HCC, iCCA and mixed HCC-iCCA cancer cells have led to the emergence of a new hypothesis that considers the existence of a common cell of origin of primary liver cancers. Indeed,

mature hepatocytes and cholangiocytes are each derived from the same progenitor cells during liver development, further reinforcing the hypothesis of a unified cellular origin for the primary liver cancers, the liver cancer stem/progenitor cells.(19)

The existence of liver stem cells had been a matter of debate for many years until their definitive isolation and identification in 2006.(62) In a healthy liver, hepatic stem cells are located in the canals of Hering, nestled within a specialized niche featuring a unique microenvironment.(63) This microenvironment delicately balances the slow, self-renewing, quiescent state with the active, proliferative state, during which these cells differentiate into hepatocytes, cholangiocytes or other specialized cells.(49)

As previously discussed, chronic inflammation driving a continuous cycle of cellular destruction and regeneration could lead to the accumulation of genetic mutations in normal liver stem cells, potentially transforming them into CSCs.(49) Indeed, liver normal and CSCs share some common traits; both have the ability of self-renewal and pluripotency and are identified and classified using the same cell surface markers, such as epithelial cell adhesion molecule (EpCAM)(64), leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)(65), cluster of differentiation 133 (CD133)(66), and CD24(67).

Nevertheless, liver CSCs do not exclusively originate from alterations to normal liver stem cells. Differentiated liver cells, such as mature hepatocytes and cholangiocytes, have the capacity to acquire stem cell-like properties during liver regeneration.(68) This process implies that, during the cycle of cellular damage and regeneration, differentiated liver cells could undergo a dedifferentiation program, leading to their transformation into CSCs. Holczbauer *et al.* demonstrated that all transduced hepatic lineage cells, including hepatic progenitor cells, hepatoblasts, and adult hepatocytes could be reprogrammed into CSCs through genetic or epigenetic alterations.(52)

Overall, these findings suggest that primary liver cancers can emerge from distinct cellular origins. HCC and iCCA can emerge directly from the malignant transformation of mature epithelial liver cells, hepatocytes and cholangiocytes, respectively.(69–71) However, evidence also supports that primary liver cancers, including mixed HCC-iCCA, can also originate from liver CSCs.(72,73) These tumours exhibit stem-like features and are associated with higher tumour aggressiveness and poorer prognosis.(74,75) In turn, CSCs can be originated from malignant mutations in normal liver stem cells, or they can result from mature epithelial liver cells and differentiated liver cancer cells, thought the

processes of dedifferentiation.(68) (Figure 3 - ***Multiple cells of origin of primary liver cancers.*** *Adapted from* (76,77)

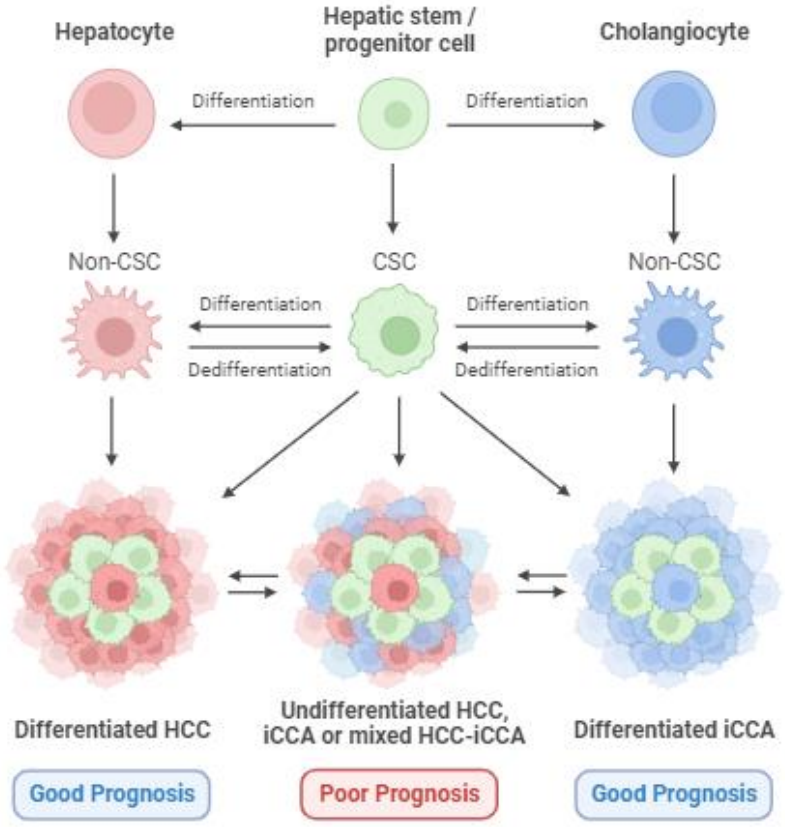

Figure 3 - Multiple cells of origin of primary liver cancers. Adapted from (76,77)

3.2. Liver microenvironment and the CSC niche

The liver microenvironment plays a crucial role in the development and progression of liver cancer. Within the liver microenvironment exists a specific niche known as the hepatic CSC niche. Despite the limited understanding of the hepatic CSC niche, it is believed that the liver microenvironment supports the growth and maintenance of CSC, by promoting their stemness and sustaining the liver CSC subpopulation.(78)

