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TOPIC HIGHLIGHT

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Genetic and epigenetic aspects of initiation and progression of hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is a primary cancer of the liver that is predominant in developing countries and is responsible for nearly 600000 deaths each year worldwide. Similar to many other tumors, the development of HCC must be understood as a multistep process involving the accumulation of genetic and epigenetic alterations in regulatory genes, leading to the activation of oncogenes and the inactivation or loss of tumor suppressor genes. Extensive research over the past decade has identified a number of molecular biomarkers, including aberrant expression of HCC-related genes and microRNAs. The challenge facing HCC research and clinical care at this time is to address the heterogeneity and complexity of these genetic and epigenetic alterations and to use this information to direct rational diagnosis and treatment strategies. The multikinase inhibitor sorafenib was the first molecularly targeted drug for HCC to show some extent of survival benefits in patients with advanced tumors. Although the results obtained using sorafenib support the importance of molecular therapies in the treatment of HCC, there is still room for improvement. In addition, no molecular markers for drug sensitivity, recurrence and prognosis are currently clinically available. In this review, we provide an overview of recently published articles addressing HCC-related genes and microRNAs to update what is currently known regarding genetic and epigenetic aspects of the pathogenesis of HCC and propose novel promising candidates for use as diagnostic and therapeutic targets in HCC.

Key words: Hepatocellular carcinoma; Oncogene; Tumor suppressor gene; MicroRNA; DNA methylation

Core tip: Despite the large number of studies dedicated to the molecular diagnosis of hepatocellular carcinoma (HCC), highly sensitive biomarkers of the initiation and progression of HCC still need to be identified. At the same time, the development of novel molecular targeting agents that can surpass the effect of the

multikinase inhibitor sorafenib is much-anticipated. This review aimed to update our knowledge of genetic and epigenetic aspects of HCC by providing an overview of novel HCC-related genes and microRNAs as candidates for use as diagnostic and therapeutic targets in HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the most common cancers worldwide and is the third leading cause of cancer death, accounting for approximately 600000 deaths annually[1,2]. Although the prevalence of HCC remains highest in eastern Asia and Africa, the incidence is steadily increasing in Western countries[3]. Controlling HCC is difficult, as recurrence or metastasis is quite common in patients, even after the application of successful topical therapies or curative hepatectomy[4,5]. In addition to the high prevalence of hepatitis C virus (HCV) infection as the main reason for the increasing incidence of HCC in Western countries, multiple etiologic factors, including chronic viral hepatitis B, alcohol consumption and aflatoxin, have been reported to lead to liver damage and to an increased incidence of HCC[6-8]. This situation leads not only to the requirement for a more complex clinical approach for HCC but also to high molecular variability of the disease[6].

Complex genetic and epigenetic alterations, chromosomal aberrations, mutations, and altered molecular pathways lead to the development of HCC[9,10]. Analyses of genetic and epigenetic alterations and different molecular pathways involved in the development of HCC help to identify potential new diagnostic tools and druggable targets[10-12]. The establishment of a robust molecular classification will pave the way for a more personalized treatment scheme for HCC[10,13]. In addition, targeted therapies for HCC are currently under intensive investigation, and accumulating evidence suggests that combination therapy targeting different pathways will potentiate anti-tumoral effects and will become the therapeutic approach adopted in the future[14,15]. The success of sorafenib has proven the concept that targeted therapy can confer survival benefits on patients with HCC, although these benefits have been limited thus far[10,16]. Although the heterogeneity of HCC makes it difficult to clarify the mechanism of cancer development and to develop effective therapeutics, molecules that are potentially responsible for the initiation or progression of HCC have been reported in succession[6,7,17]. In this article, we review current, updated knowledge regarding the molecular pathogenesis of HCC relevant to the development of novel diagnostic tools and therapeutic targets by providing an overview of known genetic and epigenetic alterations without confinement to specific gene groups, functions and pathways. Additionally, some representative genes were selected from each category by the following criteria: (1) highly-innovative genes or microRNA (miRNA); (2) data from relatively large number of patients; and (3) solid data by functional analyses or *in vivo* study, and important findings were summarized.

FACILITATORS OF HCC TUMORIGENESIS; PUTATIVE ONCOGENES

Abrupt changes in hepatocytes due to viral infection or exposure to hepatotoxic stress cause activation of oncogenes *via* point mutations, gene amplification, or changes in the promoter region, resulting in tumor development[18,19]. Subsequently, increased expression of several oncogenes can influence the survival of cancerous cells by suppressing apoptosis and regulating the cell cycle[18,20]. Thus, artificial inhibition of oncogenes or their upstream genes could be a novel therapeutic approach. Recently reported genes that are upregulated in HCC are summarized in Table 1[12,21-51]. Here, we cull some studies from the list and introduce cogent findings.

