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The regulation of microRNA expression by DNA methylation in hepatocellular carcinoma†

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Emerging evidence indicates that microRNAs (miRNAs) are often dysregulated and play a fundamental role in hepatocellular carcinoma (HCC). However, the mechanism underlying miRNA dysregulation is still elusive. In the present study, we adopted an integrated analysis strategy combining data from genome-wide methylated DNA immunoprecipitation chip and miRNA expression microarray to study the regulation of DNA methylation on miRNA expression in HCC. We first characterized 864 differentially methylated regions (DMRs) located in 236 miRNA regions between cancerous and normal hepatocytes in HCC. We observed that the occurrence of miRNA DNA hypomethylation was more common than its hypermethylation while miRNA DNA hypermethylation was usually found in CpG islands. Then through correlation analysis between miRNA methylation and expression data, we identified 10 dysregulated miRNAs under the potential regulation of DNA methylation in HCC. Five of them (miR-148a, miR-375, miR-195, miR-497 and miR-378) were in hypermethylation and down-regulation status, while another five (miR-106b, miR-25, miR-93, miR-23a and miR-27a) were in hypomethylation and up-regulation status in HCC. Bioinformatics analysis showed that miR-148a may form a negative feedback loop with its targets DNMT1 and DNMT3B and the expression of the miR-195/497 cluster may be affected not only by their hypermethylated promoter region but also by their hypermethylated transcription factors NEUROG2 and DDIT3. *Conclusion*: our preliminary data and bioinformatics analysis suggest that DNA methylation plays an important and complex role in the regulation of miRNA expression in HCC, which may provide insights into the pathogenesis of HCC and thus may be used for diagnosis and intervention.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide.[1] Up to now, surgical resection or liver transplantation remains the first choice for the treatment of HCC; however, only about 10 to 20% of patients with HCC are eligible for surgical intervention at the time of diagnosis.[2] Moreover, patients who underwent curative resection often have a high frequency of relapse, and postoperative 5-year survival is only 30–40%.[3] Because of the high rate of recurrence and low detection rate for the curable stages, HCC is a cancer with poor prognosis. So further studies of the molecular mechanism underlying this disease are urgently needed for the development of novel interventions.

MicroRNAs (miRNAs) are evolutionarily conserved, small non-coding RNAs (18–25nt) that are believed to play a fundamental role in various biological processes through the regulation of gene expression at the level of post-transcription. Emerging evidence indicates that miRNAs are often dysregulated in HCC and some specific miRNAs are associated with the initiation and progression of HCC. Murakami *et al.* firstly reported that HCC had an abnormal expression pattern of miRNAs, of which four miRNAs,

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† Electronic supplementary information (ESI) available: Fig. S1. The regulatory network between hypermethylated TFs (left) and hypomethylated TFs (right), dysregulated miRNAs regulated by these TFs, and the miRNA targets. Table S1. Dysregulated miRNAs between cancerous and normal hepatocytes. Table S2. GO and KEGG pathway terms enriched by targets of the ten dysregulated miRNAs and miR-148a. Table S3. HCC or cancer related genes got from differently methylated genes; targets (protein-coding genes) of differently methylated TFs (DMTFs); targets of miRNAs which are targeted by differently methylated TFs. Table S4. DNA methylation status of three categories of intragenic miRNAs harboring differentially methylated regions (DMRs) and their host genes. See DOI: 10.1039/c4mb00563e

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miR-92, miR-20, miR-18, and precursor miR-18, were inversely correlated with the degree of HCC differentiation.[4] Since then, accumulating researches confirmed that miRNAs play an important regulatory role in hepatocarcinogenesis and malignant transformation. Based on the analysis of clinical samples, Budhu *et al.* identified a 20-miRNA metastasis signature that can predict HCC with venous metastases.[3] Of note, different subsets of miRNAs may be involved in distinct biological characteristics of HCC. For instance, miR-375 could markedly suppress the migration and invasion of HCC cells by targeting the oncogene AEG-1;[5] while miR-221 influences the cell cycle and apoptosis by regulating targets p27, p57 and BMF.[6–8] Besides, studies indicate that miR-26b and miR-23a are involved in regulating the chemosensitivity of HCC cells.[9,10] Although miRNAs play an important role in hepatocarcinogenesis, the mechanism underlying miRNA dysregulation is poorly understood.

