EUR. J. ONCOL.; Vol. 19, n. 3, pp. 130-143, 2014

Targeting cancer stem cells in hepatocellular cancer: a review

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Summary. In connection with the self renewal property of stem cells, surprising similarities can be found in cancer cells. Tumors are composed of rapidly proliferating cells, postmitotic and differentiated cells. Since, however, the latter two types cannot self renew, the idea has arisen that tumors contain self renewing stem cells termed "Cancer Stem Cells" (CSCs) occupying the top of the cancer cell hierarchy. CSCs may have molecular signaling pathways responsible for self renewal as in stem cells. CSCs are often considered resistant to chemotherapy and targeting such cells and eradicating them may improve the prognosis of cancer patients. Hepatocellular carcinoma (HCC) is the sixth most diagnosed cancer, representing the second leading cause of world mortality and thought to be driven by CSCs. There are several antigenic markers and molecular signaling pathways such as Hedgehog, TGF-β, Wnt & Notch which modulate CSCs and account for tumorigenecity and hence progression of HCC. Additional factors such as micro-RNA may also contribute to hepatocarcinogenesis by regulating stemness of CSCs. Thus, understanding and characterizing the initiating CSCs and identifying the molecular targets will be helpful in preventing and treating HCC. Such molecular pathways and targets are discussed in this review.

Key words: cancer, stem cells, hepatocellular cancer, micro-RNA, molecular marker

«Colpire le cellule staminali nel cancro epatocellulare: una recensione»

Riassunto. Rispetto alle proprietà di auto-rinnovamento delle cellule staminali è sorprendente osservare le numerose somiglianze delle stesse con le cellule tumorali. I tumori sono composti da cellule in rapida proliferazione, cellule in fase post-mitotica e cellule differenziate. Poiché però gli ultimi due tipi cellulari non hanno capacità di auto-rinnovamento, l'ipotesi che si è sviluppata è che i tumori contengano cellule staminali totipotenti chiamate " Cancer Stem Cells" (CSCs) che occupano l'apice gerarchico delle diverse cellule tumorali. Si ipotizza che le CSCs abbiano una via di segnalazione molecolare responsabile del loro auto-rinnovamento analoga a quella delle cellule staminali. Le CSCs vengono spesso considerate cellule target resistenti alla chemioterapia e la loro eradicazione può migliorare la prognosi del paziente tumorale. Il Carcinoma Epatocellulare (HCC) è il sesto tumore più diagnosticato e rappresenta la seconda principale causa di mortalità nel mondo; si ipotizza che la sua origine sia associata alle CSCs . Diversi markers antigenici e vie di segnalazioni molecolari, come Hedgehog, TGF-β, Wnt & Notch, modulano le CSCs e sono alla base della cancerogenicità e della progressione dell'HCC. Altri fattori come i micro-RNA possono inoltre contribuire alla epatocarcinogenesi regolando il potere staminale delle CSCs. Lo studio e la caratterizzazione dell'innesco delle CSCs e l'identificazione dei possibili bersagli molecolari, potrebbero essere utili e fondamentali nella prevenzione e nel trattamento del HCC. Tali aspetti vengono discussi in questa review.

Parole chiave: cancro, cellule staminali, carcinoma epatocellulare, micro-RNA, marker molecolari

Introduction

Cancer stem cells (CSCs) are a distinct subpopulation of tumor cells that exhibit features like self renewal and differentiation found in normal stem cells. CSCs are also capable of driving metastasis, and thus giving rise to various types of cancer. CSCs were originally characterized in hematological malignancies and then identified in various solid tumors such as liver, breast, prostate, brain and colon (1, 2). These are identified on the basis of specific markers - e.g CD 133, CD 44, CD 13 CD 24 etc. - and are said to cause extensive tumorigenicity when transplanted *in vivo* (in NOD/SCID mice) which is often followed by establishing tumor growth *in vitro* in the 'sphere forming assay' which measures the frequency of their clonogenicity (3-5).

CSCs are distinct populations of tumor cells characterized by self renewal and multilineage differentiation potential. They possess the ability to perpetuate tumors when transplanted into immune-deficient mice, thus containing metastasizing potential.

Cancer stem cells in solid tumors are characterized by a distinctive cell surface marker (Table 1); the identification of CSCs via combinations of different antigenic markers has shown that there can be multiple phenotypic variations of cancer stem cells within a given tumor (6).

Such CSCs were originally identified in hematological malignancies and then discovered in a variety of solid tumors such as liver, breast, prostate, brain, colon and pancreatic tumors. Recent studies have revealed tumors comprised of distinct sublclones that contribute to intratumoral heterogeneity, which is to say, some cells are capable of long term self-renewal and have a higher tumorigenic potential than other cells (7, 8).

CSCs may either originate from normal stem cells or from progenitor cells in response to environmental cues or genetic modification. The interaction between CSCs and stromal cells, which are bone marrow residing non-hematopoietic stem cells and contribute to the stem cell niche, is known to play a major role in tu-

Table 1.Types of cancer stem cell markers in different types of cancers and animal models.