Interleukin-6

A relationship between chronic hepatic inflammation and HCC pathogenesis has been identified in previous epidemiologic studies[52]. Following infection with hepatitis viruses, Kupffer cells activate nuclear factor-kB and secrete inflammatory cytokines, including Interleukin (IL)-*6*[53]. Chang *et al*[43] investigated the roles of IL-6 in HCC and found that high serum levels of *IL-6* were significantly correlated with high expression levels of *OCT4*/*NANOG*, which is a pluripotent transcription factor. IL-6 stimulated the expression of an autocrine insulin-like growth factor-I and its receptor, depending on signal transducer and activator of transcription 3, which in turn stimulated stemness-related properties in both cell lines and xenografted mouse tumors. Inhibition of the insulin-like growth factor-Ⅰ receptor *via* RNA interference or the use of an inhibitory agent significantly suppressed the *IL6*-induced stemness-related properties, both *in vitro* and *in vivo*[43]. Hence, it was indicated that *IL-6* plays an important role in the pathogenesis of HCC, which is initiated by chronic inflammation.

Metastasis associated with colon cancer 1

The metastasis associated with colon cancer 1 (*MACC1*) gene was first identified as the critical pro-metastatic factor in human colon cancer and has been demonstrated to activate the hepatocyte growth factor

Table 1 Genes upregulated in hepatocellular carcinoma; putative oncogenes

Symbol (location)	Biological function	Expression	*n*	Relevant clinical factors	Functional analyses	Interacting molecules	*In vivo*	Ref.
AURKA (20q13)	Cell cycle-regulated kinase	IHC	44	OS, RFS, number, stage	Chemoresistance, apoptosis	NF-κB, miRNA-21, PTEN	Yes	40
BIRC7 (20q13.3)	Inhibitor of apoptosis protein family	mRNA, IHC, WB	61	None	Proliferation, migration, invasion, apoptosis	CDK	-	21
Brachyury (6q27)	Embryonic nuclear transcription factor	mRNA, IHC	112	OS, size, stage	Migration, invasion	PTEN, Akt, Snail, EMT	-	22
CCL20 (2q36.3)	Cytokine displaying chemotactic activity for lymphocytes	IHC	62	OS, RFS, AFP, size, mumber, vascular invasion, differentiation	Proliferation, migration	pAKT, β-catenin, EMT	-	45
CCT8 (21q22.11)	Transport and assembly of newly synthesized proteins	IHC	102	OS, grade, size	Proliferation, cell cycle	-	-	30
CTSL (9q21.33)	A lysosomal cysteine proteinase for intracellular protein catabolism	mRNA, IHC, WB	82	OS, RFS, stage, differentiation	Proliferation, tumorigenesis	-	Yes	36
CYTH3 (7p22.1)	Mediator of protein sorting and membrane trafficking	mRNA, IHC	202	OS, size, vascular invasion	Proliferation, migration	-	-	24
DLX4 (17q21.33)	Forebrain and craniofacial development	mRNA, IHC, WB	226	OS, size, differentiation, AFP	-	-	-	27
GOLPH3 (5p13.3)	Peripheral membrane protein of the Golgi stack regulating Golgi trafficking	mRNA, IHC	173	OS, stage	Chemoresistance, apoptosis, angiogenesis, proliferation	NF-κB	Yes	44
GSC (14q32.1)	Autoregulatory transcription factor	mRNA, IHC, WB	112	OS, distant metastasis	Migration, invasion	EMT	-	39
HSF1 (8q24.3)	Heat-shock transcription factor rapidly induced after temperature stress	IHC	67	OS	Proliferation	-	-	34
IL-6 (7p21)	Cytokine that functions in inflammation and the maturation of B cells	mRNA, IHC, WB, ELISA	120	RFS, vascular invasion, number	Stemness	IGFIR, OCT4, NANOG	Yes	43
ISG15 (1p36.33)	Chemotactic activity towards neutrophils	mRNA, IHC	50	OS, differentiation, distant metastasis	Proliferation, migration	Survivin	Yes	33
KPNA2 (17q24.2)	Nuclear transporter of proteins and VJ recombination	mRNA, IHC	314	OS, RFS, size	Proliferation, metastasis	PLAG1	-	29
LGALS3 (14q22.3)	Member of the galectin family of carbohydrate binding proteins	mRNA, IHC, WB	165	OS, microvessel density	Proliferation, migration, invasion, apoptosis	-	-	31
MACC1 (7p21.1)	Regulator of *HGF-HGFR* pathway	mRNA, IHC, WB	50	OS, grade, stage	Proliferation, apoptosis	HGF, c-MET, PI3K, AKT, Caspase 9	Yes	47
MAGED2 (Xp11.21)	Promotor of the cancer cell adhesion to the vascular epithelium	mRNA, IHC	151	OS	-	-	-	51
MAGED4 (Xp11.22)	Unknown	mRNA, IHC	94	OS, RFS, AFP, vascular invasion, differentiation	-	-	-	50
MCT4 (17q25)	Catalyzing lactic acid and pyruvate transport across plasma membranes.	IHC	318	OS, RFS, AFP, size	Proliferation, migration, invasion	pAKT, HIF-1α	-	26
MRC2 (17q23.2)	Extracellular matrix remodeling by mediating the internalization	IHC	96	OS, number, vascular invasion	Migration, invasion	TGFb	-	25
PDGFRα (4q12)	Cell surface tyrosine kinase receptor for platelet-derived growth factor family	mRNA	57	OS, RFS, vascular invasion, microvessel density	Proliferation, migration, invasion	EMT	Yes	38
PKM2 (15q22)	Pyruvate kinase generating ATP and pyruvate	mRNA, IHC, WB	721	OS, RFS, size, vascular invasion, differentiation	Proliferation, migration, invasion	pAKT, HIF-1α	Yes	46
PLK1 (16p12.2)	Controlling mitotic entry, centrosome maturation, and bipolar spindle formation	IHC	67	Differentiation, capsule invasion	Proliferation, cell cycle, apoptosis	caspase 3/8, Bax, Bcl-2	-	37
PTP4A1 (6q12)	Regulator of cellular processes, including cell proliferation and migration	IHC	167	OS	Migration, invasion	PI3K/AKT/GSK3β pathway	-	32

