Liver Cancer

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Introduction

HCC affects more than half a million individuals annually and is the fifth leading cause of cancer, and ranks third in cancer mortality worldwide [1]. Despite some progress in cancer treatment, existing therapies are limited in their ability to cure malignancies and to prevent metastases and relapses. Surgery, radiofrequency ablation therapy, and chemotherapy are all directed at reducing the bulk of the tumor mass. However, in the majority of cases, tumor regrowth and relapse of disease occurs on completion of therapy. Although the concept of tumor stem cells has been proposed for a number of decades, the demonstration of their existence has only occurred within the last decade. Recently, progression of HCC is thought to be driven by cancer stem cells (CSC) through their capacity for self-renewal, the production of heterogeneous progeny, and their ability to limitlessly divide. CSCs with such characteristics have been reported for many haematological and solid human tumors. Furthermore, many potentially biologically significant surface markers and pathways that modulate these stem/progenitor cells in cancer tissue have been identified since they have dual roles in embryogenic stem cell development and tumor activation or suppression. In this review, we demonstrate a brief and uptodate review of molecular signaling in liver CSCs and present insights into new therapeutic strategies.

Liver Stem Cells in Human Liver Regeneration and Cancer Stem Cells

The liver is both an exocrine and an endocrine gland that performs complex functions and has the capacity to regenerate. This process enables the recovery of lost mass without endangering the viability of the entire organism and many studies suggest the existence of two basic types of liver regeneration. Acute liver injury is often observed in patients without liver disease, although sustaining such an injury may result in rapid liver dysfunction. Several different factors appear to be primarily responsible for injury, including drugs, toxins, chemicals, ischemia/reperfusion, and viral hepatitis. During extensive acute liver injury, there is wide-spread necrosis and apoptosis with release of cytokines, which far exceeds the capacity of the remaining healthy hepatocytes to replicate and restore the liver function. As a result, resident liver progenitor cells within the canals of Hering are activated to support or take over the role of regeneration [2].

By contrast, liver regeneration after the loss of hepatic tissue does not depend on these cell types, but rather on the proliferation of existing mature hepatocytes, the parenchymal cells of the organ. Liver regeneration in this non-toxic model of injury is a multi-step process with at least two important phases: 1) transition of quiescent hepatocytes into the cell cycle and, 2) their progression beyond the restriction point in the G1 phase of the cell cycle. Control of this process depends on a complex interaction of cytokine and growth factors released in response to liver injury. Three main growth factors: hepatocyte growth factor (HGF), epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) underpin normal hepatic regeneration through their potent mitogenic action on hepatocytes via stimulation of DNA synthesis. Termination of hepatocyte proliferation at the end of regeneration is an important part of this process which is regulated by TGF-β and activin, which serve as negative feedback mechanisms. Termination of hepatocyte proliferation is regulated by the ratio of liver to body mass rather than liver mass *per se*, thus providing a remarkable check on the extent of liver regeneration [3].

Hepatocytes are capable of large-scale clonal expansion within a diseased liver. Following very extensive liver damage or in situations in which hepatocyte regeneration after damage is compromised, a potential stem cell component located within the smallest branches of

the intrahepatic biliary tree is activated. HPCs amplify a biliary population of transit amplifying cells that are at least bipotential, namely, capable of differentiating into either hepatocytes or cholangiocytes. HPCs are induced during chronic liver inflammation, replacing damaged hepatocytes and cholangiocytes in various liver diseases including alcoholic and non-alcoholic fatty liver disease, and HBV- and HCV-induced hepatitis. HPCs are almost always accompanied by an inflammatory reaction, which is located directly adjacent to the inflammatory cells. HPCs are strongly associated with liver regeneration following acute and chronic damage through cellular interactions with liver immune cells involving paracrine signals, especially from growth factors that are released as part of the regeneration process. However, during regeneration, HPCs are considered a dangerous target in hepatocarcinogenesis by the interaction or modulating inflammation niche involved in tissue repair. HPCs have also been reported to initiate HCC and intrahepatic cholangiocarcinoma (ICC), and their function in carcinogenesis is supported by a histological investigation of liver cancer that exhibits features of both HCC and ICC accompanied by the presence of numerous HPCs. Detailed immunophenotyping of HCCs has revealed that 28-50% express markers of progenitor cells such as cytokeratin 7 and 19, respectively. These tumors also consist of cells that have an intermediate phenotype between progenitors and mature hepatocytes. In fact, patients with HCCs that express hepatocyte and biliary cell markers have a significantly poorer prognosis and a higher recurrence rate after surgical resection and liver transplantation. A small subset of cancer cells with CSC properties has been identified and characterized from HCC cell lines, animal models, and human primary HCCs, which can be identified by several cell surface antigens including CD133, CD90, CD44, EpCAM, and CD13 [4, 5], respectively.

Stem cells in the liver are proposed to be from two origins, either endogenous or intrahepatic, and exogenous or extrahepatic. Included in the intrahepatic stem cell category are the numerous HPCs with short-term proliferative capacity that localize within the canals of Hering and interlobular bile ducts.

Extrahepatic stem cells include cells derived from bone marrow and peripheral blood cells; these cells are limited in number but have a long-term proliferation capacity [6].

Molecular Signaling of Liver Cancer Stem Cells

Liver CSCs are likely to require a multitude of signals to maintain a phenotype characterized by self-renewal and pluripotency. These signals include the EpCAM, Wnt/β-catenin pathway, the Sonic Hedgehog pathway, and the Notch pathway, which play a decisive role in the regulation and maintenance of stemness and in tumor formation. The uncontrolled activation of these and other pathways is thought to lead to the initial formation of liver CSCs, and therefore tumorigenesis in general. As these pathways are frequently involved in the regulation of various stem cell phenotypes, it is tempting to speculate that gain of function mutations of members of these pathways are instrumental in the formation of liver CSCs. Wnt pathway proteins are a group of evolutionarily conserved intracellular signaling molecules that regulate the cellular fate and are implicated in the self-renewal of stem cells. The evolutionarily conserved Notch pathway is involved in many developmental processes such as differentiation, fate decision, proliferation, apoptosis, and cell adhesion. In the liver, Notch signaling contributes to the formation of a network of transcription factors involved in cholangiocyte differentiation [7, 47].

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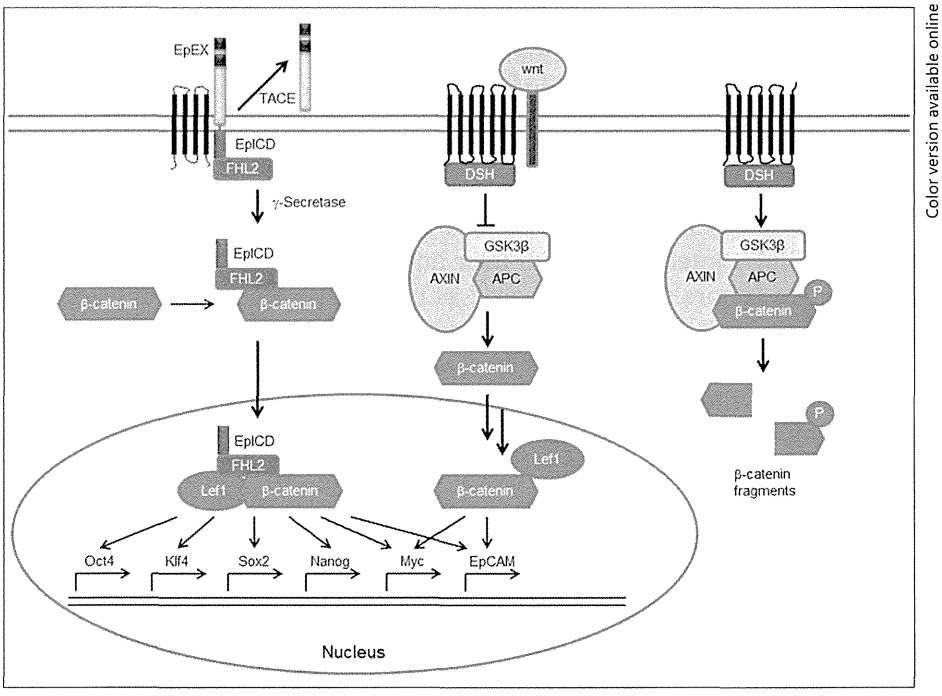

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Fig. 1. Overview of cross-talk between EpCAM signaling and the Wnt pathway. Following cleavage by TACE/PS-2, EpICD translocates to the nucleus in a multiprotein complex. This nuclear complex binds the promoters of genes involved in cell cycle regulation and stemness. EpCAM regulates Nanog, Oct4, Klf4, Sox2, and Myc.