Stromal cells, such as cancer-associated fibroblasts (CAFs), adipocytes, endothelial cells, and diverse types of immune cells, encompassing TAMs, tumour-associated neutrophils (TANs), T cells, B cells, natural killer (NK) cells, DCs, and MDSCs are some of the essential cellular components of the CSC niche.(78)

Particularly, CAFs secrete a range of growth factors and cytokines that modulate molecular pathways in HCC, enhance the expression of stemness markers and oncofoetal proteins, confer tumour-initiating properties to HCC cells and promote self-renewal, tumorigenicity, chemoresistance, epithelial-mesenchymal transition (EMT) and metastasis.(79) EMT is a biological process in which normal, polarized epithelial cells undergo phenotypical changes to assume a mesenchymal cell state, characterized by enhanced migratory and invasive capacities, high resistance to apoptosis and increased production of extracellular matrix components.(80) Some of the cellular modulators secreted by CAFs include hepatocyte growth factor (HGF)(81), interleukins (IL-6 and IL-8)(82), cartilage oligomeric matrix protein (COMP)(83), cardiotrophin-like cytokine factor 1 (CLCF1)(84), transforming growth factor-beta 1 (TGF-β1)(85), and a newly identified protein, follistatin-like 1 (FSTL1)(86). Interestingly, HCC cells themselves secrete chemokines such as chemokine (C-X-C motif) ligand 6 (CXCL6) and TGF-β, which in turn stimulate the expression and secretion of CLCF1 by CAFs, creating a positive feedback loop.(84) Given the diverse pro-stemness effects of CAFs on liver CSCs, these cells present a promising target for the development of anti-CSC therapies.

Adipocytes and endothelial cells are also key components of the tumour stroma.(78) The tumour microenvironment includes a vascular niche that generates angiocrine factors to support CSCs.(85) Studies have reported that chemokine (C-X-C motif) ligand 9 (CXCL9) secreted by vascular endothelial cells enhances the migration and invasion of CSCs.(87) Furthermore, lymphatic endothelial cells can also secrete IL-17A which promotes self-renewal and tumorigenesis properties of CSCs.(88) Additionally, adipocytes differentiated from mesenchymal stem cells have been found to secrete IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP1), which boost stemness properties in HCC cells.(89)

TAMs are the most extensively studied immune cell type present in the tumour microenvironment, which have been shown to produce IL-6, a well-established driver of CSCs stemness, and a regulator of the TGF-β and CC chemokine ligand 17 (CCL17)-induced EMT process.(90–92) TANs, another important immune component of the tumour microenvironment, have been implicated in liver cancer initiation, development, and progression, through the secretion of TGF-β2 and bone morphogenetic protein 2 (BMP2).(93) Regulatory T cells can also induce stemness properties in HCC cells by releasing TGF-β1.(94) Noteworthy, hepatic CSCs have shown the ability to evade the immunological surveillance of neighbouring immune cells, such as B cells and NK cells.(95,96) Furthermore, studies have shown that liver CSCs can

modify DC phenotype to decrease T cell activation and, therefore, to induce immunotolerance.(97,98)

Altogether, the tumour microenvironment and CSCs engage in a reciprocal interaction that plays a crucial role in tumour progression and immune evasion. The tumour microenvironment provides a nurturing niche for CSCs, promoting their self-renewal, maintenance, and resistance to therapies. Conversely, CSCs actively contribute to the maintenance of the tumour microenvironment by modulating immune responses and evading immunological surveillance.

3.3. Intrinsic regulatory mechanisms of liver CSCs

Several pathways identified to be pivotal in the normal function of hepatic stem cells and in maintenance of the self-renewal potential and pluripotency of embryonic stem cells have been implicated in the regulation of CSCs. (Figure 4) Prolonged and aberrant activation of these pathways contributes to the unique characteristics of CSCs in liver cancer.

3.3.1. Wnt/β-catenin pathway

The Wnt/β-catenin pathway is a complex signalling cascade that controls various cellular processes, both during embryonic development and adult tissue homeostasis. At the core of the Wnt/β-catenin pathway are the Wnt ligands, which bind to cell surface receptors known as Frizzled (FZD) receptors. Upon ligand binding, FZD receptors associate with co-receptors called low-density lipoprotein receptor-related proteins (LRP5/6). This interaction triggers a sequence of intracellular events that lead to the activation of the pathway. β-catenin serves as the key effector protein of this signalling pathway. When the Wnt/β-catenin signalling pathway is inactive, β-catenin is continuously targeted for degradation by a "destruction complex" composed of axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). This degradation process ensures that β-catenin levels remain low in the cytoplasm. However, when Wnt ligands bind to their respective receptors, the destruction complex is inhibited, allowing β-catenin to accumulate and translocate into the nucleus. Once in the nucleus, β-catenin interacts with transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family, leading to the activation of Wnt

target genes. These target genes, in turn, regulate various cellular processes and contribute to the diverse functions of the Wnt/β-catenin pathway.(99,100)

The disruption of the Wnt/β-catenin signalling pathway following genetic and epigenetic alterations has been reported in various tumours, including in primary liver cancers.(101) This signalling pathway appears to play a role in maintaining stemness features in both embryonic and CSCs.(102)

β-catenin activation has been reported in approximately 20-35% of HCC cases. The most common mechanisms potentially involved in the activation of β-catenin in HCC are *CTNNB1* and *AXIN* mutations.(103) Moreover, β-catenin was discovered to be expressed in about 58% of iCCA and mutated in 8.3% of the cases.(104) Hence, the perturbation of the Wnt/β-catenin pathway appears to be a significant factor in the development and progression of HCC and CCA.