Specific Marker	Type of cancer	Animal	References
CD133	Glioblastoma	NOD/SCID	(13)
	Laryngeal Cancer	NOD/SCID	(14)
	Colorectal Cancer	NMRI (nu/nu) mice	(15)
	Ovarian Cancer	NOD/SCID	(16)
	Colon Cancer	NOD/SCID	(17)
	Pancreatic Cancer	NOD/SCID	(18)
	Gastric Cancer	NOD/SCID	(19)
	Non-Small-Cell Lung Cancer	NOD/SCID	(20)
	Melanoma	athymic NCr-nu/nu mice	(21)
CD44+	Head and Neck Squamous Cell Carcinoma	Nude mice	(22)
CD44+/CD24-	Breast Cancer	NOD/SCID	(23)
CD44+CD133+	Gallbladder Carcinoma	NOD/SCID	(24)
	Adenocarcinoma	Nude	(25)
	Colon cancer	NOD/SCID	(26)
$EpCAM)^{high}/CD44^{+}$	Colorectal cancer	NOD/SCID	(27)
$EpCAM^{+}/CD44^{+}$	Gastric cancer	NOD/SCID	(28)
CD44 + CD90+	Breast cancer	NOD.CB17-Prkdc(scid)	(29)

mor progression (3, 9-11). There is accumulating evidence that CSCs in sqamous cell carcinoma, colorectal cancer, bladder cancer, leukemic cancer and brain cancer require support from the microenviroment for establishing tumorigenicity (12). Cancer stem cells share many factors found in normal stem cells such as Wnt, Notch, Hedgehog & Transforming growth factor-beta, etc. The eradication of cancer stem cells responsible for cancer could aid in successful cancer therapy. Targeting signaling pathways and their constituent molecules could offer an effective approach to treating various cancers such as hepatocellular carcinoma (12). Table 1 lists some cancer stem cell markers expressed in various cancers.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most prevalent cancer and the second leading cause of cancer-related death world-wide (30) while susceptibility to it increases sharply at the cirrhotic stage of liver injury (31). The major factors which contribute to this disease development are viral hepatitis B (HBV) and/or C (HCV), aflatoxin and alcoholism and factors that vary greatly with respect to geographical location. Other factors which may contribute to disease prevalence are obesity and diabetes. HBV is the most prevalent cause in China and Africa and HCV infection accounts for the majority of liver cancer in Japan and western countries (32).

Curative treatments for HCC at an early stage include surgical resection, radio-frequency ablation and liver transplantation, but these are of limited use in patients with advanced disease stage. Hepatocellular carcinoma seems resistant to systemic chemotherapy except for a multikinase inhibitor, sorafenib, which seems to prolong the survival rate in patients with advanced hepatocellular carcinoma. Furthermore, HCC is largely incurable due to the highly resistant nature of cancer stem cells within the tumor and its high recurrence rate (31, 33, 34). However advances in biomarker approaches and molecular targeted therapy might significantly improve treatment of liver cancer.

Markers for identification

The exact marker identifying cancer stem cells has not yet been identified although many putative markers are known whose characterization and identification could aid in the prospective isolation of CSCs in HCC as well as in understanding their role in metastasis. Numerous reports have focused on identification of several putative stem cell markers. The first evidence of a cancer stem cell population in the liver was identified as side population (SP) cells in Huh-7 and Hep-3B cell lines (35). These SP cells display features of cancer stem cells such as tumorigenic potential, clonogenicity and significant resistance to chemotherapy (36, 37).

Oncofetal markers for identification of HCC

NOPE

It has been shown that alteration in glycosylation of serum glycoproteins is linked to development of HCC, so it might be used for diagnosis of early stages of HCC even when used independently, while increased prognostic value is achievable when it is used in combination with other serum oncofetal markers such as alpha-fetoprotein, and Golgi protein-73 GP73 for detecting HCC (38). Recently NOPE (Neighbor of Punc E 11) has been characterized as an oncofetal marker of murine and human liver cancer. NOPE is a transmembrane protein of the immunoglobulin superfamily and exhibits close sequence similarity with punc and the axonal guidance receptors deleted in colorectal cancer (DCC) and Neogenin. Its expression is evident during embryonic development in skeletal muscle cell and in the ventricular region of the nervous system. It is expressed in the hippocampus of the adult brain (39). NOPE is significantly upregulated in the oncogenic murine and human hepatoma cell line of HCC but is barely detectable in normal liver or at a pre-neoplastic stage of hepatocellular carcinogenesis. Furthermore, NOPE expression progressively increases with later and more advanced HCC stages, suggesting its potential role as a prognostic factor. It is associated with a high detection rate in tumors that were not recognized by Afp and Gpc-3, making it a more promising marker for curative therapy (40).