EpCAM Signaling Pathway

EpCAM is a type 1 transmembrane glycoprotein consisting of a large extracellular (EpEX), a single transmembrane and a short intracellular (EpICD) domain. Three independent glycosylation sites in the EpEX part dictate the stability of the whole protein at the cell surface. Liver CSC markers such as EpCAM, CD44, and CD133 share a number of entities and represent the most frequently used markers for the enrichment of tumor-initiating cells from primary human cancer. As is the case for many cell adhesion molecules, EpCAM has dual properties in that it can mediate cell-to-cell contact as well as transmit signals from the plasma membrane to the nucleus in order to regulate gene transcription [8]. In addition, EpCAM is not solely expressed in epithelial cells, but is also strongly expressed in various tissue stem cells, precursors, and in embryonic stem cells of murine and human origin [9]. Its mode of signaling proceeds via regulated intramembrane proteolysis and is activated by regulated intramembrane proteolysis (RIP) and the shedding of its EpEX [8] Sequential cleavage of EpCAM by tumor necrosis-factor alpha converting enzyme (TACE/ADAM17) and a gamma-secretase complex containing presenilin 2 (PS-2) results in the release of EpEX into the culture medium, and the release of the EpICD into the cytoplasm (fig. 1). EpICD then becomes part of a large nuclear complex containing transcriptional regulators β-catenin and Lef, which are both components of Wnt/β-catenin signaling. Four and one-half LIM domain protein 2 (FHL2) is essential for signal transduction by EpCAM, and FHL2 further regulates the localization and activity of TACE and PS-2. Through its function as a co-activator of β-catenin, FHL2 links EpICD with specific DNA sequences and gene regulation. FHL2 also has the potential to serve as a scaffolding protein for various signaling proteins used by EpCAM [10].

Wnt/β-CateninSignaling Pathway

The Wnt/β-catenin pathway is evolutionarily well-conserved and is essential for normal cellular processes such as development, growth, survival, regeneration, and self-renewal [11]. Disruption of Wnt/β-catenin signaling results from both genetic and epigenetic changes and is associated with a range of diseases including many cancers, especially colonic cancer and HCC. Disrupted Wnt/β-catenin signaling by mutational and non-mutational events is observed in around one third of all HCCs, emphasizing the importance of this pathway in hepatocarcinogenesis [12]. The Wnt pathway diversifies into two main branches, canonical (β-catenin-dependent) and non-canonical (β-catenin-independent), which play critical roles in specifying cellular fates and movements, respectively, during both embryonic development and adult tissue regeneration [13].

Wnt ligands signal through binding to seven transmembrane Frizzled (Fzd) receptors and single transmembrane lipoprotein receptor-related protein (LRP) 5 or 6 co-receptors. Canonical signaling mediated by ligands such as Wnt3a inhibits a multiprotein degradation complex consisting minimally of axin, adenomatous polyposis coli, and glycogen synthase kinase 3β. This inhibition culminates in the nuclear translocation of β-catenin, enabling it to interact with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors to regulate gene expression. The resulting accumulation of β-catenin in the cytoplasm allows for its transfer into the nucleus, where it interacts with transcription factors of the LEF1 family. This functional complex induces the transcription of prominent targets like CD44 [14], cyclin D1 [15], and c-myc [16], which is also a major target of EpCAM signaling [17]. Moreover, c-myc can trigger the induction of a stem-like transcriptional profile in normal and cancer cells and represents the central switch from adult to embryonic stem cells [18].

Thus far, it remains unknown at which point in the signaling cascades of EpCAM and Wnt/Frizzled cross-talk occurs. However, EpICD does not only interact with β-catenin and Lef-1, it also binds to Lef-1 consensus sites in the promoter of Wnt target genes such as cyclin D1. EpICD appears to be essential for the formation of one of the two major nuclear protein/DNA complexes formed at Lef-1 consensus sites in EpCAM-positive carcinoma cells [10]. This suggests that EpICD can provide additional levels of regulation to Wnt target genes, which are central in cell cycle regulation, and thus could play important roles in self-renewal. Since Wnt signaling is reportedly instrumental in tumor-initiating cells (TICs), and because TICs rely on Wnt pathway-inducing signals from their microenvironment for the maintenance of their phenotype [19], it is tempting to speculate that EpCAM overexpression and signaling are also instrumental in this.

In addition to c-myc, other key factors such as Nanog, Klf4, Sox2, and Oct4, which are central to the conversion of somatic cells into induced pluripotent stem cells (iPS), have also been described as direct targets of EpCAM in human embryonic stem cells [20]. EpCAM possesses a crucial role in the induction and/or maintenance of the phenotype of tissue precursors, stem cells, iPS cells, and TICs. This function most likely relates primarily to the proliferation and the maintenance of an undifferentiated state. In the liver, EpCAM expression and Wnt signaling are both associated with a tissue stem cell phenotype and regenerative capacity of cells [21]. It is important to note that EpCAM expression is only detected in regenerating cells such as hepatobiliary stem cells and progenitor cells [21]. The interrelationship of EpCAM and Wnt in HCCs has been further substantiated upon by the finding that the EpCAM gene becomes transcriptionally activated by Tcf-4, a member of the Lef family of transcription factors. EpCAM is a marker for TICs with a stem/progenitor phenotype in HCC [22].

Non-canonical signaling, which is much less defined, is mediated by ligands such as Wnt11 that use the same Fzd receptors. The Wnt-Fzd-G protein complex can also stimulate p38 kinase and activate phosphodiesterase 6, which hydrolyzes cyclic GMP and results in the inactivation of protein kinase G and an increase in intracellular calcium. Wnt-mediated

increases in intracellular Ca^{2+} activate calcineurin and subsequently the nuclear factor of activated T-cells (NF-AT) family of calcineurin-dependent transcription factors, as well as TAK1-Nemo-like kinase (NLK) kinases. Signaling through the TAK-NLK kinases is proposed to inhibit canonical Wnt signaling, stimulating the Jun NH2-terminal kinase [23], calcium-calmodulin-dependent protein kinase II and protein kinase C pathways. These pathways interact with each other, and, in some cases, non-canonical signaling antagonizes the canonical pathway [24].

SALL4 Signaling Pathway

The human homologue of the Drosophilia spalt homeotic gene, SALL4, encodes a C2H2 zinc-finger transcription factor. It is one of the key factors for maintenance of pluripotency and self-renewal of embryonic stem cells, potentially through the interaction with Oct4, Sox2, and Nanog. SALL4 is known to encode two isoforms, namely SALL4A and SALL4B, and recent studies have suggested the important role of SALL4B on maintaining the stemness of embryonic stem cells [25]. In the liver, SALL4 is expressed at high levels in fetal-liver progenitor cells but not in adult hepatocytes, and it plays a critical role in hepatic cell lineage commitment. Recently, this oncofetal gene was identified as a marker of a subtype of HCC with progenitor-like features and is associated with a poor prognosis [26, 27].

SALL4 affects phosphatase and tensin homologue (PTEN) and phosphatidylinositol 3-kinase (PI3 K)-AKT signaling thorough the interaction with NuRD (nucleosome remodeling and histone deacetylase (HDAC)) complex. Since SALL4 is a known inhibitor of PTEN, the silencing of it reduces pAKT levels and blocks PI3 K survival signaling in HCC cells highly expressing SALL4. Furthermore, SALL4-positive HCC cells tend to show high HDAC activity and chemosensitivity to HDAC inhibitors such as suberic bis-hydroxamic acid and suberoylanilide hydroxamic acid. Consistently, HDAC inhibitors might be useful for the eradication of SALL4-positive HCC cells through their inhibitory effects on histone deacetylation of NuRD.

TGF-β Family

The TGF-β family plays a vital role in the control of proliferation and cellular differentiation in both stem cells and cancer cells. Impaired TGF-β signaling through the activation of interleukin-6 in hepatic stem/progenitor cells can contribute to altered differentiation patterns and HCC development [28]. TGF-β inhibits cell proliferation and promotes tumor cell invasion by inducing epithelial-mesenchymal transition (EMT). Reduced expression of the TGF-β receptor might be a key factor in shifting to the late response to TGF-β. Many studies have reported a reduction of TGF-β receptors in up to 70% of HCCs. Moreover, reduced TGF-β receptor 2 expression in HCC has been correlated with intrahepatic metastasis. TGF-β levels in the serum and urine are increased in HCC patients, while up to 40% of HCCs have increased TGF-β expression based on immunohistochemical analysis. In addition, high TGF-β levels have been correlated with advanced clinical stages of HCC. This dual role of TGF-β signaling in HCC is explained by its effect on the tumor tissue microenvironment and on the selective loss of the TGF-β-induced antiproliferative pathway. Tumor cells that have selectively lost their growth-inhibitory responsiveness to TGF-β, but retain an otherwise functional TGF-β signaling pathway may exhibit enhanced migration and invasive behavior in response to TGF-β stimulation. Recently, loss of the TGF-β adaptor and signaling molecule embryonic liver fodrin in the liver was found to cause cancer through deregulated hepatocyte proliferation and stimulation of angiogenesis. More recently, it was reported that HCC cells positive for signal transducers and activators of transcription-3/Oct4, have dysfunctional TGF-β signaling, and are likely cancer progenitor cells with the potential to give rise to HCC [29].

