Search for useful biomarkers in hepatocellular carcinoma, tumor factors and background liver factors (Review)

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Abstract. Hepatocarcinogenesis is a complex and multistep process that involves the accumulation of genetic and epigenetic alterations in regulatory genes. To understand the development of hepatocellular carcinoma (HCC), current research has utilized improved array technologies. The identification of cancer-related molecules could lead to the development of novel molecular targets for treatment and biomarkers for predicting prognosis. However, prognostic prediction is insufficient when considering only tumor factors, since hepatocarcinogenesis is also greatly influenced by the status of the background liver. Clinical background liver factors, such as the presence of chronic active hepatitis or cirrhosis, are well known as risk factors for developing HCC. In contrast, genetic or epigenetic background liver factors remain unknown, albeit those are important to understand the developing process of HCC. Investigating background liver factors could contribute to the development of carcinogenic markers of HCC and to the prevention of the development of HCC. In the present study, we review the currently identified tumor factors and background liver factors from a molecular biological viewpoint and also introduce our combination array analysis.

Contents

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death worldwide. HCC is associated with a high recurrence rate after curative resection. There is currently no effective adjuvant chemotherapy available for HCC, and treatment options for advanced or recurrent HCC are limited. Additionally, the current tumor markers for HCC, α-fetoprotein and prothrombin induced by vitamin K absence or antagonist-2, are not ideal owing to their relatively low sensitivity and specificity. Thus, identification of novel molecular targets for treating recurrence and biomarkers for predicting prognosis are urgently required.

Investigation of the genetic and epigenetic alterations in the hepatocarcinogenic stage which lead to the activation of oncogenes and the inactivation or loss of tumor-suppressor genes may further our understanding of the development of HCC. Many HCC-related molecules have been recently identified as tumor factors, in part as a result of the improvement of the array technology that was first established by Grunstein and Hogness in 1975 (1). The genes that are upregulated or downregulated in HCC tissue may become the novel molecular targets for treatment or biomarkers for predicting prognosis.

HCC recurs in residual liver in 80% of patients who undergo curative resection (2). Postoperative recurrence in the residual liver arises from either a monoclonal origin caused by intrahepatic metastasis (IM) or multicentric occurrence (MO). IM develops from tumor cells that spread into the remnant liver via the portal vein before or during hepatic resection. MO is a unique recurrence pattern from the background liver status such as liver cirrhosis secondary to infection with hepatitis B virus or hepatitis C virus, alcoholic liver disease and non-alcoholic steatohepatitis. Several studies from developed countries have shown that MO recurrence is more common than IM recurrence (3-7). However, other studies have shown that IM recurrence is more common than MO recurrence (8,9). The incidence of IM and MO recurrence may depend on the balance of tumor malignancy and background liver status (10). For example, advanced stage primary HCC lesions may have more accumulated epigenetic alterations and the rate of IM rate increases. Pervasion of HCC screening for high-risk patients increases the number of patients diagnosed in the early stage. HCC diagnosed at an early stage may be cured by surgical procedures, in which case MO recurrence becomes the major issue. This may be one of the reasons why the incidence of

MO recurrence is more common than IM recurrence in developed countries. Although the incidence of HCC is highest in eastern Asia and Africa, the incidence is steadily increasing in Western countries. Thus, epigenetic understanding of MO is critical. However, most epigenetic studies concerning HCC have mostly focused only on tumor factors. Recent studies have initiated the investigation of background liver factors in HCC, and we also pursued the detection of background liver factors using combination array analysis. In the present review, we review the recent literature regarding tumor factors as well as background liver factors in HCC patients from a genetic and epigenetic viewpoint.

2. Tumor factors

Numerous studies have revealed the genetic and epigenetic alterations in HCC tissue. The ongoing development and improvement in array technology have contributed to the steady increase in these findings. Furthermore, some researchers have combined existing array technologies to establish combination array analysis to effectively extract tumor factors. Moreover, it is expected that the establishment of next-generation sequencing may accelerate the identification of HCC-related factors. The investigation into tumor factors is necessary for the discovery of novel molecular targets for treatment and biomarkers for predicting prognosis. In the present study, we review the upregulated molecules in HCC as oncogene candidates and downregulated molecules as tumor-suppressor gene (TSG) candidates. Oncogenes have the potential to become therapeutic targets directly or tumor markers for liquid biopsy, thus the exploitation of oncogenes is very important. TSGs are not conducive for use as direct therapeutic targets, although novel therapeutic strategies can be developed by investigating the suppression mechanism of a TSG and the downstream pathway of the TSG. We also review the current findings on microRNAs (miRNAs), which are often reported as negative regulators in post-transcriptional processes.

Oncogenes in HCC. Oncogenes are frequently upregulated in HCC tissues and their expression levels correlate with poor prognosis or malignant phenotypes of HCC cells. In generally, oncogenes function to resist apoptosis, drive cell cycle progression and inhibit TSG expression or activities, enabling the acquirement of enhanced proliferation, migration and/or invasion ability by neoplastic cells. The identification of critical oncogenes in HCC could lead to the development of treatment targets for these unfavorable phenomenon. Additionally, when the protein encoded by an oncogene is overexpressed in serum, it may be a useful tumor marker. We listed the recently reported oncogenes in HCC in Table I (11-43) and below we discuss the current findings.