SERPINB3 (18q21.3)	Ovalbumin family of serine proteinase inhibitors	mRNA, IHC	67	RFS	-	HIF-2α	-	41
SIRT1 (10q21.3)	Regulating epigenetic gene silencing and suppress recombination of rDNA	mRNA, IHC, WB	99	OS, size, number, stage	Migration, invasion	EMT	Yes	28
		mRNA	-	-	Proliferation, cell cycle	miR29c	-	58
		IHC	248	RFS	Proliferation, cell cycle	p53, AMPK, mTOR	-	59
TPX2 (20q11.2)	Microtubule organization during mitosis	mRNA, IHC	130	OS, RFS, stage, number, differentiation	Migration, invasion	MMP2/9, pAKT	-	35
		mRNA, IHC	86	OS, RFS, vascular invasion, stage	Proliferation		Yes	62
TREM1 (6p21.1)	Receptor belonging to the Ig superfamily that is expressed on myeloid cells	mRNA, IHC, WB	322	OS, RFS, age, AFP	Proliferation, invasion, cell cycle, apoptosis	IL-1β, TNF-α, MCP-1, p65, STAT3, ERK	-	23
UBE2Q1 (1q21.3)	Member of the E2 ubiquitin-conjugating enzyme family	IHC	86	OS, grade	Proliferation, cell cycle	p53, p21	-	42
UBE4B (1p36.3)	Conjugation factor E4 involved in multiubiquitin chain assembly	mRNA, IHC, WB	149	OS, grade, stage	Proliferation, migration, invasion, apoptosis	p53, Bcl-2, caspase 3	-	48
VEGF-B (11q13)	Regulator of angiogenesis	mRNA	48	Number, vascular invasion, capsule invasion, stage	-	-	-	12
YY1AP1 (1q22)	Unknown	Microarray	76	OS	Proliferation, apoptosis, spheroid-formation	EpCAM	Yes	49

IHC: Immunohistochemistry; WB: Western blotting; OS: Overall survival; RFS: Recurrence free survival; AFP: Alpha-fetoprotein; PIVKA: Protein induced by vitamin K antagonists.

(*HGF*)/cellular MET proto-oncogene (*c-MET*) pathway through binding to the promoter of *c-MET*[54,55]. Yao *et al*[47] reported that *MACC1* expression was upregulated in HCC tissues and correlated with poor patient outcome. In functional analyses, *MACC1* repressed HCC cell apoptosis and promoted cell growth, effects which were abolished by the knockdown of *c-MET*. *MACC1* activated phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT signaling by sensitizing *HGF*/*c-MET* signaling and enhanced the *HGF*-driven phosphorylation of *BCL2*-associated agonist of cell death, caspase-9 and forkhead box O3 and inhibited their pro-apoptotic functions in HCC cells[47]. These authors also demonstrated that *MACC1* inhibited cell apoptosis and promoted HCC growth in an *in vivo* study[47]. Thus, *MACC1* may be a key factor in resistance to apoptosis in HCC.

Sirtuin 1

Sirtuin 1 (SIRT1) is a member of the mammalian sirtuin family, which plays a critical role in the regulation of critical biological processes such as metabolism, aging, oncogenesis, and cancer progression[56]. *SIRT1* is the most well-characterized member of the sirtuin family and plays a key role in both cell death and survival, together with other p53 family members, forkhead box, sub-group O, transcription factors, and the nuclear factor-κB family[57]. Hao *et al*[28] found that SIRT1 was overexpressed in HCC cells and tissues and significantly promoted the migration and invasion ability of HCC cells by inducing the epithelial-mesenchymal transition. An *in vivo* study supported the oncogenic functions of *SIRT1* in enhancing metastasis[28]. Bae *et al*[58] found that knockdown of *SIRT1* inhibited cell growth by transcriptional deregulation of cell cycle proteins, leading to hypophosphorylation of *pRb*, which inactivated *E2F/DP1* target gene transcription, and thereby caused the G1/S cell cycle arrest. In addition, miR-29c was identified as a suppressor of *SIRT1* by comprehensive miRNA profiling and ectopic miR-29c expression recapitulated *SIRT1* knockdown effects in HCC cells[58]. On the contrary, Zhang *et al*[59] reported that *SIRT1* has anti-carcinogenic effects in HCC *via* the *AMPK*-mammalian target of rapamycin (mTOR) pathway. They evaluated the relationship between p53 mutations and activation of *SIRT1* in 252 patients with hepatitis B virus-positive HCC and found that activated *SIRT1* was associated with a longer recurrence free survival in HCC tissues harbouring mutant *p53*. Inhibition of *SIRT1* increased cell growth, bearing mutated *p53*, by suppressing AMPK activity and enhancing *mTOR* activity[59]. The conflicting results from different papers indicated that *SIRT1* is multi-functional gene and its biological features are left unsolved.

