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REVIEW

DNA methylation, microRNAs, and their crosstalk as potential biomarkers in hepatocellular carcinoma

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Abstract

Epigenetic alterations have been identified as a major characteristic in human cancers. Advances in the field of epigenetics have contributed significantly in refining our knowledge of molecular mechanisms underlying malignant transformation. DNA methylation and microRNA expression are epigenetic mechanisms that are widely altered in human cancers including hepatocellular carcinoma (HCC), the third leading cause of cancer related mortality worldwide. Both DNA methylation and microRNA expression patterns are regulated in developmental stage specific-, cell type specific- and tissue-specific manner. The aberrations are inferred in the maintenance of cancer stem cells and in clonal cell evolution during carcinogenesis. The availability of genome-wide technologies for DNA methylation and microRNA profiling has revolutionized the field of epigenetics and led to the discovery of a number of epigenetically silenced microRNAs in cancerous cells and primary tissues. Dysregulation of these microRNAs affects several key signalling pathways in hepatocarcinogenesis suggesting that modulation of DNA methylation and/or microRNA expression can serve as new therapeutic targets for HCC. Accumulative evidence shows that aberrant DNA methylation of certain microRNA genes is an event specifically found in HCC which correlates with unfavorable outcomes. Therefore, it can potentially serve as a biomarker for detection as well as for prognosis, monitoring and predicting therapeutic responses in HCC.

Key words: DNA methylation; MicroRNA; Epigenetics; Hepatocellular carcinoma; Biomarker

Core tip: A comprehensive review of the literature revealed that epigenetic inactivation of microRNA genes is a frequent event in hepatocellular carcinoma (HCC). Hypermethylation of microRNA genes can discriminate HCC from benign liver tumors and correlates with poor prognosis, representing a promising new diagnostic and prognostic marker in HCC. Aberrant DNA methylation of microRNA genes affects several key signaling pathways important in hepatocarcinogenesis and for maintenance of cancer stem cell phenotype.

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INTRODUCTION

Human cancers develop through gradual accumulation and mutual interaction of genetic and epigenetic alterations[1]. Genetic alterations, both germline and somatic mutations, have been recognized as an important aspect in carcinogenesis[2]. Epigenetics, on the other hand, refers

to inherited modifications that influence gene expression and phenotype manifestation without any changes in the DNA sequence. Epigenetic mechanisms consist of several processes *i.e.,* DNA methylation, histone modification, and expression of non-coding RNA. Recent findings suggest that epigenetic alterations occur at much higher rates and more diverse in cancer cells compared to DNA mutations[3]. Among other epigenetic factors, aberrant DNA methylation is the longest known and best studied. DNA methylation cooperates with other epigenetic marks such as histone modifications and non-coding RNAs in the complex regulation of gene expression[4].

MicroRNAs, a major class of small non-coding RNAs, are well-conserved very small RNA molecules (20-22 nucleotides) that can negatively modulate gene expression post-transcriptionally. Accumulating evidence over the past decades highlights the importance of microRNAs as key regulators of many important physiological processes such as cell proliferation, differentiation, apoptosis, and embryonic development[5]. Dysregulation of microRNA expression has been inferred in numerous diseases as well as in cancer. It has later been found that the expression of certain microRNAs is regulated by DNA methylation. This points out that several layers of epigenetic mechanisms are involved in the regulation of gene expression[6,7]. Aberrations of DNA methylation and microRNA expression have been inferred to play an important role in the initiation and progression of human hepatocellular carcinoma (HCC)[8,9].

HCC is the most common type of primary liver cancer that ranks as the fifth most frequent cancer and the third leading cause of cancer mortality worldwide[10]. Epidemiological studies have revealed several key risk factors for the development of HCC such as hepatitis B virus infection, chronic hepatitis and cirrhosis, chronic alcoholic consumption, exposure of dietary aflatoxin, and cigarette smoking[11]. HCC is frequently diagnosed at a late stage in individuals with severe liver dysfunction. Therefore, options for chemotherapeutic and adjuvant therapies are often limited. In addition, lack of early detection markers and drug-resistance may contribute to the high mortality rate in HCC[12]. Although surgical resection and liver transplantation provide improvement of the 5-year survival up to 65%, most HCC cases are diagnosed at an intermediate or advance stage when surgical procedure is not an option anymore[11,12]. The understanding of cellular and molecular mechanisms leading to overt malignant liver tumors is very important in order to develop early detection markers as well as to improve clinical outcome and develop new therapeutic targets for patients with HCC. Over the last decade, the involvement of DNA methylation and microRNAs in liver carcinogenesis has been shown in many studies. DNA methylation at certain genomic loci has been established as a potential marker for sub-classification, diagnosis, prognosis, and therapeutic targets in HCC (as previously reviewed[9,13,14]) and so does microRNA expression patterns (as reviewed in[8,15,16]). In this paper, we focus on the interplay between DNA methylation and microRNA dysregulation, the involved pathways during liver carcinogenesis, and their potential benefits for certain clinical applications in HCC.

DNA METHYLATION IN LIVER CARCINOGENESIS

In the mammalian genome, covalent addition of a methyl group to nucleotides takes place at cytosine located next to guanine (CpG dinucleotide)[17]. CpG dinucleotides are frequently enriched in certain genomic regions[18] that span the range of 0.5-5 kb known as "CpG islands". Nearly 70% of annotated gene promoters in the human genome are characterized by a high CpG content[19]. DNA methylation at CpG islands located upstream of gene promoter is associated with differential expression of the gene. DNA methylation can mediate gene silencing through direct inhibition of the binding of methylation-dependent transcriptional activators or indirectly by altering the affinity of proteins involved in the chromatin remodeling[4,20]. During embryogenesis, DNA methylation plays a role in the regulation of expression of some genes involved in the differentiation of pluripotent cells[21]. However, recent evidence shows that non-CpG methylation is prevalently observed in embryonic stem cells and during neuronal development[22,23]. Nearly 25% of DNA methylation in embryonic stem cells is in non-CpG nulceotides that disappears upon differentiation[22].

Aberrant DNA methylation is frequently found in cancer cells in comparison to healthy cells[24]. Studies in primary tumor specimens showed that differential DNA methylation can be found in almost every type of cancer[25]. Methylation changes are manifested as hypomethylation and/or hypermethylation. Loss of methylation primarily affects repetitive genomic elements and gene bodies while hypermethylation mostly occurs at the promoters of tumor suppressor genes. Both loss and gain of DNA methylation are often found concurrently in cancer and are likely to be driven by different mechanisms involving chromatin reorganization and DNA replication timing[26].