Recent studies suggested that epigenetic alteration including abnormal DNA methylation was involved in the dysregulation of miRNAs, especially, the hypermethylation of CpG islands in the miRNA promoter region could lead to the down-regulation of miRNAs in various cancers.[11–13] Although several studies have reported some aberrant DNA methylation profiles in HCC,[14,15] a global DNA methylation profile of miRNA genes in HCC is still lacking to date. Methylated DNA immunoprecipitation chip (MeDIP-chip) is one of the high-throughput technologies in DNA methylation studies, which is genome-wide, less biased, reproducible and more efficient.[16] In this report, we identified a global DNA methylation profile of miRNA genes in HCC through analysis of custom-designed MeDIP-chip data from cancerous and normal hepatocytes. Then combined with the miRNA expression microarray data reported in our previous study,[5] we focused on studying the correlation between DNA methylation and miRNA expression. Furthermore, based on bioinformatics analysis and the literature, we proposed a model to show the complex regulatory network among DNA methylation, miRNAs, transcription factors (TFs) and HCC related genes.

Results

Differential miRNA methylation profile between cancerous and normal hepatocytes

To identify differentially methylated regions (DMRs) between cancerous and normal hepatocytes, we first obtained genome-wide DNA methylation profiles of miRNA genes for three hepatoma cell lines (SK-HEP-1, HepG2 and MHCC97-H) and three cases of normal primary human hepatocytes (PHHC) using MeDIP-chip, which is a custom-designed methylation chip containing CpG islands and regions near miRNAs (Fig. 1A). The methylation chip data were deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/, GEO accession number: GSE49554). After finding methylation peaks and performing *T*-test statistical analysis, we identified 864 differentially methylated regions (DMRs) with a median size of 486 bp located in 236