Other Pathways

The Notch signaling pathway plays an important role in stem cell self-renewal and differentiation.

However, other signaling pathways influence whether Notch functions as a tumor suppressor or oncogene depending on the particular tissue [30]. Notch signaling plays a well-defined role in liver embryogenesis and bile duct formation. In addition, Notch family members are involved in angiogenesis and endothelial sprouting. The increased expression of genes involved in this pathway has been shown in CD133-positive liver cancer cells compared to CD133-negative cells. The activated intracellular form of Notch-3, as well as the Notch ligand Jagged, is highly expressed in HCC. Conversely, Notch-1 has been reported to function as a tumor suppressor and participate in cross-talk with other signaling pathways such as Ras/Raf/Mitogen-activated protein kinase/ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) through the regulation of the PTEN tumor suppressor. Recent evidence indicates that activation of Notch-1 signaling increases the expression level of death receptor 5 (DR5) with enhancement of TNF-related apoptosis-inducing ligand induced apoptosis *in vitro* and in vivo [31].

Conserved from Drosophilia to humans, the Hedgehog (HH) pathway has a central role in embryonic development and adult tissue homeostasis by controlling cell fate specification and pattern formation [32]. The functional importance of this pathway is illustrated by the multiple birth defects and malignancies associated with mutations and/or aberrant activation of the pathway. Three HH ligands Sonic, Indian, and Desert have been identified in mammals that can bind interchangeably to two related twelve-pass membrane patched receptors. After ligand stimulation, Gli, like β-catenin, accumulates in the nucleus and induces transcription of genes related to the cell cycle and growth including insulin-like growth factor-2, cyclins, and β-catenin. Sonic is the predominant isoform in the liver. Up to 60% of human HCCs express Sonic, and the concomitant downregulation of Gli-related target genes is observed after the specific blockade of this pathway. Furthermore, tumorigenic activation of Smo can mediate overexpression of c-myc, a gene known to play an important pathogenic role in liver carcinogenesis. Recent studies have also shown that activation of Hedgehog signaling is critically related to CSCs and EMT features in many types of cancers including colonic, gastric, esophageal, hepatic, and others [33, 34].

microRNAs

Micro-ribonucleic acids (miRNAs) play critical roles in many biological processes including cancer by directly interacting with specific messenger RNAs (mRNAs) through base pairing, then inhibiting the expression of target genes through a variety of molecular mechanisms. MiRNAs can undergo aberrant regulation during carcinogenesis, and can act as oncogenes or tumor suppressor genes. Disruption of miRNA expression levels in tumor cells may result from distorted epigenetic regulation of miRNA expression, abnormalities in miRNA processing genes and proteins, and the location of miRNAs at cancer-associated genomic regions. Consequently, abnormal miRNA expression is a ubiquitous feature of solid tumors, including HCC. In liver carcinogenesis, miRNAs have been shown to have both tumor suppressive (miR-122, miR-26, miR-223) and oncogenic (miR-130b, miR-221, miR-222) activity [35–39]. Clearly, miRNAs play a critical role in carcinogenesis and oncogenesis. Emerging evidence suggests that certain abnormal miRNA expression levels cause cancer stem cell dysregulation, resulting in unlimited self-renewal and cancer progression. Therefore, miRNA expression is a vital key to CSC dysregulation. The let-7 miRNA precursor, which binds to the mRNA Lin28 (a marker of human embryonic stem cells), is regulated by the product of the oncogene c-myc. Let-7 family members are downregulated in malignancies, including HCC, and are associated with CSCs. The family members Lin28 and Lin28B

each target and inhibit let-7, and Lin28 and Lin28B are overexpressed in primary human tumors and human cancer cell lines, with an overall frequency of 15%. The mammalian homologs of Lin28 bind to the terminal loop of the precursors of let-7 family miRNAs and block their processing into mature miRNAs. Let-7 suppresses the expression of c-myc, which inhibits the transcription of let-7. Loss of such a negative feedback loop appears to be a common event in cancer cells from advanced-stage tumors such as HCC. MiR-181 regulates the Wnt/β-catenin signaling pathway in a positive feedback loop within stem cells. MiR-181 family members are highly expressed in embryogenic livers and isolated hepatic stem cells.

MiR-181 promotes the stem-cell-like features of HCC cells by targeting mRNAs that encode caudal type homeobox transcription factor 2 (CDX2) and GATA6, which are hepatic transcriptional regulators of differentiation. It also inhibits the mRNA that encodes NLK, an inhibitor of Wnt/β-catenin signaling, and maintains HCC stemness by inhibiting CDX2, GATA6, or NLK. Hepatic transcriptional regulators of differentiation and an inhibitor of Wnt/β-catenin signaling are directly targeted by miR-181. This type of positive feedback loop might be used by cancer cells to continuously self-propagate and contribute to metastasis and drug resistance.

Epigenetic Regulation of Hepatic Stem/Progenitor Cells

Although various genes have been identified as stem cell related, the control of stem cells is likely to arise from an integrated expression pattern of multiple genes involved in proliferation and differentiation rather than decimal gene expression [40]. In the self-renewal process of stem cells, it is important that the gene expression pattern is inherited in daughter cells after cell division. Therefore, chromatin regulation is a newly considered parameter that controls and integrates the expression of multiple genes. Chromatin modifying enzymes regulate the expression of target genes by manifesting structural changes in chromatin. As an epigenetic code, this forms the basis of stem cell identity and determines its responsiveness to extrinsic signals at successive developmental stages. In fact, progression from undifferentiated stem cells toward their differentiated progeny is characterized by alterations in the epigenetic landscapes of regulatory and coding regions of genes. The enzyme complex responsible for histone modification regulates activation and inactivation of transcription through methylation and acetylation of lysine residues in histone H3 and H4 [41]. In particular, histone modifications have been shown to affect polycomb group proteins such as Bmi1 and Ezh2 involved in stem cell regulation.

Recently, the bivalent domains, consisting of active modification H3K4me3 and repressive modification H3K27me3, have been shown to play an important role in the mechanism of action of histone modification proteins in stem cells [42]. Functional analyses of these molecules during liver development have advanced the understanding of several complex chromatin-modifying enzymes involved in cell lineage commitment [43]. In addition, it is reported that the expression of liver-specific transcription factors is changed by the administration of histone deacetylase inhibitors *in vitro* [44]. Special attention is being paid to their role in controlling both the growth and differentiation of stem cells *in vitro*.

Therapeutic Target of Molecular Signaling

The successful eradication of malignancy requires anticancer therapy that affects the differentiated neoplastic cells and the potential CSC population [45–47]. At present, conventional anticancer therapies include chemotherapy, radiation, and immunotherapy that kill

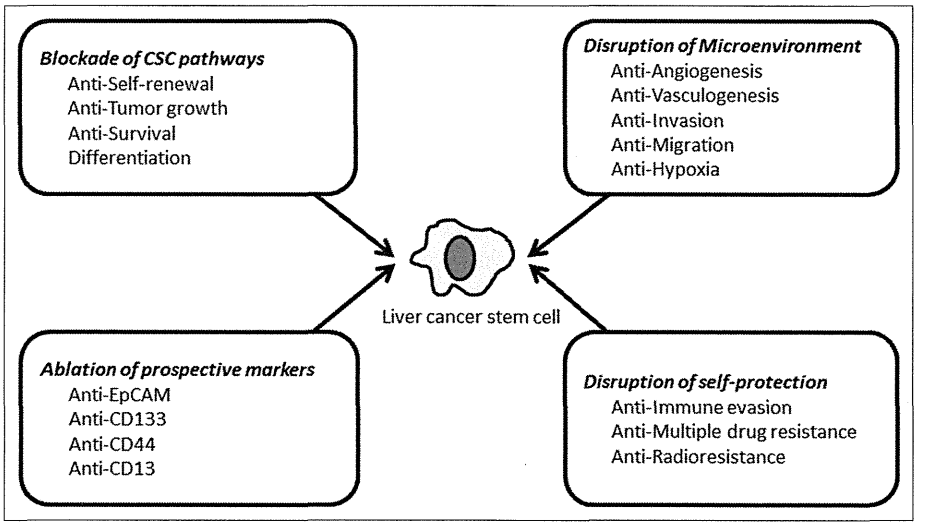

Fig. 2. Strategies to eradicate liver CSCs. CSCs are protected from conventional therapies by changing their microenvironment and self-protection. Specifically targeting any of these areas may lead to the eradication of CSCs.