Almost half of the human genome consists of repetitive elements, in which long interspersed nucleotide element-1 (*LINE-1*) and *ALU* already contribute to nearly 17% and 11%, respectively[27]. CpG dinucleotides located within repetitive transposable elements are typically methylated in healthy tissues. DNA methylation at the repetitive sequence is a natural protective mechanism to suppress their activation. Global loss of methylation contributes to the transformation from dysplastic to malignant nodules and is reported to be gradually altered during colorectal cancer progression[28,29]. Demethylation can cause reactivation of transposable elements and insertion to a new location leading to genetic translocations[30], insertions, exon deletions, and chromosomal loss[31]. Alterations of DNA repair pathways and error-prone DNA replication have also been described as a result of *LINE-1* demethylation[32,33].

In liver carcinogenesis, hypomethylation of *LINE-1*, *ALU*, and *SAT2* seems to play a significant role. Loss of methylation at *SAT2* precedes *LINE*-1 and *ALU* demethylation and occurs at an early stage of HCC[34]. DNA methylation levels of *LINE-1* are lower in hepatitis virus and aflatoxin associated HCC[35,36]. Genome-wide loss of methylation correlates with chromosomal instability and poorer prognosis in HCC[37]. In addition, hypomethylation of *LINE-1* elements in circulating DNA of HCC patients correlate with the advanced disease and worse survival[38]. A report involving 305 HCC cases and 1254 healthy individuals revealed that hypomethylation of *SAT2* detected in white blood cells was associated with increased susceptibility for HCC[39]. Recently, Shukla *et al*[40] demonstrated that endogenous *LINE-1* retrotransposons can propagate oncogenic activation in HCC. L1 insertion caused *MCC* (mutated in colon cancer) ablation leading to activation of β-catening/Wnt signaling and interrupted inhibition of oncogene *ST18*.

DNA methylation is mediated by a family of DNA methyltransferase enzymes (DNMTs). Being widely expressed in various tissues, DNMT1 has an ability to induce both *de novo* and maintenance of methylation. DNMT3A and DNMT3B are mainly involved in the *de novo* methyltransferase[41]. Mechanisms underlying establishment of DNA methylation have been widely known. However, how methylation is removed from DNA remains to be clarified. Multiple pathways including both active and passive mechanisms seem to be involved in DNA demethylation[42,43]. Improper functions of DNMTs and other components of the methylation machinery can lead to aberrant DNA methylation. Compelling evidences have shown that dysregulation of establishment and removal of DNA methylation is involved in hepatocarcinogenesis[34,44].

In contrast to hypomethylation, promoter hypermethylation is associated with inhibition of gene expression. CpG islands located at the gene promoters are commonly unmethylated. Increased DNA methylation at the CpG island-associated gene promoters are common features in cancer cells[45]. Hypermethylation is related to transcriptional inhibition and loss of gene function. In HCC, hypermethylation mainly affects tumor suppressor genes, particularly those that are involved in cell proliferation, cell differentiation, DNA repair, cellular metabolism, cell adhesion and metastasis. Table 1 summarizes genes that are frequently hypermethylated in primary HCC specimens[46-109]. Although alterations in gene body DNA methylation have been overlooked in the past, it seems that it is not associated with gene repression. Gene body methylation is suggested as a mechanism for silencing repetitive DNA elements and regulating exon splicing[46].

Epigenetic aberrations have been inferred as key factors during a multi-step process of HCC development. Hypermethylation of *APC*, *RASSF1A*, and *SOCS1* genes has already been detected in chronic hepatitis and cirrhosis. Both level and the frequency of methylation continuously increase in dysplastic liver nodules and HCC[9,64]. Also *GSTP1*, *CDKN2A*, *COX2*, *HIC1*, and *RUNX3* are frequently methylated in dysplastic liver nodules[9,64,90,110]. Gain of methylation at *CDH1*, *CASP8*, *MINT*, *SFRP2*, and *TIMP3* genes is observed in early and late stage of HCC[64,90]. These data support the notion that DNA methylation aberrations emerge at the early stage of hepatocarcinogenesis and gradually increase in combination with accumulation of genetic events such as *P53* mutations and copy number alterations during progression to the advanced stage of HCC.

Concurrent hypermethylation at several genes in HCC also leads to the emerging concept of CpG island methylator phenotype ("CIMP"). This concept was originally described in colorectal and gastric cancer illustrating cancer development through simultaneous inactivation of tumor suppressor and DNA repair genes by DNA methylation[111]. It was shown that CIMP can be used as an independent prognostic factor[111]. Although the concept is still under discussion in HCC given that gene panel for the classification and definition for the phenotype are not yet universally accepted, CIMP positive HCCs have been generally associated with poor clinical outcome[68,71,112,113]. Some reports have also indicated that DNA methylation profiles can be used for molecular sub-classification of HCC to improve the prognosis and the prediction of therapeutic outcomes. The future challenges for routine application in patient-based service will be not only the definition of a consensus gene panel but also the standardization of the methodology for methylation analysis. As shown in Table 1, there is high variation in the frequency of gene hypermethylation reported among different cohorts because of various techniques used for methylation analysis. In addition, many studies do not define "hypermethylation" or use dissimilar definition for hypermethylation.

MICRORNAS IN LIVER CARCINOGENESIS

The past decade has witnessed the important discovery of small non-coding single strand RNAs known as microRNAs that have revolutionized our view of regulatory networks within eukaryotic cells[114]. MicroRNAs negatively modulate gene expression through binding to target messenger RNAs, typically in the 3' untranslated region. Partial complementary binding of a mature microRNA affects the stability of the target mRNA leading to transcriptional inhibition. In contrast, complete complementary binding can lead to direct endonucleolytic mRNA cleavage. Currently, around 1600 human microRNAs are identified and registered in miRBase (www.mirbase.org) and are predicted to target almost 30% of the total human genes[115]. MicroRNAs regulate important physiological processes such as embryonic development, cell cycle checkpoint, cell proliferation, migration, differentiation, and apoptosis[116,117]. Dysregulation of microRNA expression is involved in a number of diseases including developmental disorders, neurological diseases, cardiovascular disorders, as well as cancer.