Targeting protein for Xenopus kinesin-like protein 2

Targeting protein for Xenopus kinesin-like protein 2 (TPX2) is a microtubule-associated protein that affects spindle assembly in human cells and is upregulated in multiple tumor types[60,61]. Liu *et al*[35] revealed that TPX2 expression was elevated in HCC cells and tissues. Clinical analysis indicated that TPX2 expression

in HCC tissues was evidently correlated with tumor stage, numbers and differentiation and with patient survival. TPX2 expression was positively correlated with matrix metalloproteinase (MMP) 2 and *MMP9*, and knockdown of *TPX2* using small interfering RNA prominently reduced cell invasion and migration and decreased the expression of phosphorylated *AKT*, *MMP2* and *MMP9*. TPX2 was able to activate the kinase activity of Aurora A[35]. Furthermore, TPX2 upregulated the activation of the PI3K/AKT signaling pathway and may mechanistically be associated with the activity of MMP2, ultimately causing proliferation of cancer cells[35]. Huang *et al*[62] also investigated the expression and functions of TPX2 in HCC. They found that *TPX2* was overexpressed in HCC cells and tissues from 86 patients, and positive *TPX2* expression was significantly associated with vascular invasion, advanced stage and shortened overall and recurrence-free survival. Knockdown of TPX2 reduced cell proliferation and viability in HCC cells and slowed down tumor growth in a mouse xenograft model[62]. Taken together, these studies indicated that TPX2 may serve as a prognostic marker and promotes tumorigenesis and metastasis of HCC.

Triggering receptors expressed on myeloid cells 1

Triggering receptors expressed on myeloid cells 1 (TREM1) is an approximately 30-kDa transmembrane glycoprotein belonging to the immunoglobulin superfamily[63]. TREM-1 consists of a single extracellular Ig-like region, a transmembrane domain, and a cytoplasmic domain with no signaling motifs. Activation of TREM-1 increases the secretion of tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating-factor and certain chemokines and cytokines, suggesting that *TRM1* modulates inflammation[64]. Duan *et al*[23] evaluated *TREM1* expression in 322 patients with HCC and found that increased *TREM-1* expression was associated with recurrence and poorer survival and was identified as an independent prognostic factor for recurrence. In functional analyses, *TREM-1* significantly promoted the proliferation and invasion of HCC cells and inhibited their apoptosis[23]. The levels of the proinflammatory cytokines *IL-1b* and *TNF-α* were shown to be regulated by *TREM1* expression, suggesting that *TRM1* modulates inflammation in liver tissues[23]. Thus, *TREM1* may be a modulator of inflammation and serve as an important prognostic marker for HCC.

Melanoma-associated antigens D2 and D4

Melanoma-associated antigens (MAGE) are tumor-specific antigens and have increasingly been utilized as therapeutic targets for immunotherapy[65]. MAGE proteins are classified into types Ⅰ and Ⅱ. Type Ⅰ *MAGE* genes are located on the X-chromosome and include MAGE-A, B and C, which are expressed during germ cell development, but not by mature somatic cells[66]. By contrast, the localization, expression and oncological functions of type Ⅱ MAGE proteins, which include MAGE-D, E, F, G and H, are less clear[67,68]. The function of MAGE-D2 is unclear; however, increased MAGE-D2 expression may promote cancer cell adhesion to the vascular epithelium. Moreover, MAGE-D2 has been reported to protect melanoma cells from tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis[51]. MAGE-D4 is specifically expressed in normal brains and ovaries and reportedly contributes to the proliferation, migration, and invasion of tumor cells in breast cancer and oral squamous cell carcinoma[50]. We recently evaluated the clinical significance of the expression of MAGE-D2 and D4 in HCC. Increased expression of both *MAGE-D2* and *D4* was associated with shorter postoperative survival of patients with HCC and was identified as an independent prognostic factor[50,51]. The expression levels of MAGE-D2 and D4 in HCC tissues represent promising prognostic markers, and functional analyses are warranted.

GENES SILENCED IN HCC, PUTATIVE TUMOR SUPPRESSORS

Tumor suppressor genes (TSGs) are typically inactivated *via* mutation, deletion, or promoter methylation, which silences gene expression[69]. Inactivation of TSGs is a crucial process for HCC tumorigenesis and for the activation of oncogenes[70]. Table 2 provides an updated list of the genes known to be suppressed in HCC without hypermethylation[71-80], and overviews of some individual representative genes are provided.

Aryl-hydrocarbon receptor nuclear translocator 2

Aryl-hydrocarbon receptor nuclear translocator (ARNT)-2 is a transcriptional regulator and a member of the basic helix-loop-helix/Per-ARNT-SIM superfamily, which are heterodimeric transcription factors that sense and respond to environmental signals or to physiological signals through their two PAS domains[81,82]. Li *et al*[79] showed that a high expression level of ARNT2 in HCC tissues was associated with prolonged overall and recurrence-free survival. Knockdown of *ARNT2* significantly increased the cell proliferation, invasion and migration ability, whereas forced expression of *ARNT2* inhibited the activities of HCC cells[79]. The influences of *ARNT2* on tumor growth were demonstrated in an *in vivo* study[79]. Hence, ARNT2 may be a potential biomarker and therapeutic target for HCC.