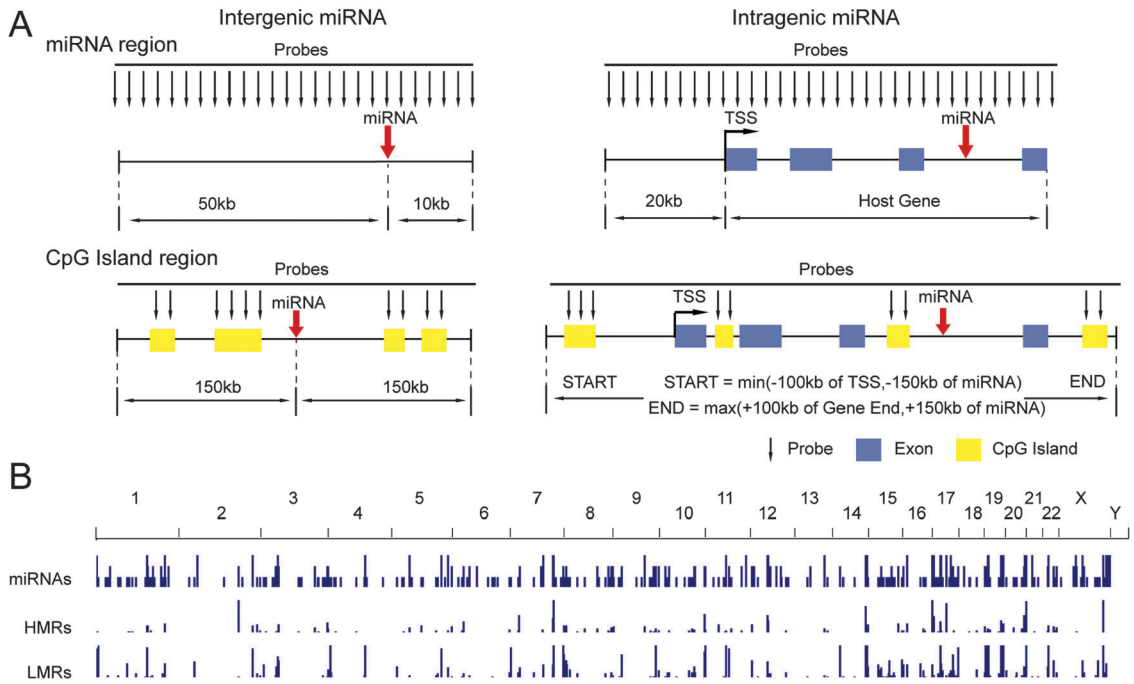

Fig. 1 Regions contained in MeDIP-chip and differentially methylated regions (DMRs) in miRNA designed regions. (A) Regions designed in human miRNA tilling array. It contains two parts: miRNA regions and CpG island regions, which are different between intragenic and intergenic miRNAs. The regions covered by probes in the array are directed by black arrows. TSS: the transcription start site of the host gene. (B) Differential methylation profile of miRNA between cancerous and normal hepatocytes: distribution and density of hypermethylated regions (HMRs) (second line) and hypomethylated regions (LMRs) (third line) of miRNA genes. The first line represents the distribution of miRNAs in the array across the whole genome. The height of the bar in each line means the density of miRNAs or DMRs. DMR includes HMR and LMR.

Table 1 Differentially methylated regions (DMRs) of miRNAs in HCC[a]

	HMRs	LMRs
DMRs	326	538
miRNAs containing DMRs	119	176
Average length of DMRs (bp)	619	406
DMRs containing CpG islands	155	18
Average length of overlapped regions between DMRs and CpG islands (bp)	590	86

[a] HMR: hypermethylated region; LMR: hypomethylated region.

miRNA regions in HCC. These DMRs were divided into two categories: 326 hypermethylated regions (HMRs) and 538 hypomethylated regions (LMRs) (Fig. 1B and Table 1). Among the 326 HMRs, 155 HMRs (47.55%, located in 70 miRNAs) were overlapped with one or more CpG islands; while only 18 (3.35%, located in 15 miRNAs) of the 538 LMRs were overlapped with CpG islands (Table 1). The percentage of HMRs containing CpG islands was significantly higher than that of LMRs ($p < 2.2 \times 10^{-16}$, Pearson's chi square test). Furthermore, the average length ($\sim$590 bp) of hypermethylated CpG islands was much longer than that of hypomethylated CpG islands ($\sim$86 bp) (Table 1). This aberrant DNA methylation profile of miRNA genes may provide insights into the pathogenesis of HCC and thus may be used for diagnosis.

Dysregulated miRNAs under potential regulation of DNA methylation in HCC

Formerly, we have identified miRNA expression profiles in seven hepatoma cell lines (SK-HEP-1, HepG2, Hep3B, Huh7, MHCC97-H, MHCC97-L, and SMMC-7721) and three cases of normal primary human hepatocytes using miRNA expression microarray, and the microarray data were deposited in the GEO database (GEO accession number: GSE20077).[5] In that report, miRNA expression of cancerous hepatocytes was compared with that of normal hepatocytes and 37 dysregulated miRNAs (Table S1, ESI†) were screened out by a 2-fold change with a significant difference ($p < 0.05$). In the present study, based on the results of the former report, we integrated data from MeDIP-chip and miRNA expression microarray to calculate the correlation coefficient between DNA methylation and miRNA expression using the Spearman rank correlation analysis. The candidate miRNAs under potential regulation of DNA methylation were screened out as their correlation coefficients were lower than -0.5. Using this analysis, we found that 13 miRNAs had DMRs in their upstream regions and identified 10 dysregulated miRNAs under the potential regulation of DNA methylation in HCC (Table 2). Five of them (miR-148a, miR-375, miR-195, miR-497 and miR-378) were in hypermethylation and down-regulation status, while another five (miR-106b, miR-25, miR-93, miR-23a, and miR-27a) were in hypomethylation and up-regulation status in HCC (Fig. 2). As shown in Fig. 2 and Table 2, the hypermethylated sites of intergenic miRNAs miR-375 and miR-148a in cancerous hepatocytes were located within the CpG islands in 2 kb upstream of their miRNA genes. Intragenic miR-195 and miR-497 as a cluster in the host gene MIR497HG exhibited two hypermethylated sites in their upstream. One was in a CpG island about 5.2 kb upstream and the other is at about 9.6 kb upstream without CpG islands. MiR-378 is also an intragenic miRNA and its hypermethylated site is located at about 2.6 kb upstream of the miRNA gene within a CpG island. The five up-regulated miRNAs were distributed in two clusters: the miR-25/93/106b cluster in MCM7 gene and the miR-23a/27a cluster in an intergenic region. The former shared a hypomethylated site in the upstream of their host gene and the latter shared three hypomethylated sites in their upstream.