Table 1 List of genes commonly silenced by DNA hypermethylation in primary hepatocellular carcinoma tumor samples

Gene	Cellular function	Freq	Ref.
14-3-3 ε	Cell cycle, mitogenic signaling	89%	[47]
APC	Cell proliferation, migration, apoptosis	44%-71%	[48-52]
ARHI	Cell proliferation and invasion	47%	[53]
ASS	Cell cycle and cell invasion	-	[54]
BASP1	Apoptosis	50%	[55]
BLU	Apoptosis	81%	[56]
CADM1	Cell adhesion and cell differentiation	41%	[57]
CASP8	Apoptosis	34%	[58]
CAV1	Cell cycle and proliferation	56%	[59]
CCND2	Cell cycle	24%-68%	[48,50]
CDH1	Cell adhesion and metastasis	34%	[52]
CDKN2A	Cell cycle	30%-70%	[49,50,60,61]
CFTR	Intercellular transport	77%-98%	[50,62]
CHFR	Cell cycle, protein degradation	35%	[63]
COX-2	Immune response, cell migration	35%	[64]
CSRP1	Cell proliferation and differentiation	56%	[59]
DKK1	Cell proliferation	36%	[52]
DKK3	Cell differentiation, embryonic development	-	[65]
DLC-1	Cell proliferation	24%-35%	[52,66]
DLEC1	Cell proliferation	71%	[67]
E2F1	Cell proliferation, migration, and differentiation	71%	[68]
FAM43B	Cell proliferation	60%	[69]
FBLN1	Cell adhesion and migration	50%	[70]
FHIT	Cell proliferation and apoptosis	65%	[56]
GSTP1	Cell metabolism and detoxification	70%-76%	[50,60,61,71]
HAI-2/PB	Cell proliferation and invasion	80%	[72]
HDPR1	Cell proliferation, differentiation, cellular signaling	51%	[73]
HINT1	Apoptosis	55%	[74]
IGFBP-3	Cell proliferation and growth	33%	[75]
KL	Cell proliferation and growth	81%	[76]
KLK10	Cell proliferation, survival, and cellular signaling	55%	[77]
LIFR	Signal transduction	48%	[78]
MAGE-A1	Cell differentiation, embryonic development	53%	[79]
MAGE-A3	Cell differentiation, embryonic development	74%	[79]
MAT1A	Cell metabolism	85%	[80]
MT1G	Cell proliferation and apoptosis	60%	[81]
MTM1	Cell differentiation	100%	[82]
MTSS1	Cell migration and metastasis	80%	[83]
MUC2	Immune system	62%	[84]
NORE1B	Cell proliferation and growth	62%	[85]
NQO1	Cell metabolism	50%	[86]
OXGR1	Cell growth and metabolism	78%	[77]
p14ARF	Cell proliferation and apoptosis	40%-42%	[68,71]
p15INK4B	Cell cycle	22%-61%	[71,87]
P16	Cell cycle	37%-83%	[48,71,87]
P21	Cell cycle	63%	[68]
P27	Cell cycle	48%	[68]
P300	Cell proliferation and differentiation	68%	[68]
P53	Cell proliferation, apoptosis, and DNA repair	14%	[68]
P73	DNA repair and apoptosis	35%	[71]
PCDH10	Cell adhesion and migration	76%	[88]
PER3	DNA repair	60%	[89]
PRDM2	Cell metabolism	-	[90]
PTEN	Cell proliferation and migration	16%-22%	[87,91]
RASSF1A	Cell proliferation, migration, and apoptosis	64%-88%	[48,51]
RB	Cell cycle, apoptosis	24%-32%	[68,87]
RECK	Cell invasion and metastasis	55%	[92]
RELN	Cell adhesion	37%	[93]
RIZ1	Cell proliferation and apoptosis	29%-67%	[48,94,95]
RUNX-3	Apoptosis	38%-48%	[50-52]
SFRP1	Cell proliferation and differentiation	37%-45%	[51,52,96]
SFRP2	Cell proliferation and differentiation	48%-54%	[97]
SFRP5	Cell proliferation and differentiation	39%	[96]
SLIT2	Cell migration	83%	[98]
SOCS1	Cell growth and survival	39%-72%	[9,48,90,99]
SOX1	Cell proliferation	57%	[100]
SOX17	Cell differentiation and embryonic development	82%	[101]

SPARC	Cell growth and invasion	75%	[102]
SPINT2	Cell proliferation and growth	60%	[50]
SRD5A2	Cell proliferation and androgenic physiology	50%	[55]
Survivin	Apoptosis and cell proliferation	33%	[58]
TFPI2	Matrix remodelling	47%	[103]
TIP30	Apoptosis and metastasis	47%	[104]
UCHL1	Cell metabolism and protein degradation	44%	[105]
UNC5C	Cell migration	26%	[106]
Vimentin	Cell migration and signaling	56%	[107]
WIF-1	Cell proliferation	49%-61%	[52,65,108]
WT1	Cell proliferation and survival	54%	[68]
ZHX2	Cell proliferation, differentiation, and development	47%	[109]

Freq: Frequency of hypermethylation.

The contribution of microRNAs in the process of malignant transformation has been well characterized. They act as oncogenes or tumor suppressors depending on the target genes and their cellular functions. In HCC, a substantial number of reports have shown frequent and extensive dysregulation of microRNA expression in different stages of liver cancer progression[118]. With the availability of tools for genome-wide expression analysis such as microarray and deep sequencing, profiling of microRNA expression has also revealed some unique signatures that are clinically valuable for diagnosis, prognosis, staging, and prediction of therapeutic responses in the majority of human cancers including HCC[16,119]. In addition, aberrant microRNA expression has also been associated with proliferative and self-renewal potential in liver cancer stem cells[120-122].

Differentially microRNA expression in primary HCC specimens has been comprehensively reported and reviewed[16,123,124]. Upregulation of miR-17-92 cluster, miR-21, miR-221, miR-222, and miR-224 is consistently reported in HCC by many studies[16,125,126]. Meanwhile, let-7 family, miR-29, miR-122, miR-124, miR-199a/b, miR-200 family are frequently downregulated in HCC[16,123]. Some important molecular networks such as Wnt/β-catenin, Ras, transforming growth factor-β (TGF-β), and JAK/STAT signaling pathways are being activated due to the changes of microRNA expression in HCC[16,127]. Recent studies using massive parallel sequencing in HCC cell lines[128] and primary specimens[129] showed basal microRNA expression in hepatocytes and healthy liver as well as the deregulation in chronic hepatitis and HCC samples. MiR-122 was most abundantly expressed in liver (approximately 50% of total microRNAs) and frequently down-regulated in HCC[128,129]. MiR-199a/b was down-regulated in all HCC patients under study (n = 40) and significantly correlated with shorter survival[129].

In addition, expression patterns of 3-6 microRNAs have been suggested to be able to discriminate HCC from the adjacent liver tissue, chronic cirrhosis, and benign liver lesions[126,130]. Furthermore, the expression profile of 20 microRNAs can be used as a metastatic predictor and correlates with survival as well as relapse rates in HCC[131]. Recent reports showed that different panels of microRNAs were differentially regulated in metastatic HCCs[118,132]. Accumulating evidence also shows that differential microRNA expression is of great use for predicting disease survival and recurrence in HCC[133-135]. Patterns of microRNA expression have also been suggested to have clinical value to predict therapeutic response to interferon[133,136,137], doxorubicin[138,139], adriamycin and vincristine[140], 5-fluorouracil[136,141], and sorafenib[142-144]. In addition, association between microRNA expression and multidrug resistance in HCC has also been reported[16,145].