B-cell translocation gene 1

B-cell translocation gene 1 (*BTG1*) is a translocation partner of the c-Myc gene in the case of B-cell chronic lymphocytic leukemia and belongs to a family of antiproliferative genes[83,84]. *BTG1* is constitutively expressed in quiescent cells, and its expression is downregulated as cells enter the growth cycle[85]. In breast and ovarian cancer, artificial expression of BTG1

Table 2 Genes suppressed in hepatocellular carcinoma; putative tumor suppressors

Symbol (location)	Biological function	Expression	*n*	Relevant clinical factors	Functional analyses	Interacting molecules	*In vivo*	Ref.
ARNT2 (15q24)	Partner for several sensor proteins of the bHLH-PAS family	IHC	195	OS, RFS, age, size, number, vascular invasion, AFP, differentiation, stage	Proliferation, invasion, migration	-	Yes	79
BTG1 (12q22)	Regulates cell growth and differentiation	mRNA, IHC	151	OS, RFS, PIVKA-II, size, differentiation, vascular invasion, stage, extra-hepatic recurrence	-	-	-	80
CDH3 (16q22.1)	Calcium-dependent cell-cell adhesion glycoprotein	IHC	69	Stage	Proliferation	-	-	71
EBP1 (12q13.2)	RNA-binding protein involved in cell growth regulation	IHC, WB	103	OS, grade, size	Growth, cell cycle	-	-	74
FBLN5 (14q32.1)	Secreted extracellular matrix protein	mRNA, IHC	86	OS, number, vascular invasion, grade, stage	Migration, invasion	MMP7	-	76
Fbxw7 (4q31.3)	Ubiquitin-mediated degradation of cyclin E	IHC	60	OS, size, vascular invasion, grade, stage	Growth, apoptosis	YAP	Yes	77
GPx3 (5q23)	Detoxification of hydrogen peroxide	mRNA, IHC, ELISA	113	OS, RFS, size, number, vascular invasion, stage	Proliferation, invasion	Erk, NFκB, SIP1	Yes	75
IGFBP-1 (7p12.3)	Mediator of the IGFs	IHC	90	OS, differentiation, cirrhosis, vascular invasion, stage	Invasion	MMP9	-	73
LGALS4 (19q13.2)	Modulating cell-cell and cell-matrix interactions	mRNA, IHC, ELISA	201	Size, differentiation, vascular invasion, stage	Migration, invasion	-	-	72
TIPE1 (19p13.3)	Unknown	IHC	50	OS, grade	Growth, apoptosis	Nec-1, Rac-1	Yes	78

IHC: Immunohistochemistry; WB: Western blotting; OS: Overall survival; RFS: Recurrence free survival; AFP: Alpha-fetoprotein; PIVKA: Protein induced by vitamin K antagonists.

mediated Bcl-2-regulated apoptosis and suppressed the proliferation of cancer cells[85]. We recently reported that BTG1 expression was frequently reduced in HCC cells and tissues without point mutations or promoter hypermethylation[80]. Downregulation of BTG1 was significantly associated with poorer survival after curative hepatectomy[80]. Thus, BTG1 can be nominated as a novel predictor of the recurrence of HCC, and elucidation of the associated regulatory mechanisms is expected.

Glutathione peroxidase 3

Glutathione peroxidase 3 (GPx3) was found to be upregulated in acute-phase injury as an anti-oxidant to protect the organ from oxidative stress by detoxifying hydrogen peroxide and other free radicals[86]. Qi *et al*[75] demonstrated tumor suppressive functions of GPx3 in HCC. Downregulation of GPx3 in HCC tissues was significantly correlated with advanced stages, vascular invasion and poor overall survival. In addition, the plasma GPx3 level was significantly associated with tumor size, the number of nodules and the recurrence rate. Artificial expression of GPx3 inhibited the proliferation and invasion of HCC cells both *in vitro* and *in vivo*. The tumor suppressive activity of GPx3 was mediated through the Erk-NFκB-SIP1 pathway[75]. Notably, GPx3 could be delivered into tumors by human-induced pluripotent stem cell-derived mesenchymal stem cells and exhibited tumor suppressive activity *in vivo*[75]. Hence, GPx3 is a candidate HCC-related TSG.

Fibulin-5

Fibulin-5 (FBLN5) is a member of the fibulin family, which is characterized by calcium-binding epidermal growth factor-like repeats and a globular carboxyl-terminal fibulin-type structure[87]. FBLN5 contains an integrin-binding arginyl-glycyl-aspartic acid motif, which binds to integrins and mediates endothelial cell adhesion[88]. FBLN5 regulates the extracellular matrix structure and has functions in fibrogenesis, angiogenesis and tumorigenesis[89]. Tu *et al*[76] revealed that reduced expression of *FBLN5* was frequently found in HCC cell lines and HCC tissues and was associated with multiple tumors, vascular invasion, advanced stages, and consequently, poorer outcomes. In addition, forced expression of FBLN5 significantly inhibited the migration and invasion of HCC cells. Knockdown of *MMP7* inhibited the migration and invasion of HCC cells, and restoring MMP7 expression abrogated the tumor-suppressive effect of FBLN5[76]. It was indicated that FBLN5 acts as a TSG through the suppression of MMP7 in HCC.