Catenin delta-1. Catenin *delta*-1 (CTNND1) encodes a member of the Armadillo protein family, which functions in cell adhesion and signal transduction. CTNND1 was reported to participate in epithelial-mesenchymal transition (EMT) (44,45), and a large amount of data have implicated CTNND1 in the regulation of cancer development and progression (46). CTNND1 was reported as an oncogene that drives migration and metastasis (47,48). Overexpression of CTNND1 has been observed in lung (49) and cervical cancer (50), pancreatic adenocarcinoma (51) and gastric cancer (52). Tang *et al* (16) showed that CTNND1 expression was significantly upregulated in HCC tissue, and the CTNND1 expression level was associated with shorter overall survival. Inhibition of CTNND1 expression promoted migratory and invasive capacities of HCC cells *in vitro* and metastasis *in vivo*. Additionally, the authors reported that CTNND1 plays an important role in regulating the EMT to mesenchymal-epithelial transition (MET) plasticity of HCC cells by interacting with E-cadherin, α-catenin, N-cadherin and vimentin and by enhancing Wnt/β-catenin signaling. These studies demonstrate that CTNND1 functions as a novel tumor oncogene in HCC and may be a potential therapeutic target for HCC management.

Galectin-1. Galectin-1 (Gal-1) is a member of the family of β-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions and regulated by HIF-1. Gal-1 has vital protumorigenic roles within the tumor microenvironment and plays a role in regulating apoptosis, cell proliferation and cell differentiation (53). Dysregulation of Gal-1 expression was found to be associated with resistance to chemotherapy through ERK pathway activation (54). Gal-1 overexpression also mediated migration and invasion in cancer cells via increased phosphorylation of AKT, mTOR and p70 kinases. Moreover, sorafenib response was impaired in HCC with dysregulated p-ERK and p-AKT activation. Zhang *et al* (22) found that Gal-1 elevated αvβ3-integrin expression, leading to AKT activation, and that Gal-1 overexpression induced HCC cell EMT via PI3K/AKT cascade activation. This led Gal-1 to promote HCC cell invasion *in vitro* and lung metastasis *in vivo*. Clinically, this study also revealed a correlation between Gal-1 overexpression and poor HCC survival outcome. Moreover, Gal-1 expression was inversely correlated with HCC sensitivity to sorafenib *in vitro*. Thus, targeting Gal-1 in a subset of HCCs may be an optimal therapeutic strategy, and Gal-1 may be a biomarker for predicting the responsiveness to sorafenib treatment and for personalized treatment.

Meprin A subunit α. Meprin A subunit α (MEP1A) encodes Meprin α, a metalloprotease that belongs to the metzincin superfamily. MEP1A cleaves a wide variety of substrates, such as basement membrane proteins, protein kinases and cytokines. Abnormal MEP1A expression has been implicated in several diseases, such as inflammatory bowel disease, nephritis and Alzheimer's disease (55). MEP1A expression has been previously observed in only colorectal cancer (56); however, the mechanisms and function of MEP1A have not been reported. OuYang *et al* (34) revealed that expression levels of MEP1A were markedly elevated in HCC tumor tissues compared with matched adjacent non-neoplastic tissues and nonmalignant liver tissues. Clinical analysis indicated that the expression level of MEP1A in tumor tissues was correlated with tumor size, microvascular invasion, portal vein tumor thrombus (PVVT), differentiation grade, BCLC stage, TNM stage and patient survival. MEP1A overexpression increased cell migration and cell invasion *in vivo* and enhanced tumor metastasis *in vitro*. The authors also investigated the mechanism of oncogenic activity of MEP1A. Overexpression of MEP1A markedly enhanced the levels of ZEB1, vimentin and matrix MMP2 and MMP9, and concomitantly reduced the expression of E-cadherin. Together these

Table I. Putative oncogenes in hepatocellular carcinoma.

Symbol (location)	Biological function	Expression	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
ACK1 (3q29)	Tyrosine kinase	mRNA, WB, IHC	150	OS, DFS, tumor number, vascular invasion, grade, stage	Proliferation, migration, invasion, apoptosis	WWOX, AKT, p-AKT, MMP2, MMP9	-	(11)
ADAM10 (15q22)	Possessing both potential adhesion and protease domains	mRNA	-	-	Proliferation, cell cycle, migration, invasion	p-P13K, p-AKT	Yes	(12)
ANGPTL2 (9q34)	Growth factor for vascular endothelium	mRNA, WB, IHC	66	Intrahepatic metastasis, cirrhosis	Migration, invasion	-	Yes	(13)
CK2a (20p13)	Serine/threonine protein kinase	mRNA, WB, IHC	47	OS, stage, distant metastasis	Proliferation, migration, invasion, apoptosis	Bcl-2, p-AKT, P53, Bax, caspase-3	Yes	(14)
CSIG (16p13.13)	Unknown	mRNA, WB	22	-	Proliferation, apoptosis, cell cycle	c-MYC	Yes	(15)
CTNND1 (11q11)	Adhesion between cells and signal transduction	mRNA, WB, IHC	289	OS, tumor size, microvascular invasion, differentiation	Proliferation, migration, invasion, metastasis	β-catenin, WNT11, cyclin D1, BMP7	Yes	(16)
CTSB (8p22)	C1 family of peptidases	mRNA, WB, IHC	168	OS, stage, metastasis/ recurrence, grade	Invasion	MMP9	Yes	(17)
DEK (6p22.3)	Transcription factor	mRNA, IHC	55	OS, tumor size, grade, portal venous invasion	-	-	-	(18)
DJ-1 (1p36.23)	Positive regulator of androgen receptor-dependent transcription	mRNA, WB	-	-	Proliferation, invasion, adhesion	PTEN, p-AKT	Yes	(19)
FAM83D (20q11.23)	Unknown	mRNA, IHC	218	OS, DFS, AFP, stage, PVTT, recurrence	Proliferation	-	-	(20)
FNDC3B (3q26.31)	Unknown	WB, IHC	242	OS, RFS	Migration, invasion, metastasis	ANXA2	Yes	(21)
Gal-1 (22q13.1)	Modulating cell-cell and cell-matrix interactions	mRNA, WB, IHC	209	OS, sorafenib response	Invasion, metastasis, EMT	Integrin αv, integrin β3, p-AKT, p-FAK	Yes	(22)
HDGFRP-2 (19p13.3)	Hepatoma-derived growth factor (HDGF)	mRNA, WB	45	-	Proliferation	IWS1, cyclin D1	Yes	(23)
HOXA7 (7p15.2)	Transcription factor	mRNA, WB	-	-	Proliferation	Cyclin E1, CDK2	-	(24)
HOXD9 (2q31.1)	Transcription factor	mRNA, WB, IHC	102	-	Migration, invasion, EMT	ZEB1	-	(25)
HSP27 (7q11.23)	Stress resistance and actin organization and translocates	mRNA, WB, IHC	167	OS	Invasion, metastasis	MMP2, ITGA7	Yes	(26)