3.3.2. Notch signalling pathway

The Notch pathway is a highly conserved cell signalling pathway that plays a fundamental role in cell fate determination, tissue development, and homeostasis. At the core of this pathway are the Notch receptors, single-pass transmembrane proteins expressed on the cell surface, (Notch1 to Notch4). These receptors have multiple domains, including an extracellular domain responsible for ligand binding, a transmembrane domain, and an intracellular domain. The pathway is activated when Notch receptors interact with membrane-bound ligands expressed by neighbouring cells: delta-like ligands (DLL1, DLL3, DLL4) and jagged ligands (JAG1 and JAG2). This triggers a cascade of events that result in the release of the intracellular domain of Notch (NICD), which translocates to the cell nucleus, where it induces the transcription of specific target genes, collectively known as Notch target genes.(105,106)

In the context of primary liver cancer, the Notch signalling pathway plays a crucial role in cancer cell differentiation, proliferation, apoptosis, as well as the maintenance of hepatic stem cells.(107) It also participates in bile duct morphogenesis, whereby malfunction of this pathway may result in abnormal differentiation of biliary precursors, ultimately promoting the development of iCCA.(108) Indeed, the expression of Notch receptors 1 and 3 has been associated with a poor prognosis and cancer progression in iCCA.(109)

In parallel, an overexpression of Notch 1, 3 and 4 has been linked to carcinogenesis in HCC.(110) Particularly, nuclear expression of Notch receptors 1 and 3 has been observed in up to 30% of HCCs, which correlated with the presence of stem cell features, further supporting the role of Notch in the expansion of the CSC niche.(111)

Given that Notch signalling can play a role in both iCCA and HCC, it may be hypothesized that this pathway might be deregulated in the bipotential hepatic progenitor cells.(111)

3.3.3. Hedgehog signalling pathway

The Hedgehog signalling pathway plays a critical role in embryonic development, cell differentiation, regeneration, and stem cell biology.(112)

At the core of the pathway are the Hedgehog ligands, which include sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). These ligands are secreted from signalling cells and bind to the patched (PTCH1) receptors on the cell surface of recipient cells. In the absence of Hedgehog ligand binding, PTCH1 inhibits the pathway by suppressing the activity of another transmembrane protein called Smoothened (SMO). Upon Hedgehog ligand binding to PTCH1, SMO is activated and initiates a signalling cascade that results in the activation of the transcription factors Gli proteins (Gli1 to Gli3), which regulate the transcription of target genes.(113)

Abnormal activation of this pathway is associated with carcinogenesis and stem cell proliferation of both HCC and iCCA.(114,115)Noteworthy, the most predominant ligands of the Hedgehog signalling pathway, Shh, Gli1, PTCH1 and SMO, have been found increased in liver tumour cells compared to normal liver cells. Additionally, the majority of tumours exhibiting an over-activation of the Hedgehog pathway were larger than 3 cm, highlighting the pathway's role in tumour growth maintenance and cancer progression. Further investigations also found an overexpression of the Hedgehog proteins in both altered precancerous tissues, which reinforces the significant involvement of the Hedgehog pathway in the early stages of carcinogenesis.(116)

3.3.4. Hippo signalling pathway

The Hippo signalling pathway plays a critical role not only in the regulation of cell proliferation, differentiation, and survival, but also in the development and maintenance of organ homeostasis.(117)

The pivotal event that triggers the Hippo pathway is a kinase cascade, in which the mammalian ste20-like kinases 1 and 2 (MST1/2) and Salvador family WW domain containing protein 1 (SAV1) form a complex to phosphorylate and activate large tumour suppressor 1 and 2 (LATS1/2). Once activated, LATS1/2 phosphorylates and activates the yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ). When phosphorylated by LATS1/2, YAP/TAZ are sequestered in the cytoplasm, preventing their nuclear translocation and transcriptional activity. However, in their unphosphorylated state, YAP and TAZ translocate into the nucleus, where they interact with transcription factors to influence gene expression.(117)

The aberrant activation of Hippo pathway effector proteins is associated with various processes during tumour initiation, such as aberrant cell proliferation.(118,119) For example, one study found that the loss of MST1/2 function is sufficient to trigger hepatocyte proliferation, leading to substantial liver overgrowth, increased resistance to pro-apoptotic stimuli, and the development of HCC.(120)

This signalling pathway is also implicated in the regulation of stem cells, including hepatic progenitor cells. One study discovered that elevated levels of YAP influence hepatic progenitor cells fate, while the overexpression of YAP in mature hepatocytes results in their de-differentiation.(121) Additionally, another study established a correlation between the expression of stemness markers in HCC and HCC-iCCA cancer cells and the overexpression of YAP1, which further suggests the involvement the Hippo pathway in cancer cell stemness.(122)