GENES THAT ARE EPIGENETICALLY SUPPRESSED IN HCC

The results of epigenetic analyses and methylation and miRNA profiling are contributing to the knowledge derived from gene expression data and should not be forgotten in the molecular diagnosis of HCC[6,90]. Unique epigenetic changes have been identified in different genes in different tumor types, revealing site-specific

Table 3 Genes epigenetically suppressed in hepatocellular carcinoma through promoter hypermethylation

Symbol (location)	Biological function	Expression	*n*	Relevant clinical factors	Functional analyses	Interacting molecules	*In vivo*	Ref.
CLDN3 (7q11.23)	Integral membrane protein and a component of tight junction strands	mRNA, IHC	114	OS	Motility, invasion, tumor formation	Wnt, EMT, β-catenin	Yes	99
DENND2D (1p13.3)	Membrane trafficking protein regulating Rab GTPases	mRNA, IHC	92	OS, RFS	-	-	-	100
DPT (1q12-q23)	Cell-matrix interactions and matrix assembly	mRNA, IHC, WB	202	OS, RFS, AFP, vascular invasion, differentiation	Migration, metastasis	RhoA, FAK, c-SRC, α3β1 integrin	Yes	98
DPYSL3 (5q32)	Cell-adhesion factor involved in the metastatic process of tumor cells	mRNA, IHC	151	OS, RFS, AFP, PIVKAII, gender, vascular invasion, serosal invasion	Migration, invasion	VEGF, FAK	-	105
EFEMP1 (2p16)	Extracellular matrix glycoproteins	mRNA	48	OS, liver damage, AFP	-	-	-	103
FBLN1 (22q13)	Secreted fibrillar extracellular matrix protein	mRNA, IHC, WB	48	number, size, stage	-	-	-	96
MEG3 (14q32)	Unknown	mRNA	72	OS, RFS, tumor size and Edmondson grade	Proliferation, apoptosis	p53, UHRF1	Yes	102
MT1G (16q13)	Preserve the homeostasis of metals	mRNA	48	none	-	-	-	11
PDSS2 (6q16.3-21)	Synthesis of coenzyme Q10	mRNA, IHC	151	OS, RFS, AFP, vascular invasion, differentiation, serosal invasion, stage	-	HNF4a, CDX2	-	101
RELN (7q22)	Secreted extracellular matrix protein	mRNA	48	RFS	-	-	-	104
TIF1γ (7p22.1)	Unknown	IHC, WB	204	OS, RFS, stage	Invasion, metastasis	TGF-β/Smad	-	97
TUSC1 (9p21.2)	Unknown	mRNA, IHC	94	OS, stage	-	-	-	106

IHC: Immunohistochemistry; WB: Western blotting; OS: Overall survival; RFS: Recurrence free survival; AFP: Alpha-fetoprotein; PIVKA: Protein induced by vitamin K antagonists.

methylation profiles[91-93]. In hepatocarcinogenesis, aberrant methylation of tumor-related genes does not only occur in advanced tumor stages, it is also recognized as a frequent and early event[1]. Promoter methylation of different types of TSGs has been demonstrated under premalignant conditions, such as chronic hepatitis or liver cirrhosis[94,95]. Moreover, the frequency of aberrant promoter methylation increases during the progression from precancerous lesions to HCC[1]. Therefore, epigenetic changes in preneoplastic or early neoplastic stages may serve as an indicator or biomarker for the screening of patients with an increased risk for HCC. Novel genes nominated as candidate methylated TSGs in HCC are listed in Table 3[11,96-106].

Transcriptional intermediary factor 1 gamma

Transcriptional intermediary factor 1 gamma (TIF1γ), alternatively referred to as tripartite motif 33 (TRIM33), is a member of the tripartite motif/RING finger, B-box proteins and is also a member of the E3 ubiquitin-ligase family[107]. *TIF1γ* plays a role in embryonic development, cell differentiation, transcriptional elongation, cell mitosis and the regulation of transforming growth factor beta (TGF-β) superfamily signaling[108]. Ding *et al*[97] investigated the expression and functions of *TIF1γ* in HCC. The expression level of TIF1γ in HCC was decreased in parallel with tumor stages and was significantly associated with patient outcome. TIF1γ inhibited the invasion and metastasis of HCC cells in both early- and advanced-stage HCC through the suppression of TGF-β/Smad signaling and Smad2/3/4 complex formation in HCC cells[97]. In addition, hypermethylation of CpG islands in the TIF1c promoter was found to be responsible for the downregulation of TIF1γ[97]. Thus, TIF1γ can be considered as a novel methylated TSG in HCC.

Dihydropyrimidinase-like 3

Dihydropyrimidinase-like 3 (DPYSL3) is a cell adhesion molecule and is actively expressed in normal tissues, including cardiac myocytes, the brain, pineal body, retina and smooth muscle, and is moderately expressed in various other tissues, including liver tissues[109,110]. DPYSL3 has been reported to be involved in the metastatic process in tumor cells in prostate and pancreatic cancer[111,112]. We found that DPYSL3 was downregulated in most HCC cell lines with DPYSL3 promoter hypermethylation, and its expression was restored after demethylation. DPYSL3 expression levels were

inversely correlated with those of vascular endothelial growth factor (VEGF) and focal adhesion kinase (FAK) in both HCC cells and tissues[105]. Knockdown of *DPYSL3* enhanced the migration and invasion of HCC cells. Patients with extra-hepatic recurrences exhibited a significantly lower expression level of *DPYSL3* mRNA in HCC compared with those without extra-hepatic recurrences[105]. *DPYSL3* was shown to be a putative HCC tumor suppressor regulated by promoter hypermethylation.