Table I. Continued.

Symbol (location)	Biological function	Expression	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
JARID1B (1q32.1)	Lysine-specific histone demethylase	mRNA, WB, IHC	38	OS, tumor diameter, microvascular invasion, tumor differentiation	Proliferation, migration, invasion, EMT	PTEN	Yes	(27)
KDM5B (1q32.1)	Lysine-specific histone demethylase	mRNA, WB	100	OS, DFS, tumor size, stage, grade	Proliferation, cell cycle	p15, p27	Yes	(28)
KMO (1q42-q44)	Catalyzes the hydroxylation of L-tryptophan metabolite	mRNA, WB, IHC	205	OS, differentiation	Proliferation, migration, invasion	-	-	(29)
LRG1 (19p13.3)	Protein-protein interaction, signal transduction, and cell adhesion and development	mRNA, WB, IHC	777	OS, DFS, tumor size, differentiation, stage, vascular invasion	Migration	-	-	(30)
MACC1 (7p21.2)	Regulator of HGF-HGFR pathway	mRNA, WB, IHC	50	OS, grade, stage	Proliferation, apoptosis	HGF, c-MET, P13K, AKT, caspase-9	Yes	(31)
MAGED2 (Xp11.21)	Promotor of the cancer cell adhesion to the vascular epithelium	mRNA, IHC	151	OS	-	-	-	(32)
MAGED4 (Xp11.22)	Unknown	mRNA, IHC	94	OS, RFS, AFP, differentiation, vascular invasion	-	-	-	(33)
MEP1A (6p12-p11)	Unknown	mRNA, IHC	212	OS, tumor size, microvascular invasion, PVTT, differentiation grade, BCLC stage, stage	Proliferation, invasion, EMT	ZEB1	Yes	(34)
NRAGE (Xp11.23)	Encode tumor-specific antigens	mRNA, IHC	151	DSF, age, AFP	-	AATF, p75NTR, PCNA	-	(35)
PIM1 (6p21.2)	Ser/Thr protein kinase family, and PIM subfamily	mRNA, WB, IHC	56	-	Proliferation, invasion	Akt	Yes	(36)
PROX1 (1q41)	Transcription factor	mRNA, WB, IHC	60	-	Proliferation	β-catenin	Yes	(37)
SETDB1 (1q21)	Histone methyltransferase	mRNA, WB, IHC	92	OS	Proliferation, metastasis	miR-29	Yes	(38)
STK33 (11p15.3)	Unknown	mRNA, IHC	251	OS, DFS	Proliferation	c-Myc	Yes	(39)
TAZ (Xq28)	Unknown	mRNA, WB, IHC	180	OS, tumor size, stage, grade, lymph node or distant metastasis, recurrent HCC	Proliferation, migration, invasion, apoptosis	-	Yes	(40)
TTK (6q14.1)	Protein kinase with the ability to phosphorylate	mRNA	152	OS, DFS, age, AFP, median size, stage, PVTT, distant metastasis	Proliferation, migration	Akt	-	(41)

Table I. Continued.

Symbol (location)	Biological function	Expression	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
UBE4B (1p36.3)	Conjugation factor E4 involved in multiubiquitin chain assembly	mRNA, WB, IHC	149	OS, grade, stage	Proliferation, migration, invasion, apoptosis	p53, Bcl-2, caspase-3	-	(42)
WWP1 (8q21)	Protein degradation, transcription, and RNA splicing	mRNA, WB, IHC	149	OS, PFS, tumor size, grade, stage, vascular invasion, tumor capsule	Proliferation, migration, invasion, apoptosis, cell cycle	-	-	(43)

WB, western blotting; IHC, immunohistochemistry; OS, overall survival; DFS, disease-free survival; AFP, serum α-fetoprotein; PVVT, portal vein tumor thrombus; RFS, recurrence-free survival; EMT, endothelial-mesenchymal transition.

studies indicate that MEP1A is a novel prognostic predictor in HCC and plays an important role in the development and progression of HCC.

Serine/threonine kinase 33. Serine/threonine kinase 33 (STK33) is a serine/threonine protein kinase that belongs to the calcium/calmodulin-dependent family of kinases and is weakly expressed in the liver (57). Recently, STK33 was found to be critical for the survival of KRAS-dependent hematopoietic cancer cell lines and epithelial cancer cell lines. The kinase activity of STK33 was inferred to be required for the survival of KRAS-dependent cancer cell lines using mutations in the ATP-binding loop (58,59). Yang *et al* (39) investigated the function and mechanism of STK33 in HCC, and STK33 expression was found to be frequently upregulated in HCC patients. Significant associations were found between increased expression of STK33 and advanced HCC staging and shorter disease-free survival of patients. Overexpression of STK33 increased HCC cell proliferation both *in vitro* and *in vivo*, whereas suppression of STK33 inhibited this effect. The authors also demonstrated that STK33 binds directly to c-Myc and increases its transcriptional activity. In particular, the C-terminus of STK33 blocked the STK33/c-Myc association, downregulated HCC cell proliferation, and reduced liver tumor cell number and tumor size. Together this suggests that STK33 plays an essential role in hepatocellular proliferation and liver tumorigenesis. The C-terminus of STK33 could be a potential therapeutic target in the treatment of patients with STK33-overexpressing HCC.