Then, bioinformatics analysis was carried out for the 10 dysregulated miRNAs to evaluate their potential functions in HCC.

Table 2 Dysregulated miRNAs under potential regulation of DNA methylation in HCC

miRNA	Locus	DMRs	CpG islands with DMRs	Correlation[a]
Hypermethylated				
miR-148a	7p15.2	chr7: 25956931–25957030 chr7: 25957582–25957681	chr7: 25956537–25957845	−0.81
miR-375	2q35	chr2: 219575324–219575923	chr2: 219574375–219576298	−0.77
miR-195/497	17p13.1	chr17: 6866560–6866959 chr17: 6870361–6871310	chr17: 6866124–6868251	−0.88/−0.70[b]
miR-378	5q32	chr5: 149089949–149090248	chr5: 149089763–149091943	−0.55
Hypomethylated				
miR-106b/25/93	7q22.1	chr7: 99539372–99539587	No	−0.87
miR-23a/27a	19p13.13	chr19: 13807249–13810855 chr19: 13814456–13814705 chr19: 13817981–13818080	chr19: 13814222–13814554	−0.76

DMR: differentially methylated region. [a] Correlation refers to the correlation coefficient between DNA methylation and miRNA expression. [b] The correlation coefficients of miR-195 and miR-497 are different: the correlation coefficient of miR-195 is −0.88 and the correlation coefficient of miR-497 is −0.70.