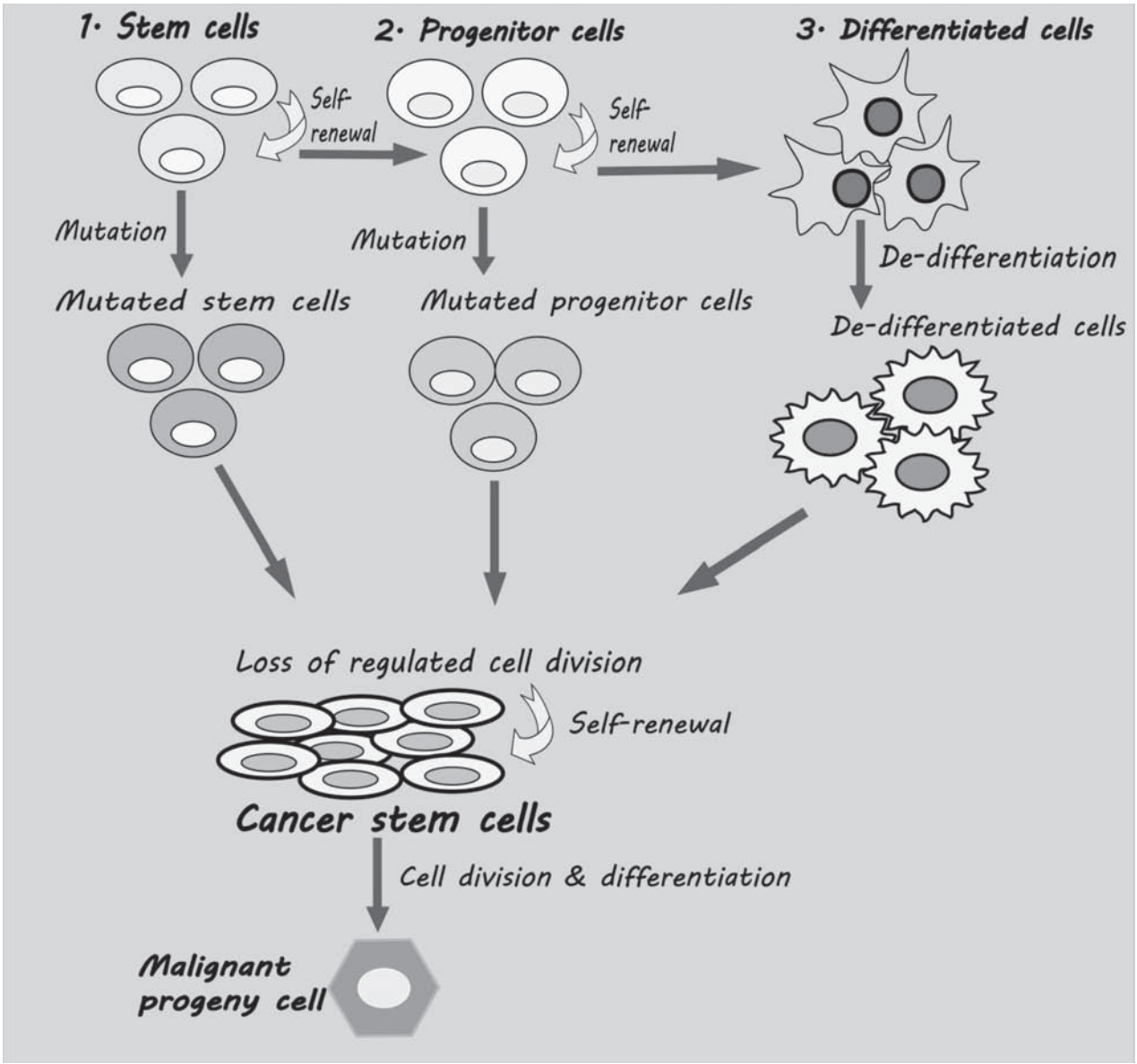

Figure 1. Diagrammatical presentation of cancer stem cells.

EpCAM

Epithelial cell adhesion molecule (EpCAM) can be used as a marker for identification of HCC at an initial stage due to its enhanced expression in premalignant liver tissue and subsets of HCC. cDNA microarray analysis of EpCAM-positive HCC cells showed specific molecular markers harboring properties of hepatic progenitor cells along with activation of the Wnt/β-catenin pathway, whereas EpCAM-negative cells displayed properties of the mature hepatocyte. EpCAM(+) and EpCAM (-) cell population can be further characterized according to how they express the biomarker α-fetoprotein (AFP), a prognostic factor for patient survival where each group reflects distinct stages of hepatic lineage (41). It has been shown that an activated β-catenin canonical pathway modulates the EpCAM expression in both normal and HCC cells, indicating it as a direct transcriptional target of Tcf4/beta-catenin. Thus both EpCAM and β-catenin are critical for regulating growth of HCC

cells and can serve as a target for therapeutic intervention (41)

Furthermore, introduction of exogenous EpCAM into an EpCAM (+) population revealed striking variations from the EpCAM (-) population in terms of HCC cell lines exhibiting high tumorigenicity. This suggests that the EpCAM (+) population is more likely to be a CSC population (42). It has been found that Huh7 cell populations co-expressing the $CD133^{+}EpCAM^{+}$ phenotype more precisely denoted HCC tumor-initiating cells than was the case with its counterparts $CD133^{+}EpCAM^{-}$, $CD133^{-}EpCAM^{+}$ and $CD133^{-}EpCAM$ (43). $CD133^{+}EpCAM^{+}$ cells have many properties of tumor initiating cells: they are rich in SP cells and possess high metastasis potential and resistance to chemotherapy. These cell populations can be used to study the role of tumor initiating cells in HCC as well as in devising therapeutic strategy.