TTK protein kinase. The TTK protein kinase (TTK) gene encodes a dual specificity protein kinase that phosphorylates tyrosine, serine and threonine. TTK is essential for the mitotic checkpoint and improper chromosome attachments (60). Elevated TTK level leads to amplified centrosomes, hyperactivated SAC and chromosome instability, thus, contributing to tumorigenesis (61). The diagnostic value of TTK has been reported in thyroid carcinoma (62), breast cancer (63) and lung cancer (64). Liu *et al* (41) investigated the clinical significance and prognostic value of TTK in HCC and the effects on cell function and signaling pathways. The authors found that TTK mRNA expression was frequently increased in HCC tissue. High expression of TTK was significantly correlated with AFP, tumor size, advanced stage, PVTT and distant metastasis, and shortened overall survival and disease-free survival. One of the regulatory mechanisms controlling TTK in HCC was the demethylation of the TTK promoter. Inhibition of TTK expression using siRNA led to a decrease in cell proliferation and migration *in vitro*. Further mechanistic studies have revealed that TTK activates the Akt/mTOR pathway. Together this shows that TTK contributes to HCC tumorigenesis by promoting cell proliferation and migration, and that TTK may serve as a novel biomarker and a potential target in HCC.

TSGs in HCC. TSGs are frequently downregulated in HCC tissue and suppressed expression levels of TSGs have been correlated with poor prognosis and malignant phenotypes of HCC cells. Although TSGs are less effective for use as target molecules due to their low levels of expression in cancerous tissue, TSG expression levels in tumor tissue are useful as biomarkers. Aberrant DNA methylation, one of the mechanisms for suppression of TSG gene transcription, was

reportedly detectable in plasma, and thus, the diagnostic significances are high (65). Additionally, TSGs serve as therapeutic targets with the use of DNA methyltransferase inhibitors or histone deacetylase inhibitors to reactivate TSGs. Moreover, downstream pathways of TSGs may provide therapeutic target candidates. We also reported several novel TSGs as potential biomarkers in HCC using a combination array analysis containing expression, single nucleotide polymorphism and methylation arrays. Table II (66-85) provides a list of putative TSGs and below we discuss several candidates.

Jumonji C domain-containing protein 5. The Jumonji C domain-containing protein (JMJD) family includes histone demethylases that can remove all methylation modifications on the lysine residues of histones (86). JMJD5 demethylates Lys-36 of histone H3. Previous studies have shown that dysregulation of JMJD5 promotes cancer cell proliferation and migration (87,88). Huang *et al* demonstrated that JMJD5 forms a complex with the tumor suppressor p53 by interacting with the p53 DNA-binding domain and negatively regulates its activity (89). Wu *et al* (73) conducted an expression analysis on the JMJD family in HCC and found that the most significantly downregulated gene is the gene encoding JMJD5. The authors found that downregulation of JMJD5 was caused by altered epigenetic histone modifications on the JMJD5 promoter. JMJD5 knockdown promoted HCC cell proliferation and *in vivo* tumorigenicity by accelerating the G1/S transition of the cell cycle, and forced JMJD5 expression had the opposite effects. JMJD5 knockdown led to the downregulation of CDKN1A, and CDKN1A knockdown abrogated the effect of JMJD5 knockdown or overexpression on cell proliferation, suggesting that JMJD5 inhibits HCC cell proliferation mainly by activating CDKN1A expression. The authors concluded that JMJD5 is a TSG in HCC pathogenesis and that epigenetic silencing of JMJD5 promotes HCC cell proliferation by directly downregulating CDKN1A transcription.

Kallmann syndrome-1. The Kallmann syndrome-1 (KAL1) gene, also named anosmin-1, encodes an extracellular matrix related protein with a role in cellular adhesion. KAL1 promotes the migration of gonadotropin-releasing hormone expressing neurons during development (90). KAL1 also induces neurite outgrowth and cell migration through fibroblast growth factor receptor 1 pathways (91). Mutations in the KAL1 gene cause the X-linked Kallmann syndrome. Decreased KAL1 expression has been observed in colon, lung and ovarian cancers compared with corresponding adjacent normal tissues (92). Conversely, KAL1 overexpression promotes brain tumor malignancy through integrin signaling pathways (93). Tanaka *et al* (74) found that KAL1 was downregulated in HCC tissues in their microarray project. The authors examined the expression and methylation status of KAL1 in HCC to clarify the function of KAL1 in HCC. KAL1 mRNA expression was downregulated in HCC cell lines with promoter hypermethylation and was reactivated by demethylation by 5-aza-dC treatment. KAL1 mRNA levels were inversely correlated with those of EZR, which is one of the key factors involved in tumor progression and metastasis in HCC (94). Downregulation of KAL1 mRNA in HCC was significantly associated with elevated AFP and PIVKA-2, larger tumor size and vascular invasion. Patients with downregulation of KAL1 were more likely to have a shorter overall survival. Multivariate analysis identified downregulation of KAL1 as an independent prognostic factor in HCC. Hence, KAL1 may serve as a biomarker of malignant phenotype of HCC.