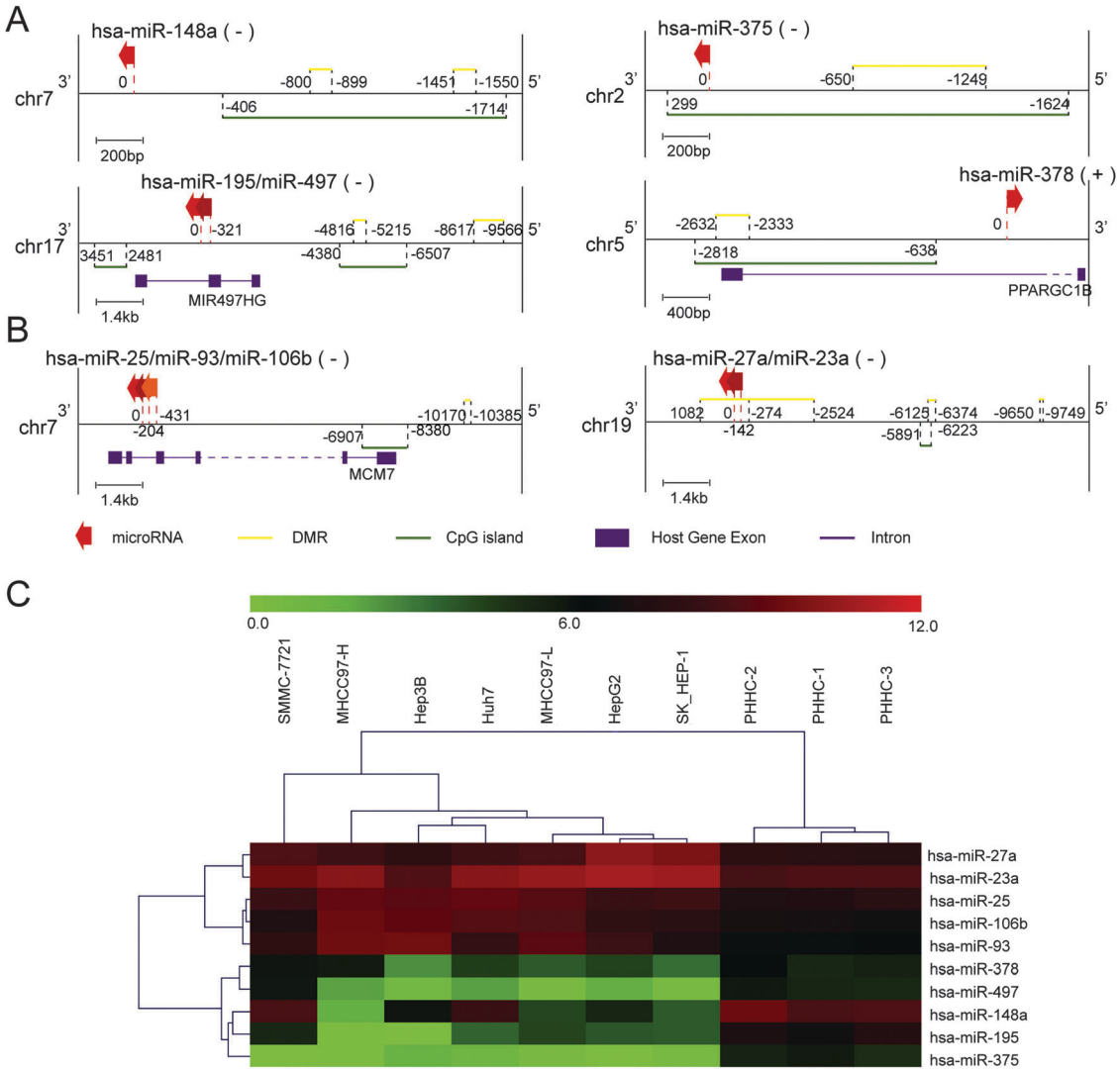

Fig. 2 Association between DNA methylation and miRNA expression. Schematic representation of the relative location of differentially methylated regions (DMRs) and CpG islands for miRNAs with hypermethylation and down-regulation (A) and miRNAs with hypomethylation and up-regulation (B). ± in the bracket represents the DNA strand of the miRNA gene. DMR: hypermethylated region (HMR) or hypomethylated region (LMR). The dotted lines in purple mean that some exons and introns are not plotted. (C) A heatmap shows the expression alteration of the 10 dysregulated miRNAs between cancerous and normal hepatocytes. Low expression is depicted in green, high expression is in red and the middle is in dark.

First, we got 947 target genes of these miRNAs from three databases: TarBase, miR2Disease and miRTarbase, which collected experimentally validated miRNA targets from the literature and high throughput experiments. Then Gene Ontology (GO) and KEGG pathway analyses were performed for these miRNA targets. We found significant enrichments ($p < 0.001$) on GO terms and KEGG pathways related with cancer development, including the cell cycle, cell proliferation, apoptotic, p53 signaling pathway and TGF-beta signaling pathway (Table S2, ESI†). Interestingly, miR-148a was previously reported to directly target DNA methyltransferases DNMT1[17] and DNMT3B.[18] Functional analysis of miR-148a target genes also displayed a significant enrichment on DNA-methyltransferase activity (Table S2, ESI†). Since DNA methyltransferase is responsible for the hypermethylation of miRNAs including miR-148a, we deduced that miR-148a and DNMT1/3B might form a negative regulatory loop, which could boost the down-regulation of miR-148a in HCC (Fig. 3A).

Therefore, these explorations on the DNA methylation status and function of the 10 dysregulated miRNAs may deepen our understanding of the mechanism underlying miRNA dysregulation and the important roles of miRNA in HCC.

Complex regulatory network among DNA methylation, miRNAs and TFs in HCC

By analyzing the methylation status of genes near miRNAs and miRNA host genes, we identified 170 differentially methylated genes (DMG) including 136 hypermethylated genes and 34 hypomethylated genes between cancerous and normal hepatocytes (Table S3, ESI†). Among them, the HOX gene family stood out due to their hypermethylation and important role in tumorigenesis[19,20] (Fig. 3B). We then obtained 1307 TF-gene