CROSS-TALK OF DNA METHYLATION AND MICRORNA EXPRESSION IN HCC

Differential expression of microRNAs in cancer cells can be caused by several mechanisms including genetic instability (amplification, deletion, or translocation). Approximately 50%-70% of microRNA genes are located at fragile genomic sites that are frequently affected by copy number alterations[146,147]. Dysregulation of microRNA expression driven by some oncogenes such as c-Myc is also evident[148,149]. Myc overexpression modulates the expression of let-7a, miR-100, miR-371, and miR-373 and the expression patterns of these four microRNAs can identify a subclass of HCC with aggressive metastatic behavior[150]. In addition, a number of transcription factors regulate microRNA transcription and their dysregulation in cancer cells affect in turn the expression of microRNA. P53, for example, has been demonstrated to mediate repression of miR-125a/b[151] and upregulation of miR-519d, miR-200 and miR-192 family members[152,153].

Mature microRNAs are biologically synthesized through multi-step processes consisting of transcription, excision, and nuclear transport as already extensively reviewed elsewhere[154,155]. Alterations of microRNA biogenesis can contribute to carcinogenesis[156,157]. Disruption of Dicer1 in a conditional knock-out mouse model revealed the critical roles of Dicer1 and microRNAs for hepatocyte survival, metabolism, and tumor suppression. Loss of Dicer1 in addition to other oncogenic stimuli can induce hepatocarcinogenesis[158]. Expression analysis of genes involved in microRNA biogenesis in primary HCC specimens has demonstrated a significant decrease in *DCGR8*, *p68*, *p72*, *DICER1*, *AGO3*, *AGO4*, and *PIWIL4* expression compared to the adjacent liver tissues. Down-regulation of those genes correlated significantly with etiological factors and shorter HCC survival[159].

Table 2 MicroRNA genes targeted by DNA hypermethylation in hepatocellular carcinoma

MicroRNA	Validated gene targets in HCC	Biological functions	Ref.
miR-1	*FoxP1, MET, HDAC4*	Regulates cell growth, replication and clonogenic survival	[164-166]
miR-10a	*HOXB3, HOXA3, HOXA1, HOXD10, USF2, HOXD4*	Regulates embryonic development and cell differentiation	[167]
miR-124	*CDK6, VIM, SMYD3, E2F6, IQGAP1*	Regulates cell cycle progression (G1-S checkpoint), apoptosis, and metastasis	[166,168]
miR-1247	*ADAM15, CIT, MMP24*	Regulates cell proliferation and migration	[166]
miR-125b	*PIGF, MMP2, MMP9, SIRT7, LIN28B*	Regulates cell proliferation, anchorage-independent growth, cell migration, invasion, and angiogenesis	[169]
miR-129-2	*SOX4, VCP, IκBa*	Regulates apoptosis	[166,170]
miR-132	*AKT1, CTNNB1, CCND1*	Regulates cell proliferation	[171]
miR-203	*ABCE1, CDK6*	Regulates cell growth and cell cycle progression	[168]
miR-320	*NRP1, CTNNB1*	Regulates cell migration, proliferation, and metasatasis	[167]
miR-335	*ROCK1, MAPK1, LRG1, MYCN*	Regulates migration and cell proliferation	[172]
miR-596	*LGALS3BP, FOXP1, IGF2BP2*	Regulates cell growth and induces apoptosis	[166]
miR-663	*JUNB, JUND,*	Regulates cell proliferation	[165]
miR-9	*MTHFD2, HOXD1, MMP14*	Regulates cell proliferation, invasion, metastasis, and angiogenesis	[166]

HCC: Hepatocellular carcinoma.

Aberrant microRNA expression in cancer has also been associated with epigenetic regulation such as DNA methylation and histone modifications. Since the first report about aberrant DNA methylation in a microRNA locus[6], several epigenetically silenced microRNAs have been reported across different type of cancers (reviewed in[160,161]). It is estimated that transcription of 10% of all microRNA species is controlled by DNA methylation[162]. However, a greater proportion of microRNAs silenced by DNA methylation has been suggested since 14.3% (218/1523) are located within 500 bp downstream of a CpG island[163]. Approaches frequently used for identification of epigenetically deregulated microRNAs in HCC cells are the treatment of HCC cell lines by epigenetic drugs such as de-methylating agents (5-aza-cytidine and 5-aza-deoxy-cytidine) and HDAC inhibitors (such as Trichostatin) as well as knockdown of DNMT family members followed by expression profiling. MicroRNAs repressed by DNA methylation in primary HCC specimens are summarized at Table 2. Among those, miR-9 and miR-124 seem to be commonly hypermethylated not only in HCC but also in other tumors[7,161].

Hsa-mir-1-1 is the first microRNA gene reported to be targeted by aberrant DNA methylation in primary HCC specimens[164]. Hypermethylation of *hsa-mir-1-1* leads to overexpression of its target genes, *FOXP1* and *MET*[164]. Transcriptional regulator FOXP1 protein plays a dual role as tumor promoting or suppressing protein depending on the tissue type[173]. *FOXP1* is commonly upregulated in leukemia but donwregulated in kidney and colon cancer. A contribution to carcinogenesis is suggested by fusion with ABL1 and PAX5 in B-ALL and ETV1 in prostate cancer (reviewed in[174]). Elevated FOXP1 expression in HCC correlates with aggressive malignant phenotypes and poor survival[175]. In addition, miR-1 also targets MET, a tyrosine kinase that interacts with hepatocyte growth factor (HGF) upon external stimuli to subsequently activate Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathways. The canonical c-MET/HGF pathway is an important player in many physiological functions including cell proliferation, growth, migration and angiogenesis and has been conveyed in liver development and regeneration as well as in hepatocarcinogenesis[176]. Overexpression of c-MET that is observed in almost 80% of HCCs correlates significantly with worse clinical outcome[177,178]. Accumulating evidence shows the efficacy of c-MET inhibitors as alternative targeted therapy in HCC in which the clinical trials are still ongoing (reviewed in[178]).

Hypermethylation of *hsa-mir-10a* in HCC has also been recently reported. It is accompanied miR-10a downregulation and elevated expression of its host gene, *HOXB4*[167]. HOXB4 is widely represented in leukemogenesis acting as a transcription factor that promotes stem cell renewal[179] and in cervical cancer as a marker for non-differentiated cells[180]. However, overexpression of miR-10a was also reported in dysplastic nodules and HCC samples[181]. A recent study shows an interesting result since upregulation of miR-10a can promote cell migration and invasion *in vitro* but inhibit hepatocellular carcinoma metastasis *in vivo*[182]. It is suggested that *in vivo*, miR-10a can restrain cell-matrix adhesion directing its ability to further suppress cell invasion and metastasis[182].