Ras association domain family member 10. The gene encoding Ras association domain family member 10 (RASSF10) is located on chromosome 11p15.2, a region that shows frequent loss of heterozygosity (LOH) in several cancer types. Hypermethylation of the RASSF10 promoter region, which inactivates the gene, is common across several cancers (79). Wang *et al* (95) examined RASSF10 expression in HCC and its role in hepatocarcinogenesis. The authors found that RASSF10 was epigenetically downregulated by promoter hypermethylation in human HCC tissue and HCC cell lines. Low RASSF10 expression was associated with poor differentiation, cirrhosis, tumor thrombus and BCLC stage and contributed to tumor recurrence and shortened patient survival. Overexpression of RASSF10 in HCC cell lines resulted in suppressed cell proliferation and apoptosis induced by Bcl-2 family proteins. *In vivo*, RASSF10 overexpression also reduced proliferation, migration and invasion of HCC cells by inhibiting EMT. Together, these findings indicate that RASSF10 may be a useful prognostic biomarker in HCC.

Synaptojanin-2-binding protein. Synaptojanin-2-binding protein (SYNJ2BP) regulates endocytosis of activin type II receptors (ActRIIs) through a Ral/Ral-binding protein 1-dependent pathway (96). Expression of SYNJ2BP enhances endocytosis of ActRIIs and suppresses activin-induced transcription. Adam *et al* (97) demonstrated that SYNJ2BP stabilizes Notch ligands and inhibits sprouting angiogenesis. Notch signaling contributes to the occurrence and development of many cancers (98). Brito *et al* (99) used gene expression profiling to show that SYNJ2BP expression was suppressed in clear cell renal carcinoma. Liu *et al* (81) indicated that SYNJ2BP acted as a tumor suppressor in HCC by inhibiting tumor growth and metastasis via activation of the DLL4 pathway. To the best of our knowledge, no studies have pursued the clinical significance of SYNJ2BP in neoplasms. Liu *et al* further showed that SYNJ2BP mRNA and protein were downregulated in human HCC tissues and HCC cell lines. Low expression of SYNJ2BP in HCC tissues was associated with tumor size, tumor nodule number, vascular invasion, TNM stage and BCLC stage, and patients with low SYNJ2BP expression had shorter overall survival and disease-free survival. Knockdown of SYNJ2BP increased proliferation, migration and invasion activities of HCC cell lines *in vitro*, and increased tumor growth and metastasis. Additionally, knockdown of SYNJ2BP decreased DLL4 expression in HCC cell lines, and forced expression of SYNJ2BP elevated DLL4 expression. This suggests that SYNJ2BP inhibited HCC growth and metastasis through activating DLL4. Hence, SYNJ2BP can be used as a potential marker for HCC and may serve as a target for HCC treatment in the near future.

Combination array analysis. To detect cancer-related genes in HCC, we developed a new technique: a combination array analysis, consisting of a gene expression array, a methylation array and a single nucleotide polymorphism array (100). Nomoto *et al* previously developed the 'double-combination array' by combining expression array analysis and SNP array analysis to effectively gain whole genome information (101).

The gene expression profile provides a snapshot of the transcriptional state of non-cancerous and tumor tissues. The SNP array is a useful tool for surveying LOH, a prominent characteristic of many human cancers. The authors' combination of these two microarrays in one representative surgical sample enabled the identification of several, novel tumor-specific gene alterations (100-105). To further evaluate hypermethylation of promoter CpG islands, a methylation array can be added to complete the triple-combination array analysis, which thus, more efficiently searches for epigenetic alterations. We identified several genes as candidates for TSGs in HCC using this combination array analysis. We listed these putative TSGs detected by combination array analysis in Table III (100-112).

Dysregulated miRNAs in HCC. As a group of small non-coding RNAs, miRNAs negatively regulate post-transcriptional processes and can function as oncogenes or TSGs. miRNAs bind to complementary sequence in the target mRNA and, as a result, negatively regulates the target gene expression. miRNAs have been reported to be involved in many types of diseases particularly malignancies including HCC. miRNAs released from cancer cells into serum can be quantified by PCR technique. Some studies have demonstrated the potential value of miRNAs as prognostic or diagnostic markers. In the present review, we introduce newly identified miRNAs that potentially represent biomarkers for HCC in Table IV (113-139).

miR-192. Yang *et al* (140) reported the association between miR192 and HCC for the first time by genomic sequence. Previous studies have shown that miR-192 inhibited HCC growth by negatively regulating HOTTIP, and HCC patients with high HOTTIP expression had a much shorter overall survival (141). Lian *et al* (125) assessed the function and clinical significance of miR-192 in resected HCC specimens. miR-192 expression was decreased and negatively correlated with vascular invasion in HCC specimens. Low miR-192 expression significantly contributed to short overall survival in HCC patients. miR-192 significantly suppressed metastasis of HCC cells *in vitro* and *in vivo*. SLC39A6, which promoted HCC cell migration and invasion, was identified as a direct and functional target of miR-192. Additionally, miR-192 decreased SLC39A6 expression, subsequently downregulating SNAIL and upregulating E-cadherin expression. Thus, miR-192 and SLC39A6 may be useful predictors for HCC patient prognosis, and the miR-192/SLC39A6/SNAIL pathway may be a therapeutic target for HCC treatment.