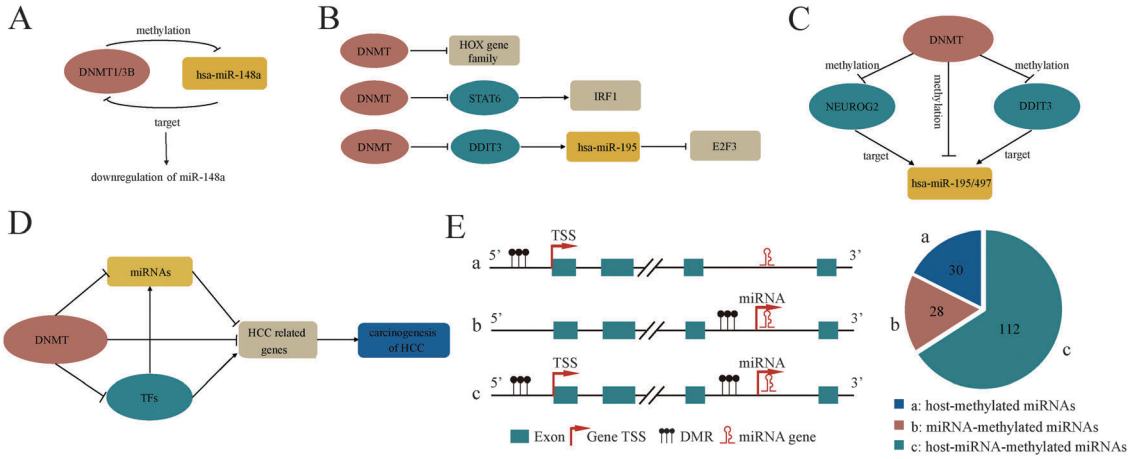

Fig. 3 Network between DNA methylation, miRNAs, TFs and HCC related genes. (A) Negative regulatory loop of miR-148a and its targets DNMT1/DNMT3B, two DNA methyltransferases. (B) Examples of association between DNA methylation and HCC related genes. (C) Network between DNA methylation, the miR-195/497 cluster and TFs NEUROG2 and DDIT3. The expression of miR-195/497 is regulated by DNA methylation directly and indirectly. (D) Patterns showing how DNA methylation affects miRNA expression using a complex regulatory mechanism and contributes to hepatocarcinogenesis by affecting the expression of miRNAs, TFs and genes. (E) Three categories of methylated intragenic miRNAs which were classified based on the location of differentially methylated regions (DMRs). TSS: transcription start site. DNMT in figures B–D represents DNA methylation.

pairs and 72 TF-miRNA pairs by analyzing the targets of TFs in the 170 DMGs using the method described in the Materials & methods section (Table S3, ESI†). Among the TF-gene pairs, 11 genes were reported as HCC related genes, such as IRF1 targeted by the TF STAT6[21] (Fig. 3B and Table S3, ESI†). For the TF-miRNA target pairs, we screened out the pairs containing dysregulated miRNAs and further analyzed the targets of these miRNAs. As a result, we obtained 7 HCC related genes, such as E2F3, a target of miR-195 which was targeted by the TF DDIT3[22] (Fig. 3B and Fig. S1, Table S3, ESI†). Surprisingly, we noticed that the miR-195/497 cluster was predicted to be targeted by hypermethylated TFs NEUROG2 and DDIT3. As we have identified that the miR-195/497 cluster was under potential regulation of DNA hypermethylation in HCC, we inferred that the down-regulation of miR-195/497 in HCC might result from its promoter hypermethylation and the regulation by TFs NEUROG2 and DDIT3 simultaneously (Fig. 3C).

Based on the above combinatorial analyses, we proposed a model to interpret the regulatory role of DNA methylation on the expression of miRNAs and HCC related genes in HCC. In this model, miRNA expression could be regulated by both DNA methylation and TFs (Fig. 3D). Meanwhile, TFs might be under the transcriptional control of DNA methylation. All of DNA methylation, miRNAs and TFs could cause the dysregulation of HCC related genes. Therefore, DNA methylation plays an important role in the hepatocarcinogenesis by forming a complex regulatory network together with miRNAs and TFs.

DNA methylation characteristics of intragenic miRNAs and their host genes

Due to the important regulatory role of DNA methylation on miRNA and gene expression, we analyzed the relative location of DMRs to intragenic miRNAs and their host genes in order to better understand the law of regulation of DNA methylation on intragenic miRNAs. We identified 170 intragenic miRNAs harboring DMRs and these miRNAs could be classified into three categories: (a) 30 miRNAs were host-methylated miRNAs, which meant the DMRs located only at the upstream/downstream of their host genes; (b) 28 miRNAs were miRNA-methylated miRNAs with DMRs that were located only at the upstream/downstream of miRNAs; (c) 112 miRNAs were host-miRNA-methylated miRNAs, which meant the DMRs distributed at both the upstream/downstream of miRNAs and their host genes (Fig. 3E and Table S4, ESI†). Further analysis revealed that 56.7% (17/30) of host-methylated miRNAs appeared in 5 miRNA clusters (<10 kb), while only 21.8% (37/170) of all the 170 intragenic miRNAs with DMRs were distributed in 14 miRNA clusters, which implied that co-methylation of miRNAs and their host genes tend to occur on miRNAs in clusters (Pearson's chi square test, $p < 2.0 \times 10^{-5}$). This aberrant clustering of host-methylated miRNAs might provide guidance for synergistic effects of miRNAs in one cluster.