The three genetic loci in the human genome that encode identical mature miR-124 (*Hsa-mir-124-1*, *Hsa-mir-124-2*, and *Hsa-mir-124-3*) are surrounded by CpG islands and are frequently targeted by DNA hypermethylation in HCC. *CDK6* and *E2F6* are confirmed as miR-124 gene targets[168]. Furthermore, an important component of mammalian target of rapamycin (mTOR) signaling pathway, *PIK3CA*, has also been reported as a novel target of miR-124 in HCC. Downregulation of miR-124 leads to over-activation of PI3K/Akt and mTOR signaling resulting in increased cell proliferation, survival, and metastasis[183]. MiR-124 is involved in feedback loop mechanism of liver inflammation mediated by hepatic nuclear factor-α and introduction of miR-124

systemically can inhibit liver carcinogenesis without significant side effects[184]. MiR 124 also regulates epithelial-mesenchymal transition (EMT) by directly targeting *ROCK2* and *EZH2* genes. Therefore, low expression of miR-124 in HCC correlates significantly with more aggressive behavior and shorter survival[185].

Hsa-miR-1247 is located at one of the largest microRNA clusters within the imprinted *DLK1-MEG3* locus. However, apart from the other microRNAs located in that cluster, miR-1247 is the only one that is transcribed from 3' to 5' therefore it might not be affected by the imprint regulation. Aberrant methylation was first described in colorectal cancer[186]. In liver tumor, hypermethylation was reported in 37% of total HCC cases[166]. *ADAM15*, *CIT*, and *MMP24* are putative miR-1247 target genes indicating miR-1247's role in cellular migration and metastasis[186].

Downregulation of miR-125b in HCC through DNA methylation has been recently described[151,169]. MiR-125b regulates cell proliferation, anchorage-independent growth, cell migration, metastasis, and angiogenesis by targeting placenta growth factor, matrix metalloproteinase (MMP)2, and MMP9. Sirtuin7 (*SIRT7*) that functions as inhibitor of $p21^{WAF1/Cip1}$ is also negatively regulated by miR-125b in HCC[151]. Tumor suppressive roles of miR-125b during liver carcinogenesis are also mediated through inhibition of LIN28B[187] and SUV39H1[188]. Downregulation of miR-125b is observed in around 70% of HCC primary samples and inversely correlated with expression of Ki-67, a cell proliferation index[187,188].

Hypermethylation at the upstream CpG island of *hsa-mir-129-2* is reported in 60%-90% of primary HCC samples[166,170]. MiR-129 exerts tumor suppressive effects in HCC by inhibiting vaccinia virus complement control protein that forms a complex with p97 for stabilization of IκBα. Reduced miR-129 expression in HCC cells can inhibit apoptosis and stimulate cell migration[189]. In addition, DNA methylation at *hsa-mir-129-2* is detected in plasma samples from 85% of stage I HCC patients rendering its potential for alternative surrogate marker for early diagnosis. In comparison, alpha-fetoprotein (AFP) that is widely used as a marker in liver cancer can be detected only in 10% of stage I HCC[170].

Repression of miR-132 expression by DNA methylation has also been reported in HCC. The hypermethylation appears to be mediated by interaction with hepatitis B virus x protein[171]. Meta-analysis of 38 microRNA profiling studies revealed widespread downregulation of miR-132 in various human cancers[190]. MiR-132 functions as an inhibitor of Akt-signaling pathway. As a result, inactivation of miR-132 caused induction of cell proliferation as well as colony formation in HCC cells[171]. Silencing of miR-132 by DNA methylation has also been documented in prostate[191] and pancreatic cancer[192]. Epigenetic silencing of miR-203 in HCC was reported by Furuta *et al*[168] *CDK6* and *ABCE1* were shown as direct target genes of miR-203 supporting the role of miR-203 as tumor suppressor. Hypermethylation was also reported in hematological malignancies[193] but not in esophageal squamous cell carcinoma[194]. However, a recent report showed no differential methylation between HCC tumors and the adjacent liver tissues[166]. Differences in methodology and the exact location of the CpG sites under study might explain the discrepancies.

Significant increased DNA methylation at *hsa-miR-320* gene has also been reported in HCC tumors[167]. Expression of miR-320 is modulated upon HCV infection leading to alterations of cellular structures and malignant transformation[195]. Downregulation of miR-320 was reported in intrahepatic cholangiocarcinoma and contributed to neoplastic transformation by targeting the oncogenes *MCL1* and *BCL2*[196]. Reduced miR-320 expression was linked with shortened recurrence free survival in colorectal cancer[197]. Loss of miR-320 functions in stromal fibroblasts was shown to cause oncogenic secretome release and reprogramming of the microenvironment in favor of tumor growth[198]. Furthermore, frequent downregulation of miR-320 in prostate cancer was associated with activation of Wnt/β-catenin pathway and stem-cell like properties[199].

Located at the intron of *MEST* gene, miR-335 is downregulated in 78% of HCC tumors by aberrant DNA hypermethylation[172]. Loss of miR-335 function is accompanied by dysregulation of the host gene *MEST*. Lower miR-335 expression correlates significantly with distant HCC metastasis[172]. Tumor suppressive effects of miR-335 were also demonstrated in breast cancer by targeting the *BRCA1* pathway and downregulation was observed in distant metastasic cases[200,201]. Frequent downregulation was also found in prostate cancer and reintroduction of miR-335 in cell lines repressed cell proliferation, invasion, and migration[202].

Aberrant methylation of the *hsa-mir-596* gene in HCC was identified by our group[165,166]. The tumor suppressing effects of miR-596 is mediated in cancer cells through negative regulation of *LGALS3BP*, *FOXP1* and *IGF2BP2* genes. MiR-596 is located at the short arm of chromosome 8 that is often affected by focal break points in cancer. A large deletion involving miR-596 was found in urothelial carcinomas[203]. Hypermethylation of miR-596 promoter was also found in oral cell squamous carcinoma lines and primary tissues. Ectopic expression of miR-596 caused apoptosis and reduced cell growth[204].

MiR-663 is also frequently targeted by DNA hypermethylation in HCC[165]. Proto-oncogenes *JUNB* and *JUND* are putative target genes of miR-663. The functions of miR-663 as an effective suppressor of tumor growth was shown in gastric cancer cell lines by Pan *et al*[205] Transient re-expression of miR-663 altered DNA content, induced cellular morphology changes and proliferative blockage[205]. However, functional analysis of miR-663 downregulation in HCC remains to be elucidated.