rapidly growing differentiated tumor cells, thus reducing tumor mass, but potentially leaving behind cancer-initiating cells. Therapies that exclusively address the pool of differentiated cancer cells but fail to eradicate the CSC compartment might ultimately result in relapse and the proliferation of therapy-resistant and more aggressive tumor cells. An ideal drug regime would kill differentiated cancer cells and, at the same time, specifically, selectively, and rapidly target and kill CSCs to avoid toxic side effects in other cell types and to disrupt the self-protection potential of CSCs. CSCs clearly have a complex pathogenesis, with the potential for considerable crosstalk and redundancy in signaling pathways; hence, the targeting of single molecules or pathways may have a limited benefit. Combinations of therapies may be needed to overcome the complex network of signaling pathways, and ultimately inhibit the signaling that controls tumor growth and survival. In addition to the factors possessed by CSCs themselves, their microenvironment is also important for their maintenance, such as angiogenesis, vasculogenesis, and hypoxia. Many new therapeutic strategies targeting CSCs at various stages of differentiation or targeting the microenvironment have been attempted, as will be discussed below (fig. 2).

Liver Stem/Progenitor Cell Markers

The identification of CSC markers and their exploitation in targeted chemotherapy is an important research goal. It has been shown that CSCs in HCC can be identified on the basis of several cell surface antigens (CD133, CD90, CD44, OV6, and EpCAM), or the presence of side population (SP) cells with Hoechst dye-staining. Given the phenotypic similarities between CSCs and normal stem cells, it is reasonable to infer that the surface phenotype of CSCs resembles that of normal hepatic stem cells.

EpCAM as a Target in Cancer Therapy

EpCAM is potentially a promising target as it is highly expressed in most cancer cells as well as on CSCs. In normal tissue, EpCAM is arranged in a complex with CD9, CD44, and Claudin-7, and is localized to basolateral membranes. Thus, the accessibility for EpCAM-binding antibodies is lower in normal cells than for cancer cells. In cancer cells, EpCAM is strongly overexpressed and so it might be partly unbound and more accessible for targeting antibod-

ies. Several chimeric (chimeric Edrecolomab), humanised (3622W94), human-engineered (ING-1), and fully human (Adecatumumab) anti EpCAM antibodies with different target affinities have also been designed. Antibodies with the highest affinities such as 3622W94 and ING-1 induced acute pancreatitis even at low concentrations (1 mg/kg body weight) [48] because of increased binding of EpCAM-specific antibodies to healthy tissue such as pancreas and the respiratory tract. By contrast, the human antibody Adecatumumab (MT201), with an intermediate affinity, has shown only minor side effects such as nausea, chills, fatigue, and diarrhea, even at high doses (2–6 mg/kg body weight) [49]. In a clinical phase 2 trial, randomization between high and low EpCAM expression in metastatic breast cancer revealed that high EpCAM levels are associated with a good prognosis in terms of overall survival after treatment with Adecatumumab. In 2009, the first antibody targeting EpCAM, Catumaxomab (Removab), obtained approval for the European market. This trifunctional antibody has the ability to bind EpCAM-expressing cancer cells as well as cytotoxic T-cells via the CD3 receptor. Clinical trials revealed humoral responses against this antibody after treatment, which might be due to the chimeric structure consisting of mouse IgG2a and rat IgG2b. The type of response against Catumaxomab correlated positively with the clinical outcome, and its use in patients with malignant ascites prolonged their overall survival [50]. Recently, the bispecific antibody MT110 was tested for its ability to target TICs derived from colorectal cancers. This antibody has binding affinities for EpCAM and CD3, which allows it to initiate the formation of a cytolytic synapse between T-cells and TICs. A combination of this antibody and peripheral blood mononuclear cells led to decreased or absent colony formation in soft agar assays. Moreover, treatment with MT110 prevented tumor formation in a xenograft model where mice were inoculated with TICs [51].

Based on the novel understanding of the functions of EpCAM, another interesting approach relies on the interface with the EpCAM signaling cascade. The knowledge of proteases involved in the activating proteolytic cleavage of EpCAM allows for the systematic testing of combinations of protease inhibitors. The inhibition of the EpICD-FHL2 interaction by small molecules generated from structure based rational design and bioinformatics is a promising and highly innovative strategy to specifically target EpCAM and its signaling. In liver cells, RNA interference targeting of EpCAM significantly decreased the CSC pool and reduced both the tumorigenicity and invasive capacity of CSCs. Since EpCAM expression is a downstream target of Wnt/β-catenin, these results may have implications for the development of novel target therapies.

Blockage of CSC Pathways

Anti-Self-Renewal

The targeting of key signaling pathways for CSC self-renewal is another approach to therapy. The Wnt/β-catenin signaling pathway is important for the self-renewal and maintenance of stem cells [52], and several studies have demonstrated decreased proliferation and increased apoptosis following its inhibition [53]. The pathway can be inhibited in a number of ways; for example, Dickkopf1 (Dkk1) binds to the low density lipoprotein receptor-related protein-6 (LRP6) and prevents the formation of the Frizzled-Wnt-LRP6 complex [54]. A new approach to antagonize Wnt signaling has been the development of small molecules (XAV939) to inhibit the enzyme tankyrase that normally destroys the scaffold protein axin, a crucial component of the β-catenin destruction complex [55]. Furthermore, many antibody-based therapeutic approaches targeting EpCAM are currently being developed that will be efficacious in eradicating EpCAM-expressing cancer stem cells.

The Hedgehog pathway is another potential target for CSC eradication. Several small-molecule modulators of Sonic hedgehog signaling have been used to regulate the activity of this pathway in medulloblastoma, basal cell carcinoma, pancreatic cancer, prostate cancer,

and developmental disorders [56]. In liver cells, the suppression of the Sonic Hedgehog pathway by small interfering RNA not only decreased HCC cell proliferation but also chemosensitized the cells to 5-fluorouracil (5-FU) and to the induction of cell apoptosis [57]. Furthermore, in hepatoblastoma, blocking Hh signaling with the antagonist cyclopamine had a strong inhibitory effect on cell proliferation of HB cell lines [58]. Overall, it is likely that the targeting of intracellular pathways associated with self-renewal of CSC will become established in the near future.

Differentiation

CSCs, which make up only a small proportion of cancer cells, have the capacity to sustain tumor growth and are more resistant to conventional chemotherapy than other more differentiated cancer cells. One approach to treat malignancies, therefore, is to induce their differentiation. Differentiation therapy could force hepatoma cells to differentiate and lose their self-renewal property. Hepatocyte nuclear factor-4α, a central regulator of the differentiated hepatocyte phenotype, suppresses tumorigenesis and tumor development by inducing the differentiation of hepatoma cells, especially CSCs [59]. Interferon therapy is effective not only for eradicating hepatitis viruses, but also for preventing the development of HCC regardless of the virological response. Interferon alpha treatment accelerates hepatocytic and biliary differentiation in oval cell lines [60], and could be used to treat HCC by targeting CSCs. In addition, oncostatin M (OSM), an interleukin-6 related cytokine known to induce the differentiation of hepatoblasts into hepatocytes, could be used to effectively induce the differentiation and active cell division of dormant EpCAM-positive liver CSCs. Moreover, a combination of OSM and conventional chemotherapy with 5-FU efficiently eliminates HCC by targeting both CSCs and non-CSCs [61]. These findings indicate that differentiation therapy combined with conventional chemotherapy may be an effective treatment of HCC.

Future Directions

The rapid development of the CSC field combined with genome-wide screening techniques has enabled the identification of important new CSC markers and pathways, which have contributed to one of the most important developments in cancer treatment. However, several important issues remain to be resolved, and little is known about CSC-directed therapies (e.g., targeting EpCAM in EpCAM-positive liver CSCs). Initial results are promising, but knowledge of the potential short- and long-term side effects of these therapies is limited. For example, if not sufficiently specific for CSCs, such therapies could lead to tissue and/or organ damage from the depletion of reserve/regenerative stem cells. This could cause acute and irreversible organ failure.

New drug discoveries for CSCs are currently underway that aim to completely eradicate cancer. Recent studies have highlighted the importance and necessity of exploring the susceptibility of CSCs to existing therapies in combination with the disruption of key pathways controlling self-renewal, pluripotency, chemoresistance, radioresistance, and angiogenesis through molecular targeted therapy.

Other novel and important directions for effective therapies include the disruption of the tumor niche that is essential for CSC homeostasis, and the depletion of CSCs by forced differentiation. However, more work is required to advance our knowledge on the role of CSCs in tumor hierarchy and to design more effective and specific anti-CSC therapy. The current state of knowledge strongly indicates the advantage of targeting CSCs to improve the limited efficiency of existing therapies, and it has provided an important framework for the develop-

ment of novel therapeutic regimens with the ultimate hope of long-term clinical benefits to the patients.

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Gd-EOB-DTPA-Enhanced Magnetic Resonance Imaging and Alpha-Fetoprotein Predict Prognosis of Early-Stage Hepatocellular Carcinoma

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The survival of patients with hepatocellular carcinoma (HCC) is often individually different even after surgery for early-stage tumors. Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced magnetic resonance imaging (MRI) has been introduced recently to evaluate hepatic lesions with regard to vascularity and the activity of the organic anion transporter OATP1B3. Here we report that Gd-EOB-DTPA-enhanced MRI (EOB-MRI) in combination with serum alpha-fetoprotein (AFP) status reflects the stem/maturational status of HCC with distinct biology and prognostic information. Gd-EOB-DTPA uptake in the hepatobiliary phase was observed in ~15% of HCCs. This uptake correlated with low serum AFP levels, maintenance of hepatocyte function with the upregulation of *OATP1B3* and *HNF4A* expression, and good prognosis. By contrast, HCC showing reduced Gd-EOB-DTPA uptake with high serum AFP levels was associated with poor prognosis and the activation of the oncogene *FOXM1*. Knockdown of *HNF4A* in HCC cells showing Gd-EOB-DTPA uptake resulted in the increased expression of *AFP* and *FOXM1* and the loss of *OATP1B3* expression accompanied by morphological changes, enhanced tumorigenesis, and loss of Gd-EOB-DTPA uptake *in vivo*. HCC classification based on EOB-MRI and serum AFP levels predicted overall survival in a single-institution cohort (n = 70), and its prognostic utility was validated independently in a multi-institution cohort of early-stage HCCs (n = 109). *Conclusion*: This noninvasive classification system is molecularly based on the stem/maturation status of HCCs and can be incorporated into current staging practices to improve management algorithms, especially in the early stage of disease. (HEPATOLOGY 2014;60:1674-1685)

Liver cancer is the fifth most commonly diagnosed cancer and the second most frequent cause of cancer death in men worldwide.[1] Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70-86% of cases of primary liver cancer.[1] Several staging systems are currently available for HCC classification and include Tumor Node

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; EOB-MRI, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced magnetic resonance imaging; FOXM1, forkhead box protein M1; Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; HCC, hepatocellular carcinoma; HNF4α, hepatocyte nuclear factor 4 alpha; IHC, immunohistochemistry; MRI, magnetic resonance imaging; NOD/SCID, nonobese diabetic, severe combined immunodeficient; OATPs, organic anion transporting polypeptides; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SI, signal intensity; TNM, tumor node metastasis.

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Metastasis (TNM) and Barcelona Clinic Liver Cancer (BCLC) staging, which are based on tumor number and size, vascular invasion, metastatic status, hepatic reserve, and performance status.[2] These systems can provide an approximate estimate of patients' survival, but patients diagnosed at the same disease stage sometimes show a different prognosis. This is most likely because these systems do not include an assessment of the malignant phenotype of the tumor, which would be especially important in those patients diagnosed at the early stage of disease. To overcome these limitations, gene expression profiling technologies have been applied to classify HCC. In particular, the stemness of HCC is currently of great interest because its gene expression profile reflects the malignant nature of the tumor.[3-7] However, the application of these new technologies still needs to be validated externally prior to their implementation in clinical practice.

The hallmark of HCC diagnosis has been image analysis based on vascularity. Gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is a liver-specific magnetic resonance imaging (MRI) contrast agent introduced specifically to improve the detection of liver lesions.[8] Gd-EOB-DTPA-enhanced MRI (EOB-MRI) has been used to evaluate liver tumors in Europe since 2004, in the USA and Japan since 2008, and in China since 2010. Gd-EOB-DTPA is characterized by its rapid and specific uptake by hepatocytes by way of organic anion transporting polypeptides (OATPs) expressed in the sinusoidal membrane. Therefore, Gd-EOB-DTPA uptake in the liver is considered to reflect hepatocyte function.[9] Among OATP1A2, 1B1, 1B3, and 2B1, only OATP1B3 expression was found to correlate with the enhancement ratio on EOB-MRI, indicating that it transports Gd-EOB-DTPA into HCC cells.[10] It is generally accepted that ~85% of HCCs show hypointensity in the hepatobiliary phase of EOB-MRI compared to the noncancerous background liver, with a reduction of OATP1B3 protein or *OATP1B3* gene expression in the tumor.[10,11] However, atypical Gd-EOB-DTPA uptake in the hepatobiliary phase is observed in the remaining 15% of HCCs, and the molecular phenotype and clinical features of these HCCs remain to be elucidated.

We hypothesized that EOB-MRI findings may vary in different tumor subtypes with distinct biology. Therefore, in this study we evaluated the molecular profiles of HCCs in a single-institute cohort determined from the EOB-MRI findings using quantitative reverse-transcription polymerase chain reaction (qRT-PCR), microarray, and immunohistochemistry (IHC) analyses. To clarify the clinical utility of the EOB-MRI findings, we also evaluated the prognosis of a multicenter cohort of patients with early-stage HCC who underwent radical resection.

Materials and Methods

Patients. A total of 417 patients who received surgical resection for HCC were enrolled in this study. Seventy patients underwent EOB-MRI for the diagnosis of HCC and received surgical resection at Kanazawa University Hospital from 2008 to 2011. Survival analysis was performed in this single-institute cohort (Cohort 1) and prognosis was evaluated every 6 months. The final evaluation of survival was performed in October 2011. From these 70 patients, 62 tumor and nontumor samples were snap-frozen in liquid nitrogen and used for qRT-PCR.

For microarray analysis, we assessed 238 patients who received surgical resection of HCC at the Liver Cancer Institute of Fudan University. EOB-MRI was not performed in these patients because Gd-EOB-DTPA had not yet been introduced in China. Their clinicopathologic characteristics and prognostic data have been described previously.[12]

To evaluate the survival of early-stage HCCs, we enrolled 109 patients who received EOB-MRI and surgical resection at Tokyo Medical and Dental University Hospital, Tokyo Women's Medical University Hospital, Nihon University School of Medicine Itabashi Hospital, Niigata University Medical & Dental Hospital, Hyogo College of Medicine Hospital, or Kurume

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University Hospital from 2008 to 2009 (Cohort 2). The prognosis of these patients was evaluated every year, and the final evaluation of survival was performed in February 2012.

This study was approved by the Institutional Review Board at each study center and all patients provided written informed consent.

EOB-MRI. EOB-MRI was performed before surgical resection using a 1.5 or 3.0 Tesla MRI system with a fat-suppressed 2D or 3D gradient echo T1-weighted sequence (relaxation time / echo time [TR/TE] = 3.2-3.6/1.6-2.3 ms, flip angle 10-15°, field of view 33-42 cm, matrix 128-192 × 256-512, slice thickness 4.0-8.0 mm). A dose of 0.025 mmol/kg Gd-EOB-DTPA (Primovist; Bayer Schering Pharma, Berlin, Germany) was injected intravenously and the hepatobiliary phase was obtained at 15-20 minutes after the injection.

All abdominal MRI data of the HCC patients were generated at Kanazawa University Hospital and image analysis was performed retrospectively by two radiologists (A.K. and O.M.) without knowledge of the clinical and pathological results. The signal intensity (SI) of the tumor was measured within the region of interest, which was determined as the maximum oval area at the largest section of the tumor. The SI of the adjacent background liver was also measured within a region of interest of the same size, while avoiding large vessels. The nodules were classified into the two following types: hypointense HCC, which was defined as showing a lower SI than that of the surrounding liver (tumor SI / background SI <1.0) in the hepatobiliary phase, and hyperintense HCC, which was defined as showing an equal or higher SI (tumor SI / background SI ≥ 1.0).

For the mouse study, EOB-MRI was performed using a 0.4 T MRI system with a fat-suppressed 3D gradient echo T1-weighted sequence (TR/TE = 66.5/4.0 ms, flip angle 40°, field of view 10 cm, matrix 224 × 192, slice thickness 1.0 mm). A dose of 0.025 mmol/kg Gd-EOB-DTPA (Bayer Schering Pharma) was injected through the tail vein, and the hepatobiliary phase was obtained at 12-20 minutes after the injection.

Xenotransplantation of Primary HCC in Immunodeficient Mice and* HNF4A *Knockdown. Primary HCC tissue was dissected and digested in 1 mg/mL type 4 collagenase solution (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C for 15-30 minutes. Contaminated red blood cells were lysed with an ammonium chloride solution (STEMCELL Technologies, Vancouver, BC, Canada) on ice for 5 minutes. $CD45^+$ leukocytes and annexin V^+ apoptotic cells were removed by an autoMACS-pro cell separator and magnetic beads (Miltenyi Biotec, Tokyo, Japan). The cells were suspended 1:1 in 200 μL Dulbecco's modified Eagle's medium (DMEM) and Matrigel (BD Biosciences) and injected subcutaneously into 6-week-old NOD/SCID mice (NOD/NCrCRl-*Prkdc*^{scid}) purchased from Charles River Laboratories (Wilmington, MA). EOB-MRI was performed to evaluate Gd-EOB-DTPA uptake in the subcutaneous tumor at the hepatobiliary phase, and the subcutaneous tumor was dissected and digested as described above, and subsequently cultured in DMEM. *HNF4A* knockdown was performed using pGFP-V-RS vectors (OriGene Technologies, Rockville, MD), allowing stable delivery of the short hairpin RNA (shRNA) expression cassette against *HNF4A* or scramble sequence into host cells by way of a replication-deficient retrovirus. Infected HCC cells were grown in DMEM containing 1 μg/mL puromycin (Sigma-Aldrich Japan) for 7 days to establish stable shRNA-expressing HCC cells. Western blotting and immunofluorescence analyses were performed using an antihuman HNF4α C11F12 antibody (Cell Signaling Technology, Danvers, MA) and a mouse monoclonal antihuman OATP1B3 MDQ/5F260 antibody (Novus Biologicals, Littleton, CO), essentially as described previously.[13] Control or Sh-HNF4A-transfected HCC cells were injected subcutaneously into NOD/SCID mice, and tumor volume and survival were evaluated every 2-3 days. The protocol was approved by the Kanazawa University Animal Care and Use Committee and the Kanazawa University Genetic Modification Experiment Committee.

Microarray Analysis. The 238 HCC cases from the Liver Cancer Institute of Fudan University with available microarray data and clinicopathologic and prognostic data have been described previously.[12] BRB-ArrayTools software (v. 3.8.1) was used for class comparison analysis. Hierarchical clustering analysis was performed with Genesis software (v. 1.6.0 beta). Canonical pathway and transcription factor analyses were performed using MetaCore software (http://www.genego.com). Interaction network analysis was performed using Ingenuity Pathway Analysis software (http://www.ingenuity.com).

qRT-PCR Analysis. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The expression of selected genes was determined in triplicate using the Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and the $-\Delta\Delta CT$ method. The following probes were used: *AFP*, Hs00173490_m1; *FOXM1*, Hs01073586_m1; *OATP1B3*, Hs00251986_m1; *CYP3A4*, Hs00430021_m1; and *18S*, Hs99999901_s1 (Applied Biosystems).

IHC Analysis. IHC was performed using Envision+ kits (Dako Japan, Tokyo, Japan) as described previously.[14] Mouse monoclonal antihuman Ki-67 antigen MIB-1 (Dako Japan), mouse monoclonal antihuman OATP1B3 MDQ/5F260 (Novus Biologicals), rabbit monoclonal antihuman HNF4α C11F12 (Cell Signaling Technology), mouse monoclonal antihuman FOXM1 0.T.181 (Abcam, Cambridge, MA), mouse monoclonal antihuman glypican-3 1G12 (BioMosaics, Burlington, VT), and mouse monoclonal antiglutamine synthetase clone GS-6 (Millipore, Billerica, MA) antibodies were used. The staining area and intensities were evaluated in each sample and graded from 0-3 (0, 0-5%; 1, 5-25%; 2, 25-50%; 3, >50%) and 0-2 (0, negative; 1, weak; 2, strong), respectively. The sum of the area and intensity scores of each marker (IHC score) were calculated. Samples were defined as marker-high (IHC score ≥3) or -low (IHC score ≤2). The Ki-67 labeling index was calculated as described previously.[14]

Statistical Analysis. Mann-Whitney, χ^2, Fisher's exact, and Kruskal-Wallis tests were used to compare the clinicopathologic characteristics and gene expression data. The correlation of the gene expression data was evaluated by Spearman's rank correlation coefficient. Kaplan-Meier survival analysis with the log-rank test was performed to compare patient survival. All analyses were performed using GraphPad Prism software v. 5.0.1 (GraphPad Software, San Diego, CA).

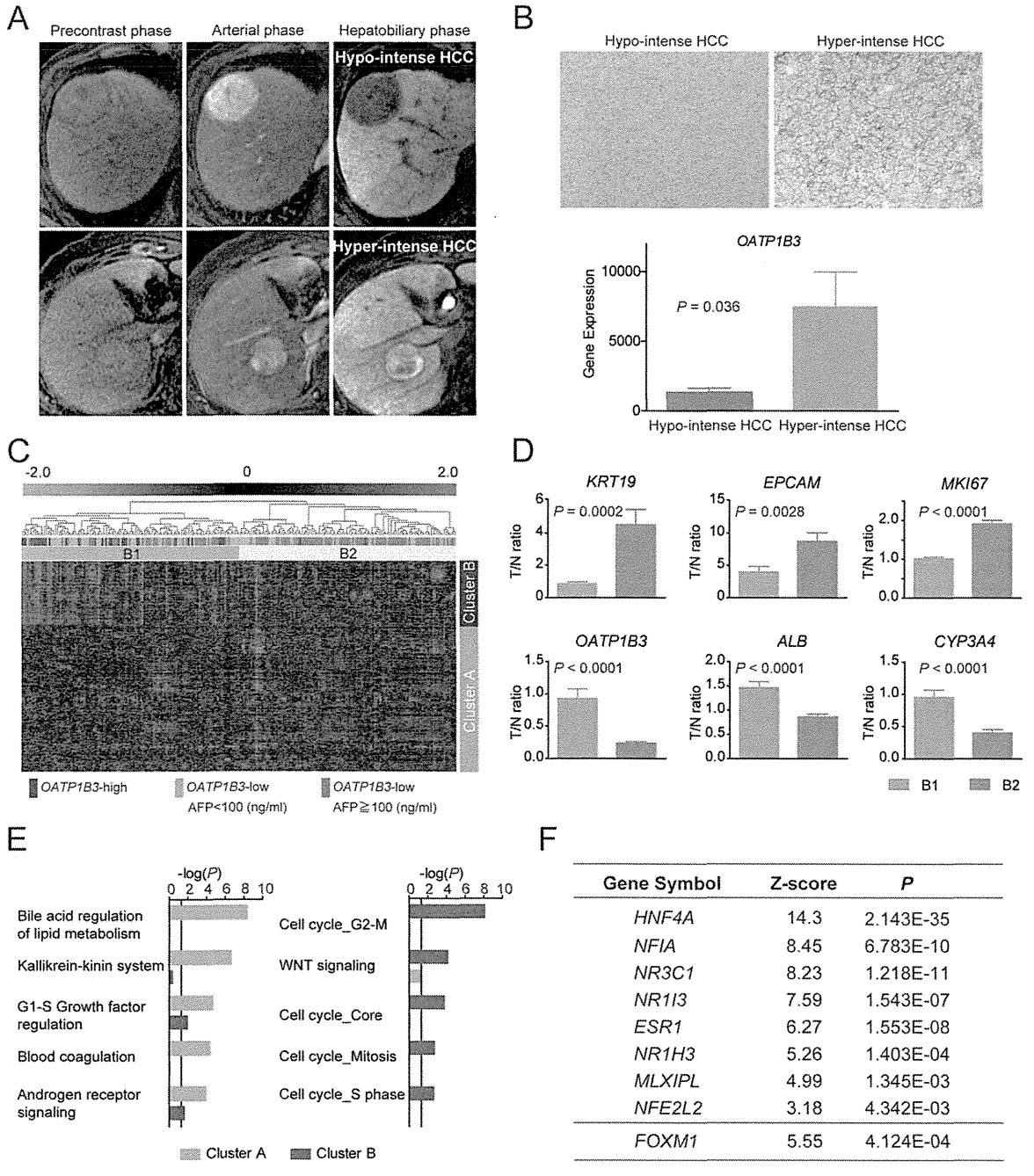
Results

EOB-MRI Findings and Molecular Characteristics of HCC. Nine of the 70 HCC cases (12.9%) in Cohort 1 were diagnosed with hyperintense HCC on EOB-MRI (Fig. 1A). Analysis of the clinicopathologic characteristics of hyper- or hypointense HCCs revealed that hyperintense HCCs were significantly associated with low serum alpha-fetoprotein (AFP) levels (Table 1). There was no significant difference between hyper- and hypointense HCCs in terms of other factors, including tumor size, number, TNM and BCLC stages, surgical procedures, and elapsed time between MRI and surgery. We confirmed the overexpression of OATP1B3, a transporter responsible for the uptake of Gd-EOB-DTPA in hepatocytes, in hyperintense HCCs by qRT-PCR and IHC (Fig. 1B).

To understand the transcriptomic characteristics of HCCs overexpressing *OATP1B3*, we analyzed the microarray data of an additional 238 HCC cases.[12] *OATP1B3*-high and -low HCCs were defined as HCCs with a T/N ratio ≥1.0 and <1.0, respectively, as used for the evaluation of hyperintense HCCs (tumor SI / background SI ≥1.0). The frequency of *OATP1B3*-high HCCs was 15.1% (36 of the 238 HCC cases), almost comparable to the frequency of hyperintense HCCs reported thus far. Class-comparison analysis yielded a total of 974 genes that were differentially expressed between *OATP1B3*-high and -low HCCs ($P < 0.001$). Hierarchical cluster analysis of this 974 gene set (*OATP1B3* gene signature) separated HCCs into two branches (B1 and B2) (Fig. 1C). Thirty-four of the 36 *OATP1B3*-high HCCs (blue box) were classified in the left branch (B1), while *OATP1B3*-low HCCs were clustered in both branches. The prognosis of HCC patients clustered in B1 was significantly better than those clustered in B2 ($P = 0.02$) (Supporting Fig. S1). Genes associated with mature hepatocyte function such as *ALB* and *CYP3A4* were significantly up-regulated in the HCCs clustered in B1, and the known hepatic stem/progenitor markers *KRT19* and *EPCAM*, as well as the G1/S cell cycle marker *MKI67*, were significantly up-regulated in the HCCs clustered in B2 (Fig. 1D).

Pathway analysis indicated that *OATP1B3*-high HCCs showed maintenance of mature hepatocyte function and decreased cell proliferation and Wnt signaling (Fig. 1E), which are known to be activated during liver development and regeneration.[15] Transcription factor analysis identified eight genes (*HNF4A, NFIA, NR3C1, NR1I3, ESR1, NR1H3, MLXIPL*, and *NFE2L2*) as candidate transcription factors that were significantly activated in *OATP1B3*-high HCCs ($P < 0.005$) (Fig. 1F). These transcription factors are known to play a pivotal role in liver development and in the regulation of hepatocyte functions including lipid, bile, carbohydrate, and xenobiotic metabolism.[16] By contrast, only one gene (*FOXM1*) was identified as a candidate transcription factor activated in *OATP1B3*-low HCCs. The forkhead box M1 (FOXM1) transcription factor is known to be activated during liver regeneration and regulation of the cell cycle.[17] We investigated the expression of the two transcription factors most strongly activated (*HNF4A* encoding hepatocyte nuclear factor 4 alpha [HNF4α]) or inactivated (*FOXM1*) in hyperintense HCCs (Fig. S2) and validated the results using microarray analyses (Fig. 2A,B).

Although the microarray data revealed distinct molecular portraits associated with liver development and the maturation programs present in hyper- and hypointense HCCs, hierarchical cluster analysis further indicated that a subset of hypointense HCCs (corresponding to the *OATP1B3*-low HCCs clustered in B1)



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Gene Symbol	Z-score	P
HNF4A	14.3	2.143E-35
NFIA	8.45	6.783E-10
NR3C1	8.23	1.218E-11
NR1I3	7.59	1.543E-07
ESR1	6.27	1.553E-08
NR1H3	5.26	1.403E-04
MLXIPL	4.99	1.345E-03
NFE2L2	3.18	4.342E-03
FOXM1	5.55	4.124E-04

Fig. 1. Molecular profiles of HCCs corresponding to the EOB-MRI findings. (A) Representative MRI scans of hypo- and hyperintense HCCs in the precontrast, arterial, and hepatobiliary phases. The T/N signal intensity ratios of the images in the hepatobiliary phase were 0.47 (upper panel) and 1.07 (lower panel). (B) Upper panel: Representative photomicrographs of IHC staining with an anti-OATP1B3 antibody in hypo- and hyperintense HCCs. Lower panel: *OATP1B3* expression in hypo- and hyperintense HCCs. (C) The expression patterns of *OATP1B3* signatures in *OATP1B3*-high (blue box), *OATP1B3*-low AFP-low (<100 ng/mL) (orange box), and *OATP1B3*-low AFP-high (≥100 ng/mL; red box) after hierarchical clustering of genes and samples, shown as a heat map image. Red indicates a high expression level; green indicates a low expression level. *OATP1B3*-high HCCs and *OATP1B3*-low AFP-high HCCs were clustered in B1 (green bar) and B2 (yellow bar), respectively. (D) Representative expression of genes in clusters A (*KRT19, EPCAM*, and *MKI67*) and B (*OATP1B3, ALB*, and *CYP3A4*). The green and orange bars indicate HCCs clustered in B1 and B2, respectively. (E) The activated pathways are identified in clusters A (orange bar) and B (blue bar). (F) Genes encoding transcription factors activated or inactivated in *OATP1B3*-high HCCs.

might show similar gene expression profiles to those observed in hyperintense HCCs. Since serum AFP levels are reportedly related to the stem/maturation subtypes of HCCs with different gene expression profiles,[12] we analyzed the characteristics of *OATP1B3*-low HCCs in 238 cases according to serum AFP levels. Interestingly, *OATP1B3*-low HCCs assigned to the left branch (B1) had low serum AFP

Table 1. Characteristics of HCCs Classified by EOB-MRI in Cohorts 1 and 2

Characteristics	Cohort 1			Cohort 2		
	Hyperintense (n = 9)	Hypointense (n = 61)	P*	Hyperintense (n = 9)	Hypointense (n = 100)	P*
Age (years, mean ± SE)	66.2 ± 3.6	64.6 ± 1.2	0.21	67.2 ± 2.0	66.2 ± 1.0	1.0
Sex (male/female)	7/2	44/17	0.72	9/0	79/21	0.13
Etiology (HBV/HCV/other)	2/3/4	14/23/24	0.95	1/6/0/2	22/56/2/20	0.52
Liver cirrhosis (yes/no)	5/4	33/28	0.94	2/7	42/58	0.25
AFP (ng/mL, mean ± SE)	12.4 ± 1.9	2,157 ± 866	0.03	7.0 ± 2.2	188.4 ± 74	0.03
Histologic grade†						
I-II	1	12		2	16	
II-III	8	38		7	74	
III-IV	0	11	0.25	0	10	0.57
Tumor size (cm, mean ± SE)	4.0 ± 0.9	4.4 ± 0.4	0.79	3.3 ± 0.4	2.6 ± 0.1	0.09
Tumor number (single/multiple)	7/2	48/13	0.95	8/1	86/14	0.81
Macroscopic portal vein invasion (yes/no)	1/8	5/56	0.58	0/9	0/100	
Microscopic portal vein invasion (yes/no)	2/7	27/34	0.21	0/9	11/89	0.59
Tumor-node-metastasis classification (I/II/III)	6/2/1	29/28/4	0.40	7/2/0	75/25/0	0.85
BCLC stage (0/A/B/C)	0/7/1/1	4/30/22/5	0.34	0/9/0/0	27/73/0/0	0.07
Elapsed time between MRI and surgery (days, mean ± SE)	47.0 ± 8.4	51.5 ± 3.2	0.73	17.3 ± 5.0	20.6 ± 3.0	0.50
Surgical procedure (partial resection or segmentectomy/ lobectomy or extended lobectomy)	6/3	35/26	0.60	8/1	86/14	1.0

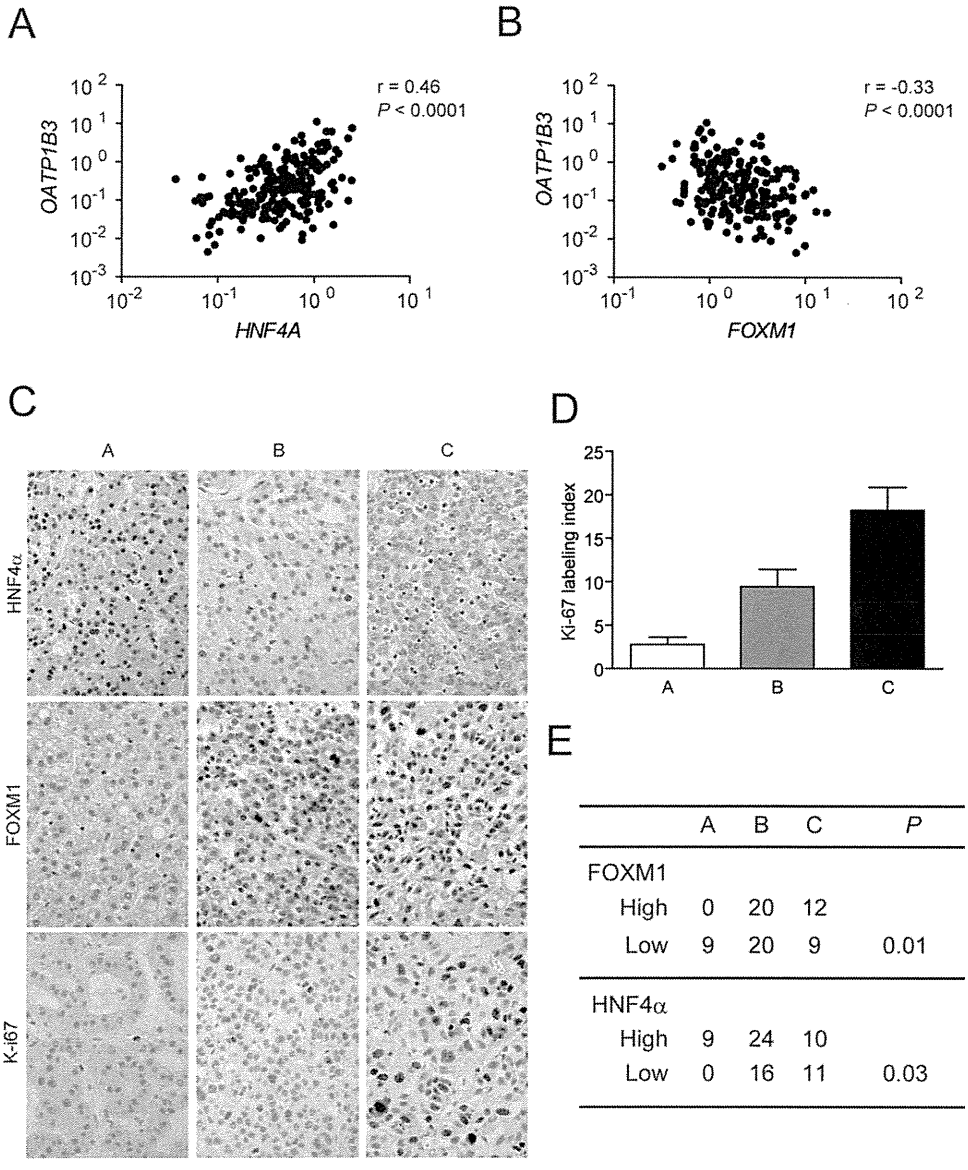
*Mann-Whitney test, Fisher's exact test, or χ^2 test.
†Edmondson-Steiner.

levels (<100 ng/mL: orange box, Fig. 1C), while the majority of AFP-high (≥100 ng/mL) HCCs (red box, Fig. 1C) were clustered in the right branch (B2). Consistently, the *OATP1B3* gene signature significantly predicted the serum AFP status of 238 HCCs ($P < 0.05$) (Tables S1-3).

OATP1B3 and AFP Expression in HCC Subtypes Related to Stem/Maturational Status. Molecular profiling of tissue samples may be useful for predicting the survival of HCC patients, as reported previously.[18,19] However, such an approach should be established before being applied routinely in a clinical setting. The above data prompted us to hypothesize that EOB-MRI findings and serum AFP levels, in place of molecular profiling techniques, have the potential to categorize HCCs (EOB-AFP classification), thus serving as predictors of survival. We categorized HCCs into three groups (class A: hyperintense HCC, class B: hypointense and AFP-low [<100 ng/mL] HCC, and class C: hypointense and AFP-high [≥100 ng/mL] HCC). The clinicopathologic characteristics of patients with class A, B, and C HCCs in Cohort 1 are shown in Table S4.

We investigated the expression of HNF4α and FOXM1 as well as the G1/S marker Ki-67 by IHC according to the EOB-AFP classification system in Cohort 1 (Fig. 2C). HNF4α was most abundantly expressed in class A HCCs, but its expression was decreased in class B and C HCCs. By contrast, the expression of FOXM1 and Ki-67 was highest in class C HCCs, significantly decreased in class B HCCs, and not detected in class A HCCs. The mean Ki-67 labeling indices in class A, B, and C HCCs were 2.8%, 9.4%, and 18.2%, respectively ($P < 0.0001$) (Fig. 2D). The differences in FOXM1 and HNF4α expression among class A, B, and C HCCs were statistically significant (Fig. 2E).

We further investigated the expression of five markers (glypican 3, GPC-3; lymphatic vessel endothelial hyaluronan receptor 1, LYVE-1; survivin; heat shock 70 kDa protein, HSP70; and glutamine synthetase, GS), known to be differentially expressed between dysplastic nodule and well-differentiated HCC,[20,21] to clarify if the molecular alterations in early-stage hepatocarcinogenesis can be detected differentially in EOB-AFP class A, B, and C HCCs. IHC analysis suggested no differential expression of LYVE-1, survivin, and HSP70 among the EOB-AFP classes (data not shown). Interestingly, GS was most abundantly expressed in class A HCCs, and its expression was relatively decreased in class B and C HCCs with borderline significance ($P = 0.06$) (Fig. S3A,B). In contrast, GPC-3 expression was highest in class C HCCs and relatively decreased in class A and B HCCs with statistical significance ($P = 0.03$). We investigated the microarray data of 238 independent HCC cases and validated the positive correlation between *OATP1B3* and *GLUL* (encoding GS) and the weak negative correlation between *OATP1B3* and *GPC3* (encoding GPC-3).



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	A	B	C	P
FOXM1				
High	0	20	12	
Low	9	20	9	0.01
HNF4α				
High	9	24	10	
Low	0	16	11	0.03

Fig. 2. Transcriptional programs of HCCs corresponding to the EOB-MRI findings and serum AFP. (A,B) Scatterplot analyses of the microarray data of 238 HCCs. (C) Representative photomicrographs of IHC staining with anti-HNF4α, anti-FOXM1, and anti-Ki-67 antibodies in class A, B, and C HCCs, according to the EOB-AFP classification. (D) Ki-67 labeling index in class A, B, and C HCCs. (E) Summary of FOXM1 and HNF4α expression in class A, B, and C HCCs.

Regulation of Gd-EOB-DTPA Uptake and Tumorigenic Capacity by HNF4α in Hyperintense HCC. Microarray and IHC analyses suggested the activation of transcription factor HNF4α in hyperintense HCC, but its role in the maintenance of hepatocyte function and Gd-EOB-DTPA uptake has not yet been clarified. To directly explore the role of HNF4α in Gd-EOB-DTPA uptake and tumorigenic capacities, we transplanted tumor cells from hyper- and hypointense primary HCC specimens into NOD/SCID mice (Fig. 3A). We confirmed on EOB-MRI that Gd-EOB-DTPA uptake capacity was relatively maintained in the secondary xenotransplanted tumors that developed in the subcutaneous lesions of the mice (Fig. 3B).

Using a retrovirus system *in vitro*, we then introduced shRNA targeting *HNF4A* (Sh-HNF4A) or scramble (Sh-Scr) into tumor cells obtained from a hyperintense HCC. We confirmed the reduction of HNF4α protein expression in Sh-HNF4A-transfected cells compared with Sh-Scr-transfected cells by western blotting (Fig. 3C, left panel). Interestingly, *HNF4A* knockdown resulted in a modest increase in *AFP* and *FOXM1* expression and a dramatic decrease in *CYP3A4* and *OATP1B3* expression (Fig. 3C, right panel). It also resulted in the loss of OATP1B3 protein expression, and striking morphological changes were confirmed by immunofluorescence and phase-contrast microscopy (Fig. 3D). Sh-HNF4A-transfected cells displayed long, thin cell shapes with neurite-like extensions, whereas Sh-Scr-transfected cells were relatively smooth and round. Sh-Scr- or Sh-HNF4A-transfected cells were further injected subcutaneously into NOD/SCID mice, and aggressive tumor growth accompanied with the loss of Gd-EOB-DTPA uptake capacity was