3.3.5. PI3K/AKT signalling

The PI3K/AKT pathway mediates various cellular processes, including metabolism, proliferation, cell survival, growth, and angiogenesis, playing a critical role in various human cancers, including HCC and iCCA.(123,124) This pathway is initiated by the PI3K, an enzyme that phosphorylates phosphoinositides on the cell membrane. Upon

activation, PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3), a crucial secondary messenger that serves as a docking site for the serine/threonine kinase AKT. Following its recruitment to the plasma membrane, AKT is phosphorylated and activated, thereby modulating a range of downstream effector targets, such as mammalian target of rapamycin (mTOR), GSK-3, and Forkhead box O (FOXO) transcription factors.(125,126)

Studies have shown that the PI3K levels are particularly elevated in HCC tumour tissue and linked to enhanced HCC proliferation, while showing a negative correlation with apoptosis. Moreover, high expression of *PIK3CA* has been associated with an unfavourable prognosis in patients with HCC.(127) Furthermore, it has been suggested that the PI3K signalling pathway stimulates stem-like properties in HCC cells, participating in EMT process and, subsequently, favouring the invasion of the surrounding tissues and even distant sites through blood and lymph by cancer cells.(80)

3.3.6. MAPK/ERK signalling pathway

The mitogen-activated protein kinase (MAPK) cascades are essential signalling pathways that regulate a plethora of cellular processes, including proliferation, differentiation, apoptosis, and stress response.(128)

Generally, this pathway is composed of a small G protein (RAS) and three protein kinases (RAF, MEK, ERK). Activation of the pathway is triggered by extracellular signals, such as growth factors, receptor tyrosine kinases, or G protein-coupled receptors. The following chain of events culminates in ERK (MAPK) translocation to the nucleus, where it activates transcription factors.(129) Aberrant activation of ERK has been linked to cellular transformation and tumour development.(128)

Although the components of the MAPK/ERK signalling pathway have shown a low frequency of mutations, an over-activation of this signalling pathway has been observed in patients with HCC. This signalling pathway has been found activated in ~50% of early-stage HCC patients and nearly all patients with advanced-stage HCC. Furthermore, the activation of this pathway is linked to aggressive tumour behaviour and unfavourable prognosis in both HCC and iCCA, co-substantiating its role on liver cancer pathogenesis.(110)

3.3.7. TGF- β signalling

The TGF-β protein family trigger important signalling mechanisms involved in the self-renewal and maintenance of stem cells in their undifferentiated state, as well as in the progression of differentiation along specific lineages.(130) Thus, the dysregulation of the TGF-β family signalling could result in impaired cell differentiation, potentially facilitating the development of cancer.(131)

This signalling pathway is initiated by the binding of a TGF-β ligand to specific transmembrane type I and type II receptors on the cell surface. This triggers the oligomerization of these receptors, forming a complex on the cell surface that leads to a series of intracellular reactions, including the phosphorylation of the Smad proteins. The Smad protein family encompasses eight distinct members, classified into three functional categories: the receptor-regulated Smad (R-Smad), the co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads are directly phosphorylated and activated by the type I receptor kinase, allowing them to form complexes with the Co-Smad, Smad4. These complexes are subsequently translocated into the nucleus, where they act as transcription factors. Interestingly, the I-Smad proteins downregulate the TGF-β signalling pathway by interfering with R-Smad activation, and by modulating the activity of various co-factors and co-regulators.(132)

In the context of primary liver cancer, interestingly, it has been found that TGF-β has a dual role in tumour development, acting as a tumour suppressor during early tumour initiation, while promoting tumour growth, EMT and, eventually, metastasis at later stages.(133–135)

Regarding its tumour suppressor role, the TGF-β signalling pathway promotes hepatic cancer cell cycle arrest, namely G1 and G2 cell cycle arrest, and cellular senescence.(136,137)

On the other hand, it has also been proven that this signalling pathway plays a role in tumour development. One study has shown that the TGF-β1/Snail activation induces EMT in iCCA, leading to heightened cancer aggressiveness.(138) Additionally, TGF-β has been found upregulated in ~40% of HCCs, and its high levels have been positively correlated with advanced clinical stage of HCC.(139)

3.3.8. JAK/STAT signalling

The Janus kinase (JAK)/STAT signalling pathway governs a variety of cellular processes, such as cell proliferation, stem cell maintenance and differentiation, as well as modulation of the immune/inflammatory responses.(140)

This pathway is composed of ligand-receptor complexes, Janus kinases (JAKs) - JAK1, JAK2, JAK3, and TYK2, - as well as signal transducer and activator of transcription (STATs)- STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. The cascade of events initiates with the binding of a ligand, often cytokines, to their corresponding cell surface receptors and subsequent receptors dimerization. Activated JAKs phosphorylate tyrosine residues on the receptors, creating docking sites for STAT protein dimerization. The activated STAT dimers translocate into the nucleus, where they bind to specific DNA sequences and regulate the transcription of target genes.(141,142)

Dysregulation of this signalling pathway, often arising from either loss-of-function or gain-of-function mutations, has been observed in specific cancers and can serve as both initiating and driving factors in tumorigenesis. For instance, it has been found that STAT5 protein is upregulated in HCC patients and may exhibit pro-oncogenic properties.(143) Conversely, other studies reported hepatoprotective traits of STAT5 instead, and therefore, its downregulation is associated with tumour development. One study found that mice with hyperactive growth hormone signalling and a synthetic loss of STAT5 developed HCC.(144) Another study has shown that STAT5 might also function as a tumour suppressor by upregulating the expression of tumour supressing genes, namely *CDKN2B* and *CDKN1A* implicated in cell cycle arrest.(145,146)