CD 133

CD 133, a pentaspan transmembrane glycoprotein is a cancer stem cell marker for various solid tumors including HCC (44). CD133 HCC cells from human cell lines possess characteristics of stem/progenitor cells including self renewal and differentiation potential. The suppression of CD133(+) cells from Huh-7 HCC by anti-CD133 antibody exhibited rapid proliferation *in vitro* and downregulation of various markers including hepatocyte markers, glutamine synthetase and cytochrome P450 3A4, contrasting with its counterpart CD133(-) cells. Moreover CD133(+) cells generated tumors in an SCID mouse model. However, CD133 in HCC is expressed by a minority of the tumor cell population (45). Furthermore, $CD133^{+}$ HCC cells render patients insensitive to chemotherapeutic agents via preferential expression of survival proteins from the AKT/PKB and Bcl-2 pathway and might be responsible for tumor recurrence (46).These findings suggest that CD133(+) cells would be an efficient tool on which to study tumorigencity as well as in designing a therapeutic regime against HCC.

CD 44s

The combination (coexpression) of CD133(+) and CD44(+) cell surface marker more precisely defines the cancer stem/progenitor cell phenotype in HCC as it possesses several characteristics of CSCs including extensive growth, self-renewal and diferentiation potential as well as giving rise to tumor growth in NOD/SCID mice at a much lower number than occurs with its CD133(+)CD44(-) counterparts (40). Moreover, cells that are double-positive for CD133 and CD44 confer more resistance to chemotherapeutic agents, which is a characteristic of CSCs. Their drug resistance is mediated by upregulation of ATP-binding cassette (ABC) superfamily transporters including ABCB1, ABCC1, and ABCG2 which lends further support to the HCC origin. This suggests that the CD133(+) and CD44(+) population are true CSCs in HCC, offering an effective approach by which to study HCC tumorigenesis and devise an effective therapy targeting HCC (47).

CD 90

Yang and his colleagues attempted to identify the CSCs population in human liver samples of HCC using CD90 as a marker, and found a concomitant increase in the CD90(+) cell population with tumorigenicity. CD45(-)CD90(+) cells were discovered in almost all samples of tumor and blood specimens from liver cancer patients but remain undetected in normal, cirrhotic and neighboring non-tumorous tissues. Transplanting CD90(+) and CD45(-)CD90(+) cells isolated from liver cancer specimens into a xenograft model gave rise to tumor nodules in the existing sample and in subsequent xenografts following serial transplantation. Thus identification of CD45(-)CD90(+) CSCs in tissue and blood circulation would be helpful in determining the clinical outcome of, and therapy for, HCC (48).

Signaling pathways (molecular pathways deregulated in HCC)

Wnt/β-catenin signaling pathway

The Wnt/β-catennin pathway is crucial in liver function and is frequently deregulated in HCC. Wnt/β-catenin activation is attributed to upregulation of Low-density lipoprotein (LDL) receptor-related protein-6 (LRP6), one of the co-receptors of the Wnt/β-catenin pathway. LDL interacts with wnt ligand and surface receptor of the FZD family, resulting in increased accumulation of β-catenin (49).

Intriguingly, 40-70% of HCCs entail nuclear accumulation of β-catenin, driving Wnt/β-catenin pathway activation. Mutations affecting the Wnt/β-catenin pathway occur more frequently in HCC. Activating mutation of β-catenin gene (CTNNB1) occurs in 8–30% of tumors and appears to be a late event in hepatocarcinogenesis, while mutations in APC and Axin genes have been found in 1-3% and 8-15%, respectively. Moreover, modulation of Wnt ligands or FZD receptor expression could also lead to Wnt activation independently of mutations in CTNNB1, APC, or Axin genes. A differential response in HCC formation (hepatocarcinogenesis) is associated with mutated and non-mutated β-catenin. β-catenin-mutated HCCs display upregulation of Wnt targets, low grade and highy differentiated tumors with chromosome stability and better prognosis. In contrast, non-mutated HCCs present dys-regulation of classic Wnt targets, high levels of chromosome instability and are found to be more strongly expressed in chronic HBV infection (50).