Dermatopontin

Dermatopontin (DPT) is a tyrosine-rich acidic extracellular matrix (ECM) protein that binds to $\alpha 3\beta 1$ integrin and to a proteoglycan receptor during cell adhesion[113]. *DPT* has multiple biological functions in physiological and pathological processes. It accelerates collagen fibrillogenesis and modulates the interaction between decorin and TGF-β[114]. Fu *et al*[98] found that downregulation of DPT was frequently observed in HCC tissues and was significantly associated with metastasis and a poor prognosis. Overexpression of *DPT* inhibited migration *in vitro* and intra-hepatic metastasis *in vivo*. They also found that *DPT* was silenced mainly due to promoter hypermethylation. Inhibition of *DPT* resulted in dysregulation of focal adhesion assembly, decreases of ras homolog family member A, FAK and c-Src tyrosine kinase phosphorylation *via* integrin signaling[98]. DPT is a putative methylated TSG mediating the metastatic ability of HCC.

Prenyl diphosphate synthase subunit 2

Prenyl diphosphate synthase subunit 2 (PDSS2) was identified in 2005[115]. It encodes the second subunit of prenyl diphosphate synthase, which is an essential enzyme involved in the biosynthesis of coenzyme Q10 (CoQ10), and PDSS2 determines the side-chain length of mammalian ubiquinones[116]. CoQ10 is synthesized from mevalonic acid in the liver and plays a vital role in the mitochondrial respiratory chain, pyrimidine nucleoside biosynthesis and the modulation of cell apoptosis[93]. Aberrant expression of PDSS2 in the liver may cause DNA damage and disrupt the cell cycle through inhibition of CoQ10 synthesis, leading to the initiation and progression of HCC[101]. Recently, we reported that *PDSS2* mRNA expression was frequently decreased in HCC cell lines and tissues[101]. The expression levels of PDSS2 were significantly correlated with those of hepatocyte nuclear factor 4α. *PDSS2* transcription in HCC cells with decreased PDSS2 expression accompanying hypermethylation was reactivated after treating these cells with a methylation inhibitor. *PDSS2* expression levels, relative to those in the uninvolved liver, decreased gradually from chronic hepatitis to cirrhosis[101]. Suppression of *PDSS2* was associated with a worse postoperative outcome. Decreased levels or hypermethylation of *PDSS2* may represent a novel biomarker of HCC.

DYSREGULATED MICRORNAS IN HCC

Growing evidence indicates a direct and interdependent link between epigenetic alterations and changes in miRNA expression, illustrating the complexity of epigenetic abnormalities in HCC[10,117]. MiRNAs are small RNA molecules, approximately 22 nucleotides long, that negatively control the expression of their target genes posttranscriptionally[118,119]. The importance of their role in cancer is increasingly being demonstrated because they can act as oncogenes or tumor suppressor genes[120]. In HCC, several miRNAs have been shown to be dysregulated. Some of these miRNAs function as oncogenes and inhibit apoptosis (miR-221), promote cell invasion (miR-9) or silence c-Met and, thus, inhibit migration and proliferation (miR23b)[121]. Others appear to show a TSG-like function (miR-101, 195, 122, 338) and are silenced in HCC[10]. MiRNA profiling is expected to be a source of additional information for better understanding the complex molecular heterogeneity of HCC, and consequently, providing a rationale for new therapeutic targets in HCC[117,118]. Here, we focus on some new discoveries regarding miRNAs involved in HCC progression (Table 4)[122-132].

miR-128-2

Zhuang *et al*[132] attempted to identify serum biomarkers for HCC patients using a TaqMan® Low-Density Array. As a result, miR-128-2 was found to be significantly upregulated in patients with portal vein tumor thrombosis. Patients with low levels of serum miR-128-2 were more likely to show a favorable prognosis[132]. In addition, the expression of miR-128-2 in HCC tissues was upregulated in HCC tissues compared with the corresponding non-cancerous liver tissues[132]. MiR-128-2 represents a promising serum screening tool for HCC.

miR-148a

Li *et al*[131] conducted microarray-based miRNA expression profiling in 100 HCC tissues. In patients categorized as presenting the subtype characterized by a cancer stem cell-like signature, the expression level of miR-148a was found to be significantly lower than in other patients. MiR-148a directly suppressed activin A receptor type 1 (ACVR1), a key receptor in the bone morphogenetic protein (BMP) signaling pathway, which regulates stem cell markers[133]. Administration of miR-148a reduced the aggressiveness of HCC cells both *in vitro* and *in vivo*[131]. MiR-148a may contribute to tumor progression *via* the miR-148a-*ACVR1-BMP-Wnt* circuit in a clinically aggressive stem cell-like subtype of HCC.

miR-331-3p

It has been reported that miR-331-3p is expressed abnormally in different tumor types, including gastric cancer, lung cancer, leukemia, and prostate cancer, and