STAT3 is another component of the JAK/STAT pathway that is widely recognized as an oncogene that plays a crucial role in promoting the development of primary liver cancers.(140) The pro-inflammatory cytokine IL-6 activates the STAT3 pathway, triggering a downstream inflammatory response and promoting the transcription of target genes essential for cell growth, differentiation, and proliferation. Therefore, the IL-6/STAT3 signalling pathway is associated with liver inflammation and regeneration. Importantly, the expression of IL-6 is often increased in iCCA tissues, as well as in serum and bile from iCCA.(147) In HCC, activation of the STAT3 transcription factor has been found to promote the expression of CSC markers, such as CD24. This pathway is believed to sustain the liver CSC population through interactions with the TGF-β signalling pathway.(148)

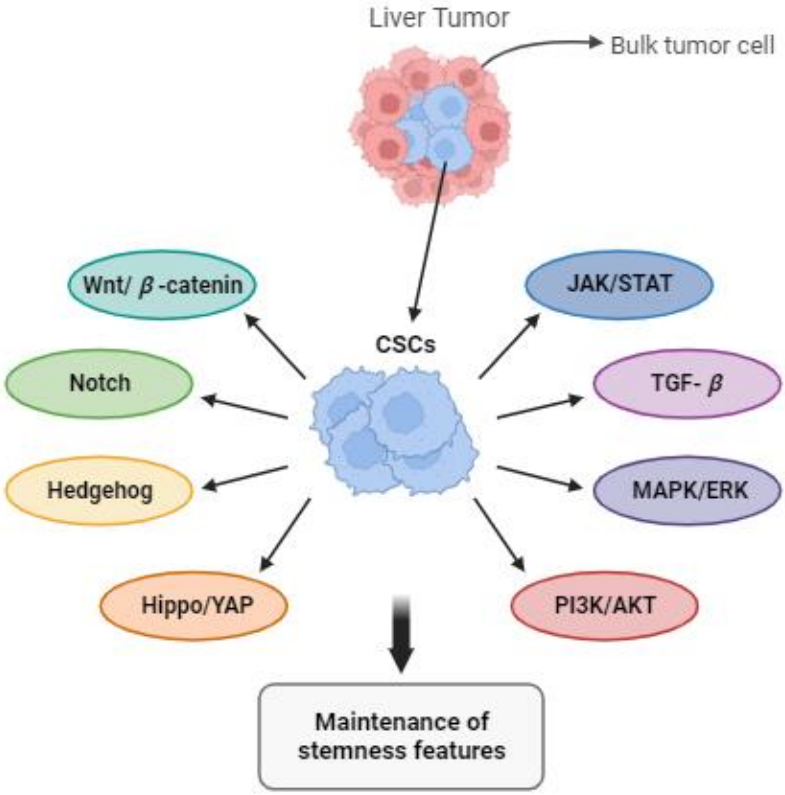

Figure 4 - Regulatory pathways of liver cancer stem cells (CSCs). Adapted from (149)

3.4. Biomarkers of liver CSCs

The identification of cell surface markers for hepatic CSCs is of great importance for several reasons. These markers can serve as potential prognostic and predictive indicators for disease progression and treatment response since CSCs have been associated with a poor prognosis in liver cancer. Liver CSC markers also provide potential targets for the development of targeted therapies. The selective targeting of CSCs, which are responsible for tumour initiation, growth, and therapy resistance, may facilitate improved treatment outcomes and reduce risk of recurrence. Thus, personalized medicine, based on the expression profile of CSC markers, may lead to more effective treatments with fewer side effects.(49,150)

Several surface markers have been identified in liver cancer stem cells, including CD133, CD44, CD90, EpCAM, CD47, CD34, C-kit, CD13, CD24, calcium channel α2δ1 isoform

5, oval cell marker (OV)-6, Delta Like Non-Canonical Notch Ligand 1 (DLK1), keratin 19 (K19), and Lgr5.(151)

CD133, also known as prominin-1, is one the most commonly reported surface markers in hepatic stem cells. Initially identified as a marker of hematopoietic stem cells, CD133 was later recognized as a marker of CSCs in various tissues, including the prostate, colon, and liver.(152–154) However, while some studies have considered CD133 a promising candidate for identifying CSCs in HCC cell lines, others have found that HCC cells that do not express this marker can still form tumours, which suggests that CD133 may not be a reliable CSC marker in HCC.(155)

In contrast, it has been found that $CD90^+$ cells from HCC tumours displayed a higher tumorigenic potential and are more likely to be highly mesenchymal cells than $CD90^-$ cells. Similarly, $EpCAM^+$ cells are more likely to be highly tumorigenic than $EpCAM^-$ cells. These observations indicate that these two surface markers could serve as potent identifiers for liver CSCs.(156)