Overexpression of constitutively active LRP6 in BEL-7402 HCC cells promoted cell proliferation and metastasis *in vitro* as well as *in vivo* when transplanted into nude mice (51). Cysteine-rich angiogenic inducer 61 (Cyr61) or CCN family member 1 (CCN1) are known to be increased in various types of cancer and play a role in tumorigenesis (52).The upregulation of β-catenin directly intensifies the expression of Cyr61 or CCN1 in HCC. Cyr61 protein was dramatically elevated in cancer-adjacent hepatic cirrhosis tissue as compared to its level in HCC and its neighboring tissues (53). One of the key regulators of cell proliferation in various cancers is Krüppel-like factor 8 (KLF8) which plays a central role in the cell cycle and metastasis (54, 55). Most recent research showed cross-talk between KLF8 and nuclear accumulation of β-catenin in HCC (56). β-catenin and KLF8 coordinatedly increased during advanced HCC (56, 57). In the HepG2 cell line, Wnt3a induces overexpression of KLF8 which in turn increases the cytoplasm and nuclear accumulation of β-catenin and recruits p300 to activate the transcriptional activity (56, 58) of β-catenin/T-cell factor 4 (TCF4) transcription complexes. This in turn induces expression of Wnt/β-catenin endogenous target genes including c-Myc and cyclin D1 which are indispensable for cell proliferation and differentiation (59) and Axin1 (56, 60).

Knockdown of KLF8 inhibits expression of these target genes (56). Targeting the Wnt pathway via antagonists of the TCf4/β-catenin complex (PKF118-310, PKF115-584 and CGP049090) exhibited antitumor activity *in vivo* and *in vitro* and is therefore a feasible therapeutic option (61).

In one study Wnt signaling was attributed to HGF/c-Met induced HepG2 cell scattering and invasiveness via E-cadherin downregulation. HGF-stimulated breakdown of the E-cadherin/β-catenin complex is accompanied by nuclear accumulation of β-catenin where it interacts with T cell factor/lymphocyte enhancer factor (TCF/LEF) to regulate the expression of target genes. It has been found that E-cadherin degradation is governed by HGF-induced MMP-7 activity (62) which is known to participate in disruption of extracellular matrix (63). Loss or reduction of E-cadherin function is known to drive tumor progression, invasion and metastasis (64, 65). Thus inhibition of the HGF/c-Met/beta-catenin/MMP-7/E-cadherin signaling pathway might represent a practical therapeutic target for preventing hepatocellular carcinoma metastasis (62).

Hedgehog signaling

There is accumulating evidence of hedgehog signaling activation in various cancers including HCC. Activation of the hedgehog pathway is a consequence of Hh ligand binding to its receptor, Patched (PTC) followed by upregulation of proto-oncoprotein Smoothened (SMO), which is positively correlated with tumor growth. Over-expression of SMO proto-oncogene mediates c-myc overexpression. Hedgehog signaling activation is accompanied by the expression of its target genes PTCH1, Gli1 and Shh (66, 67). Gli1 is transcriptionally activated by Gli2, a primary mediator of Shh signaling (68). Among the glioma-associated oncogene (Gli) transcription factors, Gli2 levels were found to be significantly higher in HCC cell lines and tumor tissues than in normal tissues. Inhibition of Gli2 with antisense oligonucleotide (ASO) resulted in decreased proliferation, whereas silencing Gli1 and Gli3 showed no antiproliferative response. Inhibitors targeting smoothened (SMO) displayed antiproliferative ef-

fects in only a subset of HCC cell lines. Moreover Gli2 ASO was able to induce anti-proliferative response in a Smo-mutated cell population (69, 70). Inhibition of hedgehog signaling by 5-fluorouracil, an anticancer drug, resulted in reduced expression of the target molecule, as well as invasion and induced apoptosis of HCC cell lines suggesting their role in therapy (71). Administration of KAAD-cyclopamine, an antagonist of SMO, induced similar effects in various HCC cell lines including SMMC-7721cells (72), Hep3B, HuH7 and PLC/PRF/5 (67) which implies there a role for them in therapy. The mechanism underlying apoptosis by cyclopamine is through dysregulation of Bcl-2 which is anti-apoptotic in nature and is known to confer resistance in various cancer treatments (73). Furthermore, Shh signaling exhibits a radioprotective response in an autocrine manner, possibly as a consequence of some failure in the DNA repair mechanism (74). Most recent research supported the idea that Hh signaling induces invasion and metastasis of HCC through ERK signaling-mediated Matrix metallopeptidase MMP-7 upregulation in human HCC. It has been seen that nuclear expression of Gli1 has a positive correlation with the progression and metastatic potential of tumors as well as with MMP-9 and phosphorylated-ERK1/2 protein expression in HCC tissue samples (75).

Transforming growth factor-beta

Transforming growth factor-beta (TGF-β) plays a role in various cellular processes during embryogenesis and is known to induce an epithelial-mesenchymal transition during tumor progression through Smad-dependent signaling (76-78). In this pathway, TGF-β dimers bind to a type II receptor which recruits and phosphorylates a type I receptor. The activated TGF-βR complex phosphorylates Smad2 and Smad3, converting them into transcriptional regulators that form a complex with Smad4 (79). TGF-β is known to play a dual role during hepatocarcinogenesis: either it acts as a tumor suppressor during the early stage or it induces tumorigenesis during the late stage (77, 80).