Mature miR-9 is encoded from 3 independent genomic loci in the human genome, *i.e.*, *hsa-mir-9-1*, *hsa-mir-9-2*, and *hsa-mir-9-3*. Simultaneous hypermethylation was frequently found in different cancers including primary HCC[165,166]. However, how miR-9 contributes to carci-

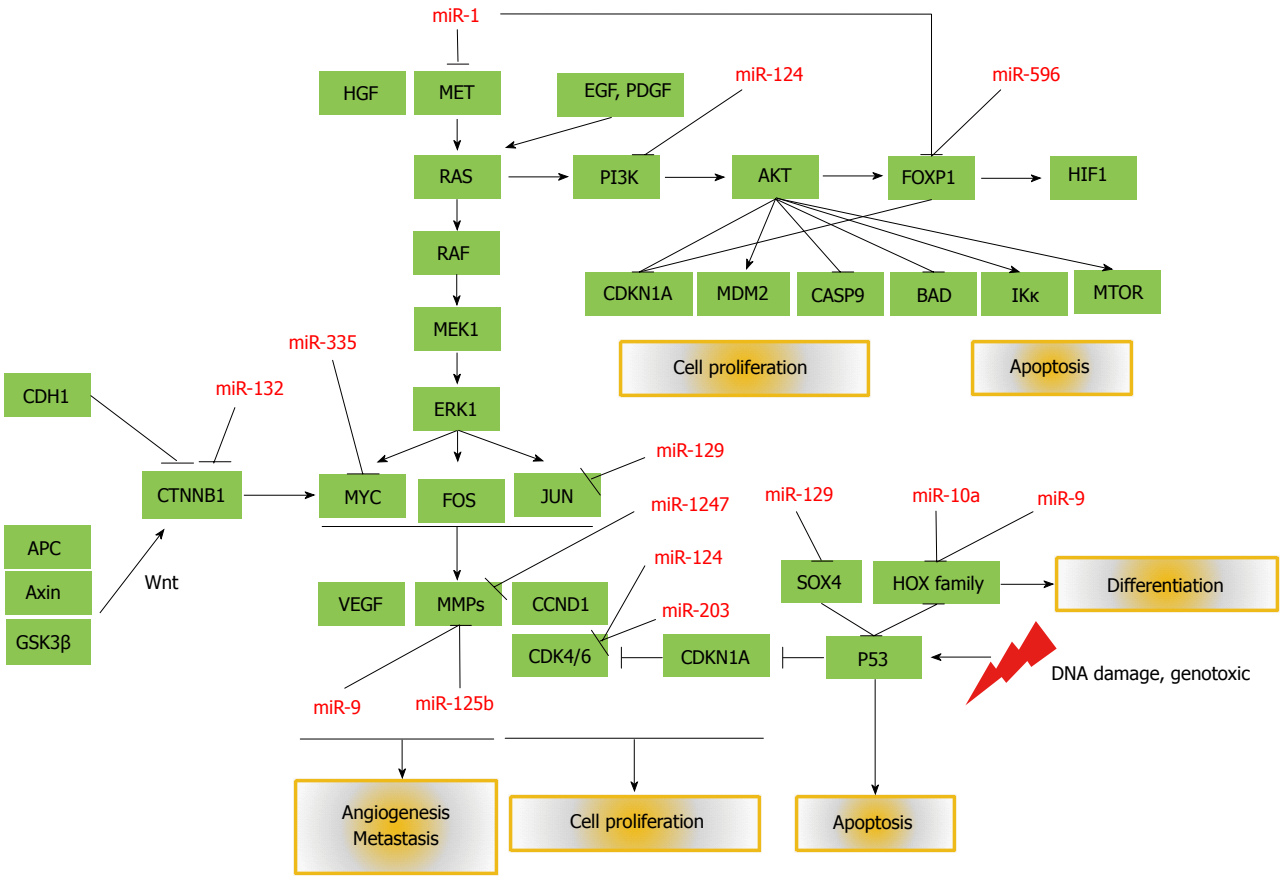

Figure 1 Mature microRNAs silenced by aberrant DNA methylation and their affected target genes and pathways that are important in the development and progression of hepatocellular carcinoma. PI3K: Phosphatidylinositol-3-kinase; MAPK: Mitogen-activated protein kinase; EGF: Epidermal growth factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; mTOR: Mammalian target of rapamycin; VEGF: Vascular endothelial growth factor; MMP: Matrix metalloproteinase; APC: Activated protein C; CTNNB1: Beta-catenin.

nogenesis remains controversial. Upregulation of miR-9 induced by c-Myc was shown to prime breast cancer cells for epithelial-mesenchymal transition by directly inhibiting E-cadherin[206]. MiR-9 overexpression in HCC cells (SK-Hep-1) led to induction of cell migration through E-cadherin suppression[207]. Differential expression of miR-9 was reported in distant metastatic breast cancer[208]. On the other hand, Selcuklu *et al*[209] reported that miR-9 was repressed in primary breast cancer specimens and ectopic miR-9 expression in MCF-7 cells induced anti-proliferative and pro-apoptotic effects. In addition, hypermethylation of miR-9 promoters is observed as a potential diagnostic or prognostic parameter in head and neck cancer[210], lung cancer[211], bladder cancer[212], gastric cancer[213] and colorectal cancer[214]. Our group showed aberrant miR-9 methylation in HCC using quantitative methylation analysis[165] and demonstrated their correlation with clinical outcomes[166]. Figure 1 summarizes gene targets and key pathways affected by aberrant DNA methylation of microRNA genes in HCC.

GENOME-WIDE STUDIES OF DNA METHYLATION IN HUMAN HCC

The development of new technologies for DNA methylation analysis have greatly contributed to the advance of epigenomic studies in liver cancer especially with the recent application of more comprehensive, high-resolution genome-wide methods. We identified 10 studies implementing genome-wide methylation analysis in primary HCC specimens: a study used methylated CpG island amplification coupled with CpG island microarray (MCAM, $n = 17$)[215], two studies used MeDIP ($n = 6$[216,217], $n = 11$[217]), 6 studies employed Illumina's Infinium Human Methylation27 ($n = 23$[218], $n = 66$[219], $n = 13$[220], $n = 13$[221], $n = 63$[89], and $n = 62$[222]), and two studies utilized Illumina's Infinium HumanMethylation450 ($n = 27$[61] and $n = 66$[223]). Among these studies, however, only Shen *et al*[167] specifically addressed aberrant DNA methylation of microRNA genes. The 27k Bead array from Illumina contains 254 assays covering 110 intragenic microRNAs located within 64 host genes. Using a panel of microRNA gene methylation assays in the 27k array, HCC tumors can be differentiated from the corresponding adjacent liver tissues. More than 20% of the 254 CpG sites showed significant differential methylation affecting 27 genes. The newly released Illumina 450k has a greater coverage with almost 99% of RefSeq genes and 96% of CpG islands are included. Using this platform, Song *et al*[61] found 10775 CpG sites located within or ad-

jacent to gene promoters were differentially methylated in HCC tumors. Of these CpG sites 493 are associated with microRNA genes. Using the same array platform, Shen *et al*[223] reported 28017 CpG sites (5.8%) to be hypermethylated in HCC tumors. These data indicate that many more microRNA genes are likely to be targeted by DNA hypermethylation in HCC and need to be further studied.