Despite the knowledge of many different hepatic CSC markers, their utility in identifying and isolating CSCs remains constrained. One major challenge lies in the inherent heterogeneity of tumour populations. Within a singular tumour, multiple clones of CSCs can coexist, each with distinct genetic and phenotypic profiles.(157) For instance, well known CSCs markers, such as CD133, ALDH1A1, EpCAM and K19, exhibit varied expression patterns among different individual HCC cancer cells.(158) This intricate diversity leads to variations in the expression of cell surface markers among CSCs, rendering the establishment of a universal set of markers for their identification. Furthermore, the utilization of stemness markers to identify CSCs encounters an additional hurdle - the lack of specificity. Many of the stemness markers commonly used to identify CSCs, such as CD133, EpCAM and CD90, are also expressed by normal stem cells or other cell types within the tumour microenvironment. This overlapping expression complicates the accurate isolation of CSCs using these markers and raises concerns about false positive identification.(159)

3.5. Therapeutical implications:

3.5.1. Treatment resistance:

Cancer relapse and subpar long-term clinical outcomes are primarily attributed to resistance to standard therapies.(160) Growing evidence suggests that tumour cells possessing stem cell-like features display higher resistance to conventional therapeutic methods compared to their non-stem-like counterparts. In fact, CSC-mediated multidrug resistance has been observed in diverse tumour types, such as leukaemia, colorectal, brain, pancreatic, melanoma, breast, cancer among others.(161–166)

The mechanisms of resistance to conventional treatments and the development of stem-like characteristics are closely linked with the properties of CSCs, such as plasticity, quiescence, increased drug efflux activity and CSC niche.(167,168)

Plasticity refers to CSCs capacity to transition between different cell states and adapt to fluctuating environmental conditions. These cells can shift between stem-like and non-stem-like states, each exhibiting distinct characteristics and active cellular pathways; whereby, plasticity may allow these cells to adapt and become less susceptible to both conventional and targeted therapies.(169)

CSCs can also shift between quiescent (or dormant) and proliferative states. Conventional cancer treatments often target actively dividing cells in a proliferative state. By adopting a metabolically inactive quiescent state, CSCs can evade these therapies and later re-enter the proliferative state to facilitate tumour regrowth, resulting in clinical cancer recurrence.(168,170)

It has also been shown that CSCs could overexpress ATP-binding cassette (ABC) transporters, which participate in the efflux of various toxic substances from cells, including drugs, directly contributing to the development of treatment resistance.(171)

Finally, as previously outlined, the CSC niche and tumour microenvironment can also contribute to tumour progression and inadequate treatment response by impacting on inflammation, angiogenesis, hypoxia, and fibrosis.(168)

In the context of primary liver cancer, liver CSCs are also linked to treatment resistance, mirroring observations seen in various other types of cancer. For instance, one study demonstrated that overexpression of the ABCB1 transporter, a specific ABC transporter, in hepatic CSCs correlates with HCC aggressiveness and reduced survival rates.(172)

However, the role of ABCB1 as a prognostic marker in primary liver cancer is still controversial. Contradictory studies have shown that the expression of ABCB1 is lower in HCC when compared to non-cancerous tissues and is not associated with cancer increased cancer aggressiveness or poorer survival outcomes.(173)

Nevertheless, the association between CSCs and treatment resistance in primary liver cancers has been established. Studies have found that cancer cells, both from HCC and iCCA, expressing stemness markers are associated with treatment resistance.(174) Specifically, overexpression of CSC markers such as CD90, CD133, CD24 and EpCAM has been linked to increased treatment resistance in primary liver cancers.(175–178)

Overall, there is an urgent unmet need to develop innovative therapeutic approaches that can specifically target and eliminate CSCs in liver cancer, while sparing normal and healthy cells.

3.5.2. Perspectives of treatment

Novel strategies are being developed to overcome treatment resistance and cancer recurrence. These include the induction of CSC differentiation, suppression of specific signalling or metabolic pathways linked to treatment resistance, or targeting CSCs through their cell surface markers.(167) The combination of these therapies with the conventional cancer therapies, including chemotherapy and radiation therapy, has the potential to efficiently eliminate the tumour.(Figure 5 **- Combination of therapies targeting hepatic CSC with conventional cancer therapeutics to reduce tumour recurrence and metastasis and promote tumour eradication.** Adapted from (181)) Although many of these strategies remain in the preclinical stage, some have been approved because they show promise in effectively eliminating CSCs, potentially reducing the risk of local cancer recurrence and metastasis.(179,180)