During tumor progression TGF-β stimulates EMT of liver cancer cells via Smad-dependent signaling. As a consequence the mesenchymal cell acquires tumor initiating stem cell traits such as marked invasion potential, expression of stem cell markers, formation of large tumor spheroids and resistance to apoptosis. In addition mesenchymals cell exhibit EMT characteristics such as loss of E-cadherin, overexpression of transcription factor Snail 1 and Nanog. Inhibition of Snail1 results in decreased tumor growth while it fails to impair tumor initiation *in vivo* (81).

It has been found that TGF-β impairs the tumor suppressor activity of HNF4α by inhibition of GSK3β, thus limiting HNF4α-mediated gene therapy of HCC (82). Most recent studies have shown that TGF-β contributes to the rapid growth of liver progenitor cells in diethylnitrosamine (DEN) -induced rat hepatocarcinogenesis and cirrhotic livers of HCC patients while a small number of liver progenitor cells (LPCs) express tumor-initiating cell markers which increase concurrently with the TGF-β level. Interestingly, constant exposure of TGF-β on the wb-f344 cell line in NOD/SCID mice attenuated their LPC potential but retained the properties of tumor-initiating cells (83). Hyperactivation of akt but not notch, STAT3 or mTor was detected in TGF-β-treated wb-f344 cells which have proved to be associated with tumor development and malignancy in various cancers. Additionally TGF-β-induced Akt activation and LPC transformation was mediated by microRNA-216a-modulated PTEN suppression, an antagonist of Akt activity which suggests that microRNA-216a could be a potential target in treating HCC (83). Again, a recent report established a potential link between the DNA methylation machinery of TICs and TGF-β. TGF-β exposure induced an increase in a proportion of CD133+ cells in liver cancer cell lines in a stable fashion and this is sustained through cellular division. This stable effect is ascribed to genome-wide changes in DNA methylation. Moreover, differential methylation signatures have been associated with CD133-negative and CD133-expressing liver cells (84).

Bmi-1 signaling

B-cell-specific Moloney murine leukemia virus integration site 1 (*Bmi-1*) is a transcriptional repressor belonging to the Polycomb (PcG) family that contributes to gene silencing through epigenetic chromatin modification. Bmi-1 is essential for regulating the self-renewal of various stem cell systems including neural, hematopoietic, intestinal (85) as well as hepatic stem

cells (86). Bmi-1 is known to be a proto-oncogene which induces lymphoma in association with c-myc and inhibits c-myc-induced apoptosis via repression of Cdkn2a locus (85). Of note, Bmi-1 regulates stem cell renewal and differentiation via repression of cyclin-dependent kinase (CDK) inhibitor, $p16^{INK4a}$, and a tumor suppressor, $p19^{ARF}$, encoded by Ink4a/Arf locus. It has been seen that repression of Bmi-1 targets contributes to oncogenic transformation of hepatic/progenitor cells (86). Furthermore, tumor-initiating SP cells with HCC phenotype showed preferential expression of Bmi-1 in Huh7 and PLC/PRF/5 HCC cell lines as compared to non-SP cells. Lentviral-mediated Bmi-1 knockdown resulted in considerable reduction in the SP subpopulation indicating that Bmi-1 is crucial for self renewal of SP cells. Interestingly, Bmi-1 knockdown completely inhibited the tumorigenic potential of SP cells *in vivo*, suggesting that Bmi-1 is a therapeutic target for the eradication of cancer stem cells in HCC (87). Subsequent studies have analyzed the correlation between Bmi-1 and ATP-binding cassette transporter B1 (ABCB1) in the HCC cell line and clinical samples. Bmi-1 was found to be upregulated in the early well-differentiated KIM-1cell line. Moreover, ABCB1 gene was upregulated together with overexpression of Bmi-1 and was downregulated along with suppression of Bmi-1. A strong statistical relation between ABCB1 and Bmi-1 mRNA expression was seen in HCC cell lines and clinical samples (88).

microRNA regulation

miRNA are small non coding RNAs of about 22 nucleotides which act as a negative regulator of various biological processes (89). This class of regulatory RNAs is dysregulated in various cancers and contributes to tumorigenicity by alteration either in miRNA genomic number and location or transcriptional regulation and probably epigenetic regulation. The point mutation, chromosomal translocation and shortening of 3'UTR ultimately alters miRNA-mRNA pairing miRNA mediated hepatic oncogenesis by either activating an oncogeneic pathway or tumor suppressive pathways (90). The metastasis-related miRNAs identified in liver cancer are miR-130b, miR-221, miR-222 and the tumor suppressors are miR-122, miR-26 and miR-223 (91).

More recently sequencing-based miRNA profiling has revealed more than 314,000 and 268,000 reliable reads from HCC and adjacent normal liver respectively (92).