Discussion

Abundant studies have disclosed that miRNAs are involved in the initiation and progression of various human cancers and proposed with potential value for diagnosis and therapy in cancer.[23–25] But the mechanisms underlying miRNA dysregulation are still not fully elucidated. Increasing evidence supports that some important dysregulated miRNAs are subjected to regulation by epigenetic alterations, including DNA methylation.[12,26,27] In this study, we detected the genome-wide DNA methylation profiles of miRNAs in HCC. On the whole, we found that HCC cells showed special global miRNA methylation profiles: miRNA hypomethylation was more common compared to hypermethylation and usually found in CpG-poor regions,

while miRNA hypermethylation was mainly found in CpG islands, which is similar to the recent observation in chronic lymphocytic leukemia.[28]

Further, through integrative analysis of DNA methylation and miRNA expression, we identified 10 dysregulated miRNAs under potential regulation of DNA methylation in HCC. The 10 miRNAs included 5 down-regulated miRNAs (miR-375, miR-195, miR-497, miR-378 and miR-148a) and 5 up-regulated miRNAs in two clusters (miR-25/93/106b and miR-23a/27a). The correlation between DNA hypomethylation and miRNA up-regulation was often underestimated in previous studies. However, considering the wide distribution of miRNA hypomethylation in HCC, we speculate that DNA hypomethylation also plays a significant role in miRNA regulation as well as in hypermethylation. For instance, recently miR-429, which can manipulate liver tumour-initiating cells (T-ICs) by targeting the RBBP4/E2F1/OCT4 axis, was reported to be up-regulated in HCC because of four abnormal hypomethylated sites at its upstream.[29] For the 5 epigenetically silenced miRNAs, miR-375 functions as a tumor suppressor by targeting AEG-1 and ATG7 in HCC.[5,30] Meanwhile, enhancing the miR-375 expression could effectively suppress the HCC cell migration and invasion[5]. The miR-195/497 cluster was reported to show a significant growth-suppressive activity with induction of G1 arrest through targeting multiple cell-cycle regulators in HCC.[31] From bioinformatics analysis, we predicted that CDK4, which is involved in chemotherapy-mediated tumor cell apoptosis,[32] is a putative target of miR-195. MiR-378 was reported to suppress HBV-related hepatocellular carcinoma tumor growth by directly targeting the insulin-like growth factor 1 receptor (IGF1R).[33] Moreover, we predicted that IGF2BP1, which is an important protumorigenic factor and a metastasis promoting factor in liver cancer,[34,35] is also a putative target of miR-378. MiR-148a was proved to play a pivotal role in the liver by promoting the hepato-specific phenotype *via* targeting DNMT1 and acted as a tumor suppressor by regulating the c-Met oncogene.[36] Interestingly, we predicted and proposed that miR-148a and its targets, DNMT1[17] and DNMT3B,[18] might form a negative regulatory loop in HCC, which could boost the down-regulation of miR-148a (Fig. 3A). This negative loop has been experimentally confirmed in breast cancer,[37] while it needs further exploration in HCC. For the 5 epigenetically activated miRNAs, the miR-25/93/106b cluster was shown to be necessary for cell proliferation and anchorage-independent growth and might suppress apoptosis by targeting the TF E2F1 in HCC.[38] Meanwhile, miR-93 was predicted to target PHLPP[39,40] and TP53INP,[41,42] which implicates that miR-93 may be associated with metastatic potential and chemo-resistant biological characteristics of HCC. The miR-23a/27a cluster was found to function as an anti-apoptotic and proliferation-promoting factor in liver cancer cells[43]. Recently, researchers have shed light on the role of miR-23a as a potential target in regulating chemosensitivity of HCC cells.[10] Therefore, the 10 miRNAs have been confirmed to be dysregulated and play a vital role in HCC. Our results here showed that DNA methylation might be responsible for their abnormal expressions and gave some clues to carry out further deeper researches on these dysregulated miRNAs.