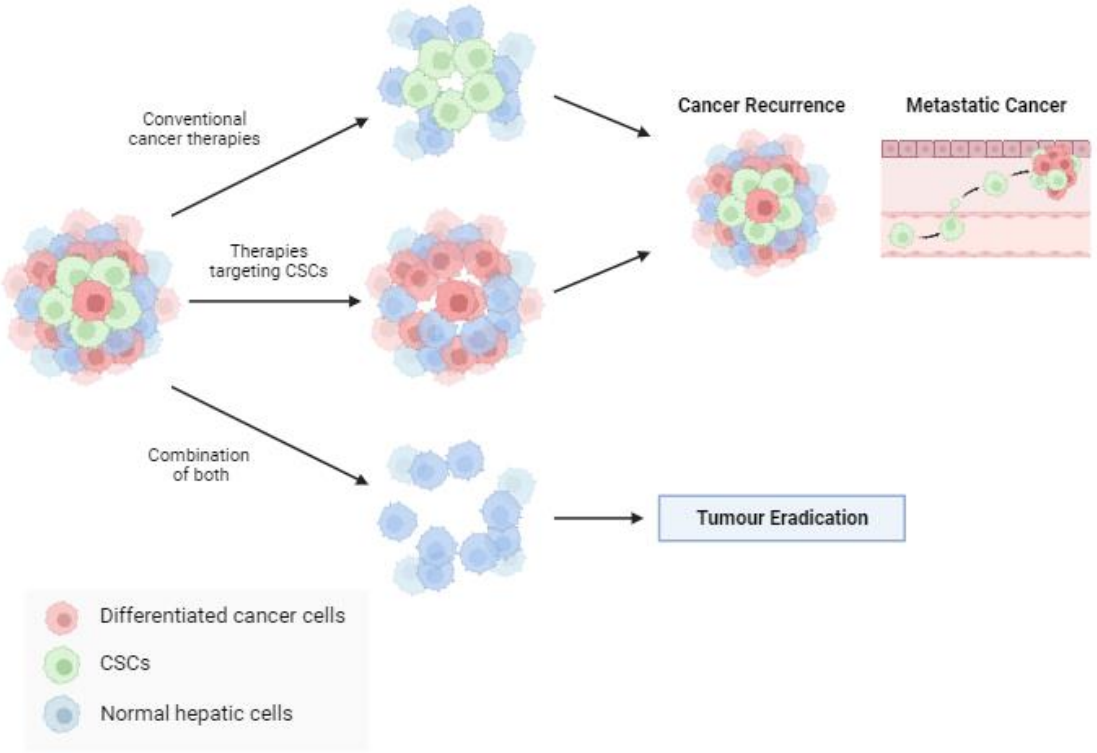

Figure 5 - Combination of therapies targeting hepatic CSC with conventional cancer therapeutics to reduce tumour recurrence and metastasis and promote tumour eradication. Adapted from (181)

In response to therapies inducing differentiation, liver CSCs may eventually differentiate into a less aggressive phenotype, losing their self-renewal properties and become susceptible to conventional therapies. Studies have demonstrated that administering high-dose exogenous bone morphogenetic protein 4 (BMP4), oncostatin M (OSM), and hepatocyte nuclear factor-4α (HNF-4α) can promote liver CSC differentiation, in parallel with the inhibition of their self-renewal capabilities and chemotherapeutic resistance.(182)

Various signalling pathways have been implicated in CSC resistance to conventional therapies. Among the most significant and well-studied are the Hedgehog, Wnt/β-catenin and Notch, described previously.(183)

Drugs targeting these mechanisms in other cancers include vismodegib and sonidegib, which are Hedgehog pathway inhibitors approved for the treatment of basal cell carcinoma, and pembrolizumab, a PD-1 inhibitor approved for various solid

tumours.(179,180,184) Pembrolizumab works by blocking the PD-1 receptor on T cells, thereby preventing its interaction with PD-L1 and PD-L2, which are often overexpressed in cancer cells, including CSCs.(184) Notably, overexpression of PD-L1 has been observed in $CD133^{+}$ liver CSCs, enabling these cells to evade immune surveillance through the PD-1 checkpoint.(185)

Several other molecules that modulate these and other pathways have been studied in clinical trials across various cancer models, including liver cancers. These studies could potentially pave the way for new treatment options for patients in the future. For instance, since γ-secretase inhibitors (GSI) have been shown to inhibit the growth of HCC cells in vitro through the downregulation of the Notch signalling pathway(186,187), several clinical trials have been initiated to evaluate the effectiveness of GSIs in different cancer models.(188–191) Although the efficacy of this intervention has not been assessed specifically in the context of primary liver cancer, the results from other cancer models suggest their potential therapeutic utility in treating liver cancer. Another clinical study studied the efficacy of the CB-103 molecule in advanced solid tumours and blood malignancies, including HCC.(192) CB-103 is a small molecule that acts as a protein-protein interaction (PPI) inhibitor, targeting the Notch transcription complex in the cell nucleus, compromising the transcription of the Notch genes.(193)

Additionally, phase I clinical trials are evaluating the efficacy of different molecules targeting the Wnt/β catenin signalling pathway in primary liver cancers. For instance, porcupine (PORCN) is a membrane-bound O-acyltransferase required for Wnt activity.(194) Several clinical studies have investigated PORCN inhibitors in cancer treatment, including applications for HCC.(195–197)

Finally, by precisely identifying and attacking CSC surface markers, therapies can be tailored to disrupt the self-renewal and survival properties of CSCs, potentially enhancing the effectiveness of cancer treatments and reducing the risk of recurrence.(198)

EpCAM-targeted therapies have shown promising therapeutical potential, being the monoclonal antibody Catumaxomab approved for the intraperitoneal treatment of patients with malignant ascites (199). However, this drug was withdrawn from the market for commercial reasons in 2014.(200) A recent study showed that EpCAM-targeted therapy enhances liver CSC chemosensitivity and decreases the likelihood of cancer relapse, suggesting a promising avenue for therapies that specifically target this CSC surface marker.(201)

Furthermore, inhibition of CD13 has also been correlated with CSC dysregulation. Ubenimex, a CD13 inhibitor, has shown anti-tumour activity against liver CSCs in pre-clinical mouse models.(202,203)

Still, given the intricate heterogeneity within tumours - marked by the presence of diverse cellular clones, each expressing unique cell surface markers - it is conceivable that future innovative approaches will aim to target more than one cell surface marker.