Mir-221 and miR-222 (miR-221/222)

Mir-221/222 is most abundant and commonly upregulate miRNA having a role in tumor progression and malignancy in various cancers (93, 94). Transfection of miR-221/222 clusters into liver cancer cell lines results in high expression of FOCUS and HLE cell lines and thus enhances proliferation as compared to PLC/PRF5 and Huh6 cells exhibiting low expression. Following transfection with antago-miRs, reduction in invasion potential has been seen in cell lines overexpressing miR-221/222 while low expressors demonstrated no effect in proliferation. Several functional targets of miR-221 have been identified such as CDKN1B/p27 and CDKN1C/p57. Overexpression of miR-221 facilitates proliferation of HCC cells in the S phase via downregulation of CDKN1B/p27 and CDKN1C/p57 protein expression which are negative regulators of cell proliferation. Furthermore, DDIT4 has also been shown to be a target of miR-221 which inhibits cell growth by regulating the TOR signaling pathway. Aryl hydrocarbon nuclear translocator (Arnt) mRNA may be a novel target of miR-221 (95-97). An increased expression of miR-221 was found to be correlated with advanced clinical stage, tumor size and capsular infiltration (95, 98).

miR-93 and miR-130

Yeung *et al.* (2008) demonstrated the role of miR-93 and miR-130b in cell growth dysregulation of adult T-cell leukemia patients who showed upregulation of these miRNAs. Knocking down these miRNAs enhanced the expression of TP53INP1 which in turn led towards enhanced apoptosis. Thus the miR-93/miR-130b-TP53INP1 axis has a role in the proliferation and survival of HTLV-1-infected/transformed cells (99).

CD133(+) cells isolated from an HCC sample exhibited greater potential for tumor spheroid growth *in vitro*, higher expression of stem cell associated genes and significant upregulation of miR-130b i*n vivo* as compared to the CD133 (-) cell population. Functional studies on miR-130b lentiviral-transduced CD133

(+) cells demonstrated high resistance to chemotherapeutic agents, increased potential for tumorigenicity and self renewal. Inhibition of miR-130b in CD133 (+) tumor-initiating cells (TICs), in contrast, yields the reverse phenomenon. MiR-130b regulates CD133 (+) liver tumor-initiating cells, in part, via silencing of its target TP53INP1 (100).

MiR-181

A MicroRNA profiling approach showed upregulation of conserved mature members of the miR-181 family in EpCAM+ AFP+ hepatic cancer stem cells (HepCSC). Inhibition of miR-181 induces a reduction in quantity and tumorigenic potential of EpCAM+ AFP and HepCSC. Intriguingly, miR-181 family members partly maintain the stemness of HepCSC by targeting nemo-like kinase (NLK), an inhibitor of Wnt/β-catenin signaling and a transcriptional regulator of differentiation including caudal-type homeobox transcription factor 2(CDX2) and GATA binding protein 6 (GATA6) (101). It has been shown that an animal model fed on a choline-deficient L-amino-acid-defined (CDAA) diet developed HCC after 84 weeks. miRNA expression profiling showed over-expression of miR-181b and miR-181d in 32 week-old mice at a preneoplastic stage and significant downregulation of its target TIMP3 which is a tumor suppressor gene. Moreover, overexpression of the TGF-β signaling pathway mediates up-regulation of miR-181b/d. TIMP3-regulated ectopically expressed miR-181b significantly promotes MMP2 and MMP9 activity, associated with enhanced clonogenic survival and metastasis of HCC cells. Suppression of miR-181b blocks tumor progression *in vivo*. In addition depletion of miR-181b sensitizes HCC cells to the anticancer drug doxorubicin (102).

MicroRNA-224

MicroRNA-224 (miR-224) is one of the most frequently upregulated miRNAs in HCC and regulate various cellular processes such as apoptosis, cell proliferation and metastasis (103). Wang *et al.*(2012) showed that miR-224 is upregulated through an epigenetic mechanism. The 100 samples of HCC were subjected to expression analysis for miR-224 and neighboring miR-452 as well as genes on chromosome Xq28 in tumor and paired neighboring nontumorous tissues, which indicated that miR-224 was coordinately overexpressed with its adjacent miRNA and genes at Xq28 locus (104). Interestingly, the miR-224-residing locus is coordinately regulated through HDACI, HDAC3 and histone acetylase protein, E1A, binding protein p300 (EP300). Thus miR-224 expression can be suppressed either through siRNA-mediated inhibition of EP300 or through C646 (104), a competitive p300 inhibitor which is a valuable anticancer target (105).

Sciscian *et al.* (2012) linked miR-224 expression to the activation of p65/ NFκB signaling. Their *in silico* experimentation revealed OR miR-122 studied *in silico* revealed that its promoter posseses multiple NFκB target sites. Exposure to LPS, TNFα and LTα inflammatory signals induces expression of miR-224 and cell metastasis in HCC cell lines. Antago-miR mediated silencing of miR-224 inhibited LPS and LTα induced HCC cell migration and invasion. The IKK inhibitor BMS-345541 blocks pre-miR-224-induced cellular metastasis (106).