miR-211. miR-211 has been reported to be dysregulated in several carcinomas. miR-211 functions as an oncogenic miRNA in colorectal cancer (142), oral squamous cell carcinoma (143), breast (144) and lung cancer (145). In contrast, miR-211 acts as tumor suppressor in glioma (146), melanoma (147) and ovarian cancer (148). Deng *et al* (126) demonstrated that miR-211 is a tumor suppressor that is pathologically downregulated in HCC tissues and cell lines. miR-211 inhibited tumor cell growth, and overexpression of miR-211 suppressed HCC cell migration and invasion *in vitro* and *in vivo*. miR-211 downregulation is associated with vein invasion, TNM stage and poor overall survival of HCC patients. Moreover, SPARC was identified as a direct target of miR-211. The authors concluded that loss of miR-211 expression and thus uncontrolled SPARC overexpression may drive progression of HCC. Together, these findings may provide a novel therapeutic target for the treatment of HCC.

miR-379-5p. Chen *et al* (130) investigated the expression level of miR-379-5p in HCC tissues and found that downregulation of miR-379-5p was associated with advanced TNM stage. In addition, miR-379-5p expression levels were markedly lower in metastatic HCC tissues than in non-metastatic HCC tissues, indicating that miR-379-5p correlates with metastasis in HCC. Overexpression of miR-379-5p inhibited HCC cell migration, invasion, EMT and metastasis both *in vitro* and *in vivo*. Moreover, miR-379-5p was found to directly target FAK and was negatively correlated with FAK in HCC tissues. Together, this indicates that miR-379-5p may represent a novel potential therapeutic target and prognostic marker for HCC.

miR-519a. Previous studies have shown that miR-519a plays an oncogenic role in breast cancer (149) and ovarian epithelial tumors (150), and acts as a tumor suppressor in glioma (151). Shao *et al* (152) reported elevated expression of miR-519a in HCC tissues compared with adjacent non-cancerous tissues. The increased expression of miR-519a was significantly correlated with adverse clinical features and was associated with a poorer overall survival and recurrence-free survival of HCC patients. Upregulation of miR-519a reduced the expression of FOXF2 mRNA, promoted cell proliferation, and inhibited apoptosis *in vitro*. Tu *et al* (135) demonstrated that upregulation of miR-519a was associated with poor prognostic features and reduced overall survival and disease-free survival of HCC patients. miR-519a promoted HCC cell proliferation and cell cycle progression. Additionally, PTEN and PI3K/AKT pathway were identified as direct targets of miR-519a. These data suggest that miR-519a may be a useful diagnostic and prognostic biomarker and a novel therapeutic target for HCC.

miR-1180. Recent studies have demonstrated that low expression of miR-1180 was associated with poor overall survival in patients with renal cell carcinoma (153). miR-1180 had suppressive effects on cell proliferation and induced p21 expression, which contributed to cycle arrest, in bladder cancer cells. Tan *et al* (139) investigated the molecular mechanisms of miR-1180 in apoptosis resistance in HCC. miR-1180 inhibition increased cell apoptosis, while miR-1180 directly targeted OTUD7B and TNIP2, which inhibited the NF-κB signaling pathway. Zhou *et al* (154) also reported that miR-1180 promoted the proliferation of HCC cells by repressing TNIP2 expression. These studies indicate that miR-1180 may act as a tumor promoter by targeting TNIP2 and resisting apoptosis via activation of the NF-κB signaling pathway.

3. Background liver factors

Unlike other carcinomas, HCC frequently recurs in residual liver after curative surgical resection. The recurrence in residual liver shows two patterns, IM and MO. IM occurs from tumor cells that spread into the remnant liver via the portal vein from the primary lesion. MO occurs from new HCC foci that develops due to the presence of HCC-relevant risk factors in non-cancerous liver tissue (155,156). Thus, hepatocarcinogenesis is greatly influenced by the state of the background liver. HCCs with MO recurrence vary in the differentiation degree and the epigenetic tumor factors in each nodule, even within a single case. However, there must

Table II. Putative tumor-suppressor genes in hepatocellular carcinoma.

Symbol (location)	Biological function	Expression	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
ASGR1	Subunit of the asialo-glycoprotein receptor	mRNA, WB, IHC	234	OS, stage	Migration, invasion	LASS2	Yes	(66)
ASK1 (6q22.33)	Mitogen-activated protein kinase	mRNA, WB, IHC	60	OS	Proliferation, migration, invasion, apoptosis	HNF4a, p38	Yes	(67)
BTG1	Regulates cell growth and differentiation	mRNA, IHC	151	OS, RFS, PIVKA-II, size, differentiation, vascular invasion, stage, extra-hepatic recurrence	-	-	-	(68)
DENND2D (1p13.3)	Membrane trafficking protein regulating Rab GTPases	mRNA, IHC	92	OS, RFS	-	-	-	(69)
FBP1 (9q22.3)	Gluconeogenesis regulatory enzyme	mRNA, WB	180	OS, tumor size, differentiation	Proliferation	-	-	(70)
FOXN3 (14q31.3)	Transcription factor	mRNA, IHC	60	-	Proliferation	E2F5	Yes	(71)
HPCAL1 (2p25.1)	Neuron-specific calcium-binding proteins	WB, IHC	90	OS	Proliferation, cell cycle	p21	Yes	(72)
JMJD5 (16p12.1)	Histone lysine demethylase	mRNA, IHC	143	OS, age, T stage cell cycle	Proliferation,	CDKN1A	Yes	(73)
KAL1 (Xp22.32)	Unknown	mRNA, IHC	144	OS, AFP, PIVKA-2, tumor size, differentiation, formation of capsule, vascular invasion	-	EZR	-	(74)
MEG3 (14q32)	Unknown	mRNA, WB	72	OS, RFS, tumor size, grade	Proliferation, apoptosis	p53, UHRF1	Yes	(75)
PBLD (10q21.3)	Unknown	mRNA, WB, IHC	108	OS, RFS, differentiation, stage	Proliferation, invasion	E-cadherin, ZO-1, N-cadherin, β-catenin, vimentin	Yes	(76)
PDCD4 (10q24)	Transcription factor	mRNA	56	OS, differentiation, metastasis	-	-	-	(77)
PDSS2 (6q16.3-21)	Synthesis of coenzyme Q10	mRNA, IHC	151	OS, RFS, AFP, vascular invasion, differentiation, serosal invasion, stage	-	HNF4a, CDX2	-	(78)

Table II. Continued.