MICRORNA AND DNA METHYLATION IN LIVER CANCER STEM CELLS

In addition to the traditional view that cancer emerges through accumulation of genetic changes in a clonal population, another theory postulates a hierarchical organization of tumor cells. The latter suggests that cancer develops from cells with particular capabilities to self-renew, produce differentiated progeny, and initiate new focal tumors. These cells are known as cancer stem cells[224]. However, rather than representing independent mechanisms, the two models are believed to be complementary and substantially contribute to the inter- and intra-tumoral heterogeneity. The features of stemness and pluripotency are ultimately formed by unique epigenetic signatures[225,226]. Hepatic cancer stem cells can be distinguished from well differentiated tumor cells through their functional properties and specific surface markers such as CD133, CG90, EpCAM, and ALDH. Expression of CD133 is regulated by DNA methylation through modulation of TGFβ/Smad pathway[227]. Side population cells that represent cancer stem cells in HCC display differential gene expression[228] and unique DNA methylation patterns[229] that regulate pathways involved in pluripotentcy and self-renewal such as WNT/β-catenin, Hedgehog, MYC, TGFβ/Smad, Notch, MET, and BMI1[229].

The involvement of microRNAs in the regulation of cancer stem cells during hepatocarcinogenesis has also been suggested. MiR-181 is upregulated in $EpCAM^+$ hepatic cancer stem cells through Wnt/β-catenin transcriptional regulation[230]. Differentiation-promoting genes, *CDX2* and *GATA6*, are revealed as miR-181 gene targets. Cairo *et al*[150] reported MYC-dependent microRNAs that featured cell renewal and stemness in HCC. Other microRNAs, miR-216a/217 were shown to increase stem-like properties by targeting PTEN and SMAD7. Consistently, miR-216a/217 upregulation in HCC tissues correlated with EMT phenotypes, early recurrence, and shorter disease-free survival[144]. In addition, overexpression of microRNA clusters at the *DLK1-DIO3* imprinted locus correlated with HCC stem cell markers, high AFP levels, and poor survival in HCC patients[120]. Since imprinting is regulated by DNA methylation, the cross-talk between DNA methylation and microRNAs during cancer stem cell de-differentiation in HCC may also be present. Some epigenetically-silenced microRNAs also target transcription factors and signaling pathways that are involved in cell-renewal and stemness phenotypes[164,168,199].

ENRICHED BIOLOGICAL PATHWAYS OF ABERRANTLY METHYLATED MICRORNA GENES IN HCC

MicroRNA target genes from the experimentally validated targets (TarBase or miRTarBase) and predicted algorithms (DIANA, miRDB, and TargetScan) of all epigenetically silenced microRNAs in HCC were used for enrichment biological pathway analysis using Kyoto Encyclopedia of Genes and Genomes and Panther. Metabolic, PIK3-Akt, MAPK, Wnt, inflammation, angiogenesis, epidermal growth factor receptor, Cadherin, and TGFβ are the most frequent pathways targeted by these microRNAs (Table 3 lists pathways and molecular functions regulated by methylation-silenced microRNA gene targets). Several biological functions such as metabolic, immune system, cell adhesion, cell communication, and developmental processes as well as molecular and cellular functions including protein binding, transcriptional regulator, catalytic and receptor activity are greatly enriched suggesting the importance of these epigenetically silenced microRNAs in the development of cancer.

DIAGNOSTIC, PROGNOSTIC, AND CLINICAL RELEVANCE OF DNA METHYLATION AT MICRORNA GENES IN HCC

Hepatic neoplasm consists of a range of benign and malignant tumors that differ in histopathology, etiology, disease progression, and clinical behavior. Aberrant DNA methylation that occurs during tumor initiation and development is relatively stable in tissues as well as serum/plasma. Additionally, dysregulation of DNA methylation and microRNA expression that are involved in the regulation of cell differentiation and developmental cell lineage is associated with poorly differentiated cancer and worse outcomes. Therefore, aberrant DNA methylation at microRNA genes is potentially a useful parameter for diagnosis as well as classification of human cancers including HCC. Single locus hypermethylation of *miR-129-2* has been shown as a highly specific marker for distinguishing HCC from chronic hepatitis and healthy liver tissues[170].

Our recent study showed that DNA methylation at microRNA genes was a specific event detectable only in malignant liver cells and tissue samples but not in adjacent liver tissue, benign liver tumors, healthy liver cells, or in hepatocyte lines. These results indicated that methylation of microRNA genes might represent a new biomarker for specific detection of malignant liver tumors. In addition, concordant DNA methylation at certain microRNA loci correlated with poor HCC survival rendering its potential to be used as prognostic marker in HCC[166]. Differential methylation at these loci appears not to be a random event but highly organized during

Table 3 Pathways and molecular functions regulated by methylation-silenced microRNA gene targets