Table 4 Dysregulated microRNAs in hepatocellular carcinoma

microRNA	Sample	*n*	Relevant clinical factors	Functional analyses	Interacting molecules	*In vivo*	Ref.
miR-9	Tissue	200	OS, vascular invasion, stage	-	-	-	122
miR-21	Tissue	109	RFS, cirrhosis, stage	Growth	AP-1	Yes	129
miR-106b	Tissue	104	OS, size, vascular invasion	-	-	-	125
miR-122, miR-29	Serum	20	Liver damage	-	-	-	127
miR-128-2	Tissue, serum	182	OS, liver damage	-	-	-	132
miR-130a	Tissue	102	OS, gender, HBsAg status, size, stage	-	-	-	124
miR-130b	Tissue	97	OS, RFS, HBsAg status, AFP, size, grade, stage	-	-	-	126
miR-138	Tissue	180	OS, stage, vascular invasion, stage	-	cyclin D3	-	130
miR-148a	Tissue	297	OS, size	Proliferation, migration, invasion	ACVR1, BMP, Wnt	Yes	131
miR-224	Tissue	130	OS, RFS, AFP, stage, grade	-	pAKT	-	128
miR-331-3p	Tissue	108	OS, RFS, size, number, vascular invasion	Proliferation, migration, metastasis	PHLPP, AKT, EMT	Yes	123

miR: MicroRNA; OS: Overall survival; RFS: Recurrence free survival; HBsAg: Hepatitis B surface antigen; AFP: Alpha-fetoprotein.

is associated with the proliferation and migration of cancers[134,135]. Chang *et al*[123] surveyed the expression of 840 miRNAs in HCC and identified miR-331-3p as one of the miRNAs that was significantly upregulated in HCC tissues. MiR-331-3p promoted the proliferation and metastasis of HCC cells *in vitro* and *in vivo* and was associated with an impaired prognosis[123]. PH domain and leucine-rich repeat protein phosphatase (PHLPP) was found to be a target of miR-331-3p. It was demonstrated that overexpression of PHLPP abrogated the influence of miR-331-3p on HCC cells, whereas silencing of *PHLPP* enhanced it. MiR-331-3p induced the phosphorylation of *AKT* and the subsequent epithelial-mesenchymal transition[123]. MiR-331-3p can be considered as a potential prognostic biomarker and therapeutic target.

CONCLUSION

HCC is a complex disease with multiple underlying pathogenic mechanisms caused by a variety of risk factors[18]. The lack of good molecular markers for HCC diagnosis and treatment assessment has posed a major challenge in health care[6,18]. The molecular profiling of genes provides powerful tools for gaining insight into the molecular mechanisms underlying hepatocarcinogenesis[136]. Knowledge obtained from such studies could be translated to develop new diagnostic, prognostic, and therapeutic targets for clinical intervention[18,137].

Sorafenib was the first compound shown to significantly improve the survival of patients with advanced HCC[10]. This multikinase inhibitor is active against Raf-1, VEGF receptor 2, B-Raf, the platelet-derived growth factor receptor and c-Kit as well as other receptor tyrosine and serine threonine kinases[16,138]. Preclinical models showed the significant anti-tumoral activity of sorafenib through reduced cell viability and induced apoptosis *in vitro* and *in vivo*[10]. The success of sorafenib is proof of the principle that molecular therapy plays an important role in the treatment of advanced HCC.

Further consideration should be given to developing more effective molecular diagnostic markers and targeted drug therapy. It is clear that genetic/epigenetic alterations are critical determinants of human hepatocellular cancer[9,118]. The progressive accumulation of genetic/epigenetic changes during the development of HCC provides a unique opportunity to use them as biomarkers in cancer detection[6,139]. However, not all aberrations may be equally important for the tumorigenic process[140]. It is unlikely that all epigenetic aberrations play a significant role in hepatocarcinogenesis. For example, some genetic/epigenetic changes may drive other events that contribute to the formation of a transformed phenotype, whereas others may be passenger events that accompany the transformation process[118].

By contrast, some compounds belonging to the group of demethylation agents have progressed to clinical trials in several human cancers[6,69]. One of the key limitations of these agents is that they do not act in a gene-specific manner; instead, they cause global hypomethylation of all genes with CpG sequences[69]. To date, the treatment outcome appears to be better in hematopoietic cancers than solid tumors[141]. The toxicity of nucleoside analogs due to the nonspecific hypomethylation of potential proto-oncogenes and retrotransposons has necessitated the development of other direct or indirect inhibitors of DNA methyltransferase (DNMT) activity, including procainamide, green tea polyphenols, epigallocatechin-3-gallate, and antisense oligodeoxynucleotides[142]. These compounds are less potent inhibitors of *DNMT* activity compared with 5-asa-dC-based compounds, but they present less toxicity and can potentially be used as an adjunct to nucleoside analog therapy[69,141,142].

Indeed miRNAs appears promising as diagnostic tools and miRNA-targeted therapy. However, the elucidation of downstream molecules is necessary to

establish highly specific diagnostic and therapeutic approaches.

Although there is still a long way to go to reach the goal, the accumulation of knowledge regarding genetic and epigenetic factors is of key importance to elucidate the biological features of HCC and overcome this disease.

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