Symbol (location)	Biological function	Expression	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
RASSF10 (11p15.2)	Unknown	mRNA, WB, IHC	288	OS, DFS, differentiation, cirrhosis, tumor thrombus, BCLC stage	Proliferation, migration, invasion, EMT, apoptosis	E-cadherin, ZO-1, N-cadherin, β-catenin, vimentin	Yes	(79)
SUSD2 (22q11-q12)	Unknown	mRNA, WB, IHC	180	Grade, stage, T status, N status, M status apoptosis	Proliferation, migration, invasion,	-	-	(80)
SYNJ2BP (14q24.2)	Unknown	mRNA, WB, IHC	98	OS, DFS, tumor size, tumor nodule number, vascular invasion, stage, BCLC stage	Proliferation, migration, invasion, metastasis	-	Yes	(81)
TIP30 (11p15.1)	Unknown	mRNA, WB	209	OS, AFP, HBV^+	Proliferation differentiation	-	-	(82)
TPD52 (8q21.13)	Unknown	mRNA, WB, IHC	154	OS, DFS, stage	-	p21, p53, MDM2, BCL2, P-GSK-3b	-	(83)
TUSC1 (9p21.2)	Unknown	mRNA, IHC	94	DSS, stage	-	-	-	(84)
ZFP191 (18q12)	Unknown	mRNA, WB, IHC	129	OS, intrahepatic metastasis, vascular invasion	Migration, metastasis	DLG1, YAP	Yes	(85)

WB, western blotting; IHC, immunohistochemistry; OS, overall survival; RFS, recurrence-free survival; PIVKA-2, protein induced by vitamin K absence or antagonist-2; AFP, serum α-fetoprotein; DFS, disease-free survival; DSS, disease-specific survival.

Table III. Candidate tumor-suppressor genes detected by combination array analysis.

Symbol (location)	Array method			No. of Pts.	Survival	Relevant clinical factors	Ref.
	Expression	SNP	Methylation				
MT1G (16q13)	Yes	Yes	-	48	-	-	(101)
EFEMP1 (2p16)	Yes	Yes	-	48	OS	Liver damage, AFP	(102)
LIFR (5p13-p12)	Yes	Yes	-	48	-	-	(103)
FBLN1 (22q13.31)	Yes	Yes	-	48	-	Tumor multiplicity, tumor size, pStage	(104)
BLMH (17q11.2)	Yes	Yes	Yes	48	-	-	(106)
RELN (7q22)	Yes	Yes	-	48	DFS	-	(100)
AKAP12 (6q24-q25)	Yes	Yes	-	48	OS	-	(105)
ESR1 (6q25.1)	Yes	Yes	Yes	48	-	-	(107)
DNM3 (1q24.3)	Yes	Yes	Yes	48	DSF	Expansive growth	(108)
DCDC2 (6p22.1)	Yes	Yes	Yes	48	OS	-	(109)
COL1A1 (17q21.33)	Yes	Yes	Yes	48	OS	Liver damage, capsule formation	(110)
PTK7 (6p21.1-p12.2)	Yes	Yes	Yes	48	OS	Age, PIVKA-II	(111)
CCNJ (10q23.33)	Yes	Yes	Yes	85	OS	-	(112)

SNP, single nucleotide polymorphism; No. of Pts., number of patients; OS, overall survival; AFP, serum α-fetoprotein; pStage, UICC pathological stage; DFS, disease-free survival; DSF, disease-specific survival; PIVKA-II, protein induced by vitamin K absence or antagonist-2.

be some shared carcinogenic characteristics in the underlying epigenetic background of non-cancerous liver tissue in cases with MO. Identifying the mechanisms of MO may contribute to the development of carcinogenic markers for HCC and to the prevention of the development of HCC. Some researchers reported various molecular changes in the background liver of HCC patients (157-161). Okamoto *et al* stated that specific gene expression profiling in non-cancerous liver tissue may predict the risk of MO recurrence (157). Hoshida *et al* showed that gene expression profiles in non-cancerous liver tissue were associated with patient outcome (158). Utsunomiya *et al* reported that specific molecular signatures, including miRNAs, in non-cancerous liver tissue contributed to hepatocarcinogenesis and recurrence of HCC (159-161). However, there are few studies that refer to individual molecules in the background liver tissue of HCC. Recently, we attempted to identify these background liver factors using our combination array analysis approach. In the present review, we summarize our methods and introduce potential background liver factors.

Methods. Control samples, termed supernormal (SN) liver, were obtained from the normal tissues of 11 patients with metastatic liver cancer who underwent liver resection. For comparison, non-neoplastic liver tissue, termed corresponding normal (CN) liver, was obtained from a typical HCC case that resulted from chronic hepatitis C. This patient was a 58-year old man with liver cirrhosis who had undergone liver resection but experienced recurrence 3 years after the primary lesion resection. Genomic DNA and total RNA were extracted from the SN and CN tissues. Expression profiling and methylation array were performed to compare the SN and CN samples and identify genes with differential expression and the methylation rate.