In order to better understand the function of DNA methylation, we put forward a network of DNA methylation, miRNAs, TFs and HCC related genes to illustrate the potential complex regulatory mechanism of DNA methylation on miRNA expression and the possible approach of DNA methylation contributing to hepatocarcinogenesis. Though lacking sufficient supporting data, this network would provide informative hints on the role of DNA methylation in HCC. We also found that co-methylation of miRNAs and their host genes tend to occur on miRNAs in clusters. To the best of our knowledge, the analysis of DNA methylation characteristic of intragenic miRNAs and their host genes has not previously been reported. However, the reasons and deep significance of this phenomenon are unknown and further investigation is needed.

In conclusion, we found that global DNA methylation profiles of miRNA genes were significantly different between cancerous and normal hepatocytes. Based on the integrated analysis of MeDIP-chip and miRNA expression microarray data, we identified 10 dysregulated miRNAs potentially regulated by DNA methylation and proposed a pattern to explain the role of DNA methylation on miRNA and gene expression in HCC. On account of the reversibility of DNA methylation and the amplified effects of miRNAs, these differentially methylated miRNAs may provide clues for the diagnosis and therapy of HCC, and a lot of validated experimental studies are worthy to be further determined.

Materials and methods

Cell lines

The hepatoma cell lines SK-HEP-1, HepG2, Hep3B, Huh7, MHCC97-H, MHCC97-L, and SMMC-7721, and normal primary human hepatocytes PHHC-1, PHHC-2, PHHC-3, and PHHC-4 were cultured and maintained as previously described.[5] This study was approved by the local Research Ethics Committee at the Tongji Hospital of Huazhong University of Science and Technology.

MeDIP-chip analysis

MeDIP-chip was processed in cancerous hepatocytes SK-HEP-1, HepG2, and MHCC97-H, and normal hepatocytes PHHC-4-1, PHHC-4-2, and PHHC-4-3. Genomic DNA from cells was extracted and sonicated to random fragments of size about 500 bp. Immunoprecipitation of methylated DNA was performed using a mouse monoclonal antibody against 5-methylcytidine and Biomag™ magnetic beads coupled anti-mouse IgG. Immunoprecipitated DNA (MeDIP DNA) was eluted and purified by phenol chloroform extraction and ethanol precipitation. The Input and MeDIP DNA were labeled with Cy3- and Cy5-labeled random 9-mers, respectively, and then hybridized to Human miRNA Tiling Microarray, which is a NimbleGen custom-designed 385k array containing 541 miRNA regions (all miRNAs in miRBase10.1) and 2700 CpG island regions (CpG islands located in regions from 150 kb upstream to 150 kb downstream of pre-miRNAs or from 100 kb upstream to 100 kb downstream of host genes) (Fig. 1A).

If a miRNA is located in an intergenic region, the miRNA region was designed from 50 kb upstream to 10 kb downstream of the pre-miRNA. If a miRNA is located in a host gene, the miRNA region is from 20 kb upstream of the host gene's transcription start site (TSS) to the end of the host gene. The array scanning was performed using an Axon GenePix 4000B microarray scanner. The experimental operation of MeDIP-chip was performed by Kangcheng Biotechnology Co., Ltd, (Shanghai, China).

Raw data containing signal intensity were extracted from array images as pair files using NimbleGen DEVA software. Then a scaled log2 ratio data for individual probe were generated using a bi-weight mean algorithm while the ratio is MeDIP/input. A modified ACME algorithm[44] was employed to get high-intensity probes ($p < 0.01$). When the distance between the nearby probes was equal to or less than 500 bp, regions of these probes were merged into a single methylated region, named as a single peak. Peaks defined by a single probe were excluded in further analysis. In consideration of precision, we only analyzed peaks in the designed regions (DRs): −10–2 kb of the start of pre-miRNAs and −20–4 kb of the start of protein-coding genes. If there is another gene in the region, the region was modified to the end of the upstream gene or to the start of the downstream gene. When a miRNA is located in a host gene, the corresponding region was amplified to the region of its host gene.

We used different strategies to obtain the DNA methylation status of miRNAs and protein-coding genes, respectively. To identify candidate DMRs on miRNAs, we integrated peaks of all samples and separated them into new peaks by dividing the overlapped peaks from different samples into segmental peaks without overlap. The methylation difference of these new peaks between cancerous and normal hepatocytes was evaluated using a *T*-test and $p < 0.05$ was used as the cutoff for DMRs. We classified DMRs into two classes: HMRs and LMRs based on their status in cancerous hepatocytes. For protein-coding genes, we divided their