No.	Panther	Genes	Gene	Contrib
GO molecular function[1]				
1	Binding (GO:0005488)	2799	40.60%	33.80%
2	Catalytic activity (GO:0003824)	2126	30.80%	25.70%
3	Transcription regulator activity (GO:0030528)	920	13.30%	11.10%
4	Receptor activity (GO:0004872)	696	10.10%	8.40%
5	Structural molecule activity (GO:0005198)	558	8.10%	6.70%
6	Enzyme regulator activity (GO:0030234)	494	7.20%	6.00%
7	Transporter activity (GO:0005215)	418	6.10%	5.00%
8	Ion channel activity (GO:0005216)	152	2.20%	1.80%
9	Translation regulator activity (GO:0045182)	57	0.80%	0.70%
10	Motor activity (GO:0003774)	53	0.80%	0.60%
GO Biological process[2]				
1	Metabolic process (GO:0008152)	3277	47.50%	20.80%
2	Cellular process (GO:0009987)	2564	37.20%	16.30%
3	Cell communication (GO:0007154)	1793	26.00%	11.40%
4	Developmental process (GO:0032502)	1315	19.10%	8.40%
5	Transport (GO:0006810)	1190	17.30%	7.60%
6	Immune system process (GO:0002376)	956	13.90%	6.10%
7	System process (GO:0003008)	927	13.40%	5.90%
8	Cell cycle (GO:0007049)	797	11.60%	5.10%
9	Cellular component organization (GO:0016043)	630	9.10%	4.00%
10	Response to stimulus (GO:0050896)	613	8.90%	3.90%
GO cellular component[3]				
1	Intracellular (GO:0005622)	486	7.10%	50.40%
2	Extracellular region (GO:0005576)	233	3.40%	24.10%
3	Plasma membrane (GO:0005886)	86	1.20%	8.90%
4	Ribonucleoprotein complex (GO:0030529)	84	1.20%	8.70%
5	Protein complex (GO:0043234)	76	1.10%	7.90%
GO protein class[4]				
1	Nucleic acid binding (PC00171)	1144	16.60%	12.60%
2	Transcription factor (PC00218)	920	13.30%	10.20%
3	Hydrolase (PC00121)	691	10.00%	7.60%
4	Receptor (PC00197)	690	10.00%	7.60%
5	Transferase (PC00220)	662	9.60%	7.30%
6	Enzyme modulator (PC00095)	634	9.20%	7.00%
7	Signaling molecule (PC00207)	481	7.00%	5.30%
8	Transporter (PC00227)	457	6.60%	5.00%
9	Cytoskeletal protein (PC00085)	402	5.80%	4.40%
10	Kinase (PC00137)	316	4.60%	3.50%
Pathway[5]				
1	Gonadotropin releasing hormone receptor pathway (P06664)	159	2.30%	4.80%
2	Wnt signaling pathway (P00057)	156	2.30%	4.70%
3	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	134	1.90%	4.00%
4	Integrin signalling pathway (P00034)	116	1.70%	3.50%
5	Angiogenesis (P00005)	98	1.40%	3.00%
6	EGF receptor signaling pathway (P00018)	84	1.20%	2.50%
7	Cadherin signaling pathway (P00012)	80	1.20%	2.40%
8	Huntington disease (P00029)	77	1.10%	2.30%
9	TGF-beta signaling pathway (P00052)	73	1.10%	2.20%
10	PDGF signaling pathway (P00047)	74	1.10%	2.20%
KEGG				
hsa01100 metabolic pathways (434)				
hsa05200 pathways in cancer (189)				
hsa04151 PI3K-Akt signaling pathway (162)				
hsa04010 MAPK signaling pathway (137)				
hsa05205 proteoglycans in cancer (134)				
hsa05166 HTLV-I infection (123)				
hsa04510 Focal adhesion (116)				
hsa04810 regulation of actin cytoskeleton (115)				
hsa05202 transcriptional misregulation in cancer (100)				
hsa04144 endocytosis (100)				

[1]Total genes n = 6893, total function hits n = 8282; [2]Total genes n = 6893, total process hits n = 15740; [3]Total genes n = 6893, total component hits n = 965; [4]Total genes n = 6893, total protein classess n = 9050; [5]Total genes n = 6893, total pathway hits n = 3313. Contrib: Contribution; PI3K: Phosphatidylinositol-3-kinase; MAPK: Mitogen-activated protein kinase; HTLV: Human T-lymphotropic virus 1; EGF: Epidermal growth factor; PDGF: Platelet-derived growth factor; TGF-β: Transforming growth factor beta.

initiation and progression of HCC. In addition, several microRNAs affected by DNA methylation in HCC are suggested to modulate therapeutic responses upon conventional chemotherapy and or treatment with sorafenib. However, using a panel of DNA methylation aberrations in microRNA genes as a new marker for CIMP in HCC is a great challenge for future research. To evaluate this, quantitative DNA methylation analysis using diverse samples of liver diseases including adenoma, chronic hepatitis, cirrhosis, early and late stage of HCC in a setting of retrospective and prospective studies involving multicenter collaboration are needed. Genome-wide DNA methylation analysis will provide extensive information not only for microRNA loci but also other regions potentially important as a marker for CIMP. Correlating methylation status with clinocopathologic and molecular profiles from large HCC cohort will also strengthen the use of CIMP as a new classifier for HCC samples.

The involvement of aberrant DNA methylation at microRNA genes in the pathogenesis and progression of HCC also provide new insight into the molecular mechanisms of the disease and emerge as a candidate for novel alternative adjuvant therapy in HCC. This is of special importance since many clinical trials for molecular targeted therapies in HCC are not yet successful[231]. Systemic administration of microRNAs that are frequently silenced by DNA methylation such as miR-124 can inhibit the HCC progression in animal models. Moreover, a therapy using microRNA mimics is relatively effective and safe without any observed side effect[184]. In addition, miR-1274 that is commonly targeted by DNA hypermethylation in HCC has been demonstrated as an important regulator in response to therapy with sorafenib[232].

Demethylating agents such as 5-azacytidine and 5-aza-2'-deoxycytidine can induce re-expression of microRNA genes silenced by methylation. So far, these demethylating agents have been approved as adjuvant therapy in myelodysplastic syndrome[233]. Therapeutic effects of demethylating drugs for HCC have been established *in vitro*[234,235]. 5-azacytidine exerts anti-tumor effects not only by reversing epigenetic aberrations but also by re-sensitizing cells to apoptotic inducing therapy such as TRAIL[236]. However, both drugs are integrated into the replicating DNA and bind to DNMT enzymes irreversibly leading to unspecific biological side effects. A second generation demethylating agent, Zebularine, provides reversible binding and exhibit less toxicity. Late stage HCC patients usually with high degree of methylation levels are predicted to benefit from zebularine therapy[218]. However, most of demethylating agents cause some adverse events including liver dysfunction[237] that need to be carefully addressed especially in HCC patients. Adjustment in dose and administration schedule is therefore required for these agents to provide optimal results in cancer therapy[238]. Another epigenetic drug, belinostat, is a potent HDAC inhibit that has already been in phase I / II clinical trial for treatment of inoperable HCCs[239].

CONCLUSION

The discovery of aberrant DNA methylation at microRNA genes during liver carcinogenesis has contributed significantly not only to an understanding of the molecular pathogenesis of the disease but also provided potential new markers for diagnosis, prognosis, and prediction in HCC. Diverse major pathways such as Wnt, mTOR, MAPK, and nuclear factor kappa B signaling seem to be affected simultaneously by hypermethylation of those tumor suppressive microRNAs. Therefore, manipulating DNA methylation at those microRNA genes and/or their expression may provide a promising strategy for alternative adjuvant therapy in HCC. In addition, DNA methylation is relatively stable in tumor tissues and body fluids under many conditions suggesting it as a new promising biomarker for diagnosis and prognosis in HCC. However, confirmation with large multicenter HCC cohorts and using robust techniques for DNA methylation analysis of microRNA genes are warranted before the application in clinical practice.

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