Thimet oligopeptidase 1. Thimet oligopeptidase 1 (THOP1) was first identified as a molecule that was related to late-onset familial Alzheimer disease by Meckelein *et al* (162). Qi *et al* (163) later found that THOP1 expression was suppressed in non-small cell lung cancer and that low expression of THOP1 in cancerous tissue was correlated with poor prognosis. Nomoto *et al* (164) identified THOP1 as a background liver factor for hepatocarcinogenesis by combination array analysis. Expression array results showed that expression of THOP1 was decreased 4.119-fold in CN. Methylation array showed a higher value for CN (0.869) than SN (0.488). Downregulation of THOP1 was shown in HCV-positive background liver as well as in hepatitis B virus-positive and non-B non-C hepatitis virus background liver. The group with higher THOP1 expression than average showed significant correlations with prolonged survival. Strongly reduced THOP1 expression was shown to be an independent prognostic factor for overall survival. The authors concluded that expression of the THOP1 gene in the background liver of HCC is likely to be a good biomarker for the risk of HCC development.

Janus kinase 2. Janus kinase 2 (JAK2), which functions in the JAK/STAT pathway, is a tyrosine kinase involved in various processes such as cell growth, development, differentiation and histone modifications. JAD2 was found to contribute to oncogenesis through activation of STAT3 in various human solid tumor cell lines (165). The activation of the JAK/STAT pathway in HCC was previously demonstrated by

Table IV. Dysregulated miRNAs in hepatocellular carcinoma.

miRNA	Sample	N	Relevant clinical factors	Functional analysis	Interacting molecules	*In vivo*	Ref.
miR-7	Tissue	18	-	Proliferation	NF90-NF45, EGFR, p-AKT	-	(113)
miR-22	Tissue	162	OS, tumor size, differentiation, stage, distant metastasis	Apoptosis	Galectin-1	-	(114)
miR-26b-5p	Tissue	23	RFS	Migration, invasion, EMT	SMAD1	Yes	(115)
miR-101	Tissue	20	-	Migration, invasion	VEGF-C	-	(116)
miR-106b	Tissue	120	OS, DFS, HBV (+)	-	MCM7, miR-93, miR-25	-	(117)
miR-127-5p	Tissue	111	Grade, vascular invasion	Proliferation	NF-κB, p65, BLVRB	-	(118)
miR-133b	Tissue	37	-	Proliferation, invasion, apoptosis	Sirt1, E-cadherin, GPC3, Bcl-2, Bcl-xL, Mcl-1, β-catenin	Yes	(119)
miR-135a	Tissue	-	-	Migration, invasion	FOXO1, MMP2, Snail, p-AKT, FOXO3a	-	(120)
miR-137	Tissue	110	OS, DSS, vascular invasion, bile duct invasion, AFP	-	-		(121)
miR-144	Tissue	100	Recurrence	Invasion, metastasis, cell cycle, EMT, chemoresistance	SMAD4	-	(122)
miR-155-3p	Tissue	45	OS	Proliferation	FBXW7	Yes	(123)
miR-186	Cell line	-	-	Proliferation, migration, invasion	YAP1	-	(124)
miR-192	Tissue	101	OS, vascular invasion	Metastasis	SNAIL, SLC39A6, E-cadherin	Yes	(125)
miR-211	Tissue	227	OS, vein invasion, stage	Proliferation, migration, invasion	SPARC	Yes	(126)
miR-214	Tissue	25	-	Proliferation	UCP2	-	(127)
miR-224	Tissue, plasma	211	Tumor size, stage, recurrence	-	-	-	(128)
miR-367	Tissue	35	-	Proliferation, migration, invasion	PTEN	-	(129)
miR-379-5p	Tissue	85	Stage, metastasis	Migration, invasion, EMT, metastasis	FAK	Yes	(130)
miR-449a	Tissue	40	-	Proliferation, migration, invasion	ADAM10	-	(131)
miR-497	Tissue	86	OS, DFS, AFP, tumor size, grade, T stage	Proliferation, apoptosis	YAP1	-	(132)
miR-502-3P	Tissue	50	-	Proliferation, invasion, metastasis, cell adhesion	SET	-	(133)
miR-503	Tissue	87	Grade, nodal metastasis, vascular invasion, stage	Proliferation, apoptosis	IGF-1R	-	(134)
miR-519a	Tissue	116	OS, DFS, tumor size, grade, stage, venous infiltration	Proliferation, cell cycle	PTEN	-	(135)
miR-613	Tissue	38	-	Proliferation, invasion	DCLK1	Yes	(136)
miR-655-3p	Tissue	84	Tumor size, PVTT, differentiation, stage, metastasis	Proliferation, migration, invasion	ADAM10	-	(137)
miR-761	Tissue	50	-	Proliferation, metastasis	MFN2	Yes	(138)
miR-1180	Tissue	7	-	Proliferation, apoptosis	OTUD7B, TNIP2, BAD	Yes	(139)

OS, overall survival; RFS, recurrence-free survival; EMT, endothelial-mesenchymal transition; DFS, disease-free survival; DSS, disease-specific survival; AFP, serum α-fetoprotein.

measuring the phosphorylation of JAK/STAT proteins (166). Sonohara *et al* (167) reported that higher JAK2 expression in CN significantly correlated with shorter overall survival while JAK2 expression in HCC did not relate to prognosis statistically. The authors suggested that higher JAK2 expression in the background liver tissue of HCC could reflect carcinogenesis potential and may be a good prognostic biomarker for resected HCC.

4. Conclusions

The improvement in array technologies and the development of next-generation sequencing have contributed to the identification of several tumor factors in HCC that may serve as novel molecular targets for treating recurrence and biomarkers for predicting the prognosis. Further research in this direction should lead to the establishment of background liver factors, which may contribute to the development of carcinogenic markers of HCC and the prevention of the development of HCC.

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