3.5.3. Hepatic CSCs as diagnostic and prognostic tools for liver cancer

In line with their association with treatment resistance and cancer recurrence, CSCs can be utilized as valuable tools for cancer diagnosis and prognosis.(204)

Although liver cancer staging systems such as BCLC or the TNM staging systems provide a rough estimate of patient survival, individuals diagnosed at the same disease stage often exhibit distinct prognoses, possibly due to variations in the malignant phenotype of their tumours.(204)

To address these limitations, researchers are using gene expression profiling technologies to identify signatures that better correlate with the aggressiveness of the tumour and, eventually, direct the therapeutic approaches, as discussed above. Gene expression profiles, which include the analysis of various CSC markers, can provide a more accurate prediction of tumour aggressiveness, recurrence likelihood, and early identification of cases with high metastatic potential. For instance, the overexpression of the hepatic CSC markers K19, CD133 and EpCAM in HCC has been correlated with increased tumour aggressiveness and poor clinical outcomes compared to low-expressing stemness-related markers HCCs.(205) Curiously, it has been reported that CD133 exhibited its most notable prognostic significance in early stages of HCC, whereas the impact of EpCAM became increasingly significant in more advanced stages.(206) In the context of iCCA, overexpression of CD133 has also been associated with poor prognosis and aggressive clinical features.(207,208) Likewise, the overexpression of other stemness markers, such as CD24(209,210), CD44(211,212) and EpCAM(213) in iCCA tumour cells has been associated with a poor prognosis and chemotherapy resistance.

Furthermore, monitoring changes in the expression of stemness markers during treatment could offer valuable insights into therapeutic response. A reduction in these markers could indicate a positive treatment response, while their persistent or increased presence might suggest resistance to the treatment.(214) Noteworthy, gene expression profiles typically represent the characteristics of the dominant cell population within a tumour, while could also have sensitivity to detect the emergence of less representative cell populations.

Another current challenge contributing to the poor clinical prognosis of primary liver cancer patients is the low rate of early diagnosis, which largely depends on serum markers, imaging tests, and tissue biopsy for diagnosing cancer, as well as for determining its recurrence and metastasis post-treatment.(215) Liquid biopsy has emerged as a promising non-invasive approach for monitoring liver cancer, offering insights into early diagnosis, disease progression and treatment response. This technique comprises the molecular analysis of tumour components in the biological fluids, including circulating tumour cells (CTCs), nucleic acids (DNA and RNAs) and exosomes. By analysing CTCs and cell-free circulating tumour nucleic acids from blood samples, liquid biopsies provide an insight into the genetic landscape of liver cancer.(216) Indeed, the presence of circulating CSCs could accurately predict HCC after hepatectomy.(217) Additionally, increased expression of CSC markers in circulation, such as K19 and EpCAM, has also been associated with liver cancer recurrence.(218)

These findings highlight the potential of liquid biopsy to identify these treatment-resistant cells and to continuously monitor their presence in the bloodstream, facilitating timely intervention, personalized treatment adjustments, and ultimately improving patient outcomes. Noteworthy, while these approaches hold promise, research in this area is still ongoing, and further studies are needed to validate the utility of hepatic CSCs for diagnosis and prognosis in HCC and iCCA. As our understanding of CSC biology advances, integrating this knowledge into clinical practice may significantly improve the management of these cancers.(219)

4. Conclusion

Hepatic CSCs are linked to a poor prognosis in liver cancer patients, largely due to their cancer aggressiveness and resistance to standard therapies (174–178), which makes compelling targets for the development of more effective therapies against liver cancer.

As discussed in previous sections, these cells present distinctive traits such as the ability to self-renew, to interconvert between stem and differentiated states and to proliferate, which contributes to tumorigenicity and chemoresistance.(51)

The cellular origins of liver cancer encompass mature epithelial cells, such as hepatocytes and cholangiocytes, and also hepatic progenitor cells, the so-called hepatic stem cells.(69–73) Liver tumours originating from hepatic CSCs exhibit stem-like features and, as expected, are associated with higher tumour aggressiveness and poorer prognosis.(74,75)

Understanding the underlying mechanisms of hepatic CSCs-like properties is critical for identifying novel potential therapeutic targets. Several factors contribute to this understanding: the liver microenvironment fosters a supportive milieu that sustains hepatic CSC self-renewal and tumorigenicity(78); regulatory signalling pathways intricately orchestrate hepatic CSC behaviour, driving their aggressive tendencies and resistance to therapy(101,107,110,114,115,121–124,133–135,143,145,146); and the identification of signature biomarkers indicative of liver CSCs holds promise, not only for improving early detection and prognosis but also for the development of tailored treatment approaches(49,150).

There is an urgent need to develop innovative strategies aimed at targeting CSCs to overcome their resilient nature and prevent cancer recurrence. These promising strategies may include 1) inducing CSCs differentiation to increase their susceptibility to standard therapies(182), 2) targeting the regulatory mechanisms involved in the differentiation, proliferation and maintenance of the stemness features of hepatic CSCs(179,180,184), or 3) tailored to disrupt the self-renewal and survival properties of CSCs. This could be achieved by targeting specific hepatic CSC biomarkers. Such approaches hold the potential to revolutionize liver cancer treatment, offering more effective therapeutic options to patients.(198)

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