Bioinformatics analysis

Computational bioinformatics identified several miRNAs modulated in HCC, including miR-122, miR-21 and miR-34a. MiR-34a and miR-21 are found to be upregulated in HCC. MiR-34a is a transcriptional target of p53 and its overexpression elicits tumor suppressive effects, including cell-cycle arrest, senescence and apoptosis (107). MiR-21 is overexpressed in HCC tumors and modulates cell migration and invasion via phosphatase and tensin homolog (PTEN) PTEN. Moreover miR-122 was downregulated and was found to be abundantly expressed in the liver where it regulates the replication of HCV (92).

Other novel ncRNAs

Computation Bioinformatics and deep sequencing have revealed the involvement of novel non-coding RNAs including new piRNA, piR-Hep1, and miR-1323 in HCC. It has been shown that a novel piRNA exhibited upregulation in HCC tumors compared to neighboring non-tumoural liver. Silencing of piR-Hep1 inhibited the potential for cell invasiveness, motility and proliferation accompanied with downregulation of the activated Phospho-AKT level, which

suggests a potential role of vPI3K/AKT signaling in the invasiveness and proliferation of novel piRNA. Furthermore, it has been revealed that miR-1323 is upregulated in cirrhotic HCC and miR-1323 overexpression is associated with poorer disease-free and overall survival by patients (108).

DNA methylation patterns in hepatocellular cancer

Aberrant methylation is the most frequent event in hepatocarcinogenesis and manifests as global hypomethylation and site-specific hypermethylation. Hypermethylation of CpG islands in promoter sequences is associated with inactivation of tumor suppressor genes, whereas hypomethylation leads to genomic and chromosome instability respectively (109).

DNA methylation is governed by enzyme methyltransferases and DNMT1, DNMT3A and DNMT3B and is found to be overexpressed in hepatic cancer where it is significantly associated with poor survival (110).

In 2003, Yang *et al.* adopted a methylation-specific polymerase chain reaction to identify the methylation status of nine TSGs, namely *SOCS-1*, *GSTP*, *APC*, *E-cadherin*, *RAR*-β, *p14*, *p15*, *p16*, and *p73*, *GSTP*, in 51 cases of HCC. Of these, 65% and 54% show methylation for *SOCS-1* and *GSTP* respectively. The tumor suppressor gene APC was found to be hypermethylated in 53%, *E-cadherin* in 49%, and *p15* in 49% while it was found that methylation of *SOCS-1*, *GSTP*, and *p15* was more frequently associated with HCV-positive HCC patients than non-infected HCC (111).

Lambert *et al.* revealed concurrent Hypermethylation of candidate genes RASSF1A, GSTP1, CHRNA3, and DOK1 in HCC tumors compared to cirrhotic and normal liver, indicating the non-random status of hypermethylation. Hypermethylation *of RASSF1* was found in *76%* and *DOK1* was silenced in 62% of HCC while its methylation statues exhibited an inverse correlation with gene expression. Hypermethylation of *GSTP* was found in (65%) of HCC and is independent of geographical area, alcohol intake and HBV/HCV infection; thus another factor such as aflatoxin B1 might contribute to methylation-associated inactivation of GSTP1 (112).

Genomewide methylation provides the bulk of information for downstream analysis. These data enable researchers to efficiently distinguish tumors from neighboring non-tumor tissue, and cluster tumors according to their specific etiological factors, such as viral infection and alcohol consumption (113). Another study by Shan at al. examined the methylation level of CpG loci in tumor and adjacent non-tumor tissues in sixty-six HCC pair samples and found methylation at 130,512 CpG sites (representing 28,017 hyper- and 102,495 hypomethylated sites) to be significantly different in tumor tissues compared to normal adjacent tissues (114).

Moreover, recent research has discovered tumor suppressor genes to be epigenetically silenced in HCC, namely *SMPD3* and *NEFH*. Knockdown of *NEFH* has been found to promote cell migration *in vitro* and tumor growth *in vivo*. *SMPD3* exhibited downregulation in early stages of HCC. Loss of *SMPD3* could potentiate tumor growth and aggressiveness and is associated with early recurrence of HCC (115).

Conclusion

The CSC field is an emerging one and scientists are still in the technically challenging stage of identifying the correct CSCs from various tumors. CSCs are a specific subset of transformed cells that are able to maintain primary tumor growth in the cancer hierarchy. These comprise a minor subpopulation of tumor cells that are relatively quiescent and capable of self renewing. Recent research suggests that tumor formation may result from the development of cancer stem cells via deregulation of normal self renewal pathways of tissue specific stem cells. However, many pathways that are crucial for CSCs are shared by normal stem cells while the complexity and crosstalk among signaling pathways limits the benefit of treatment targeting individual pathways. Mounting evidence has also shown that CSCs confer resistance to anti-cancer therapy and hence contribute to tumor recurrence, reducing patient survival. Thus, understanding the biology of normal stem cells and CSCs and identifying biomarkers which distinguish between them both will help to develop new and effective therapeutic regimes against cancer.

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Received: 9.4.2014
Accepted: 30.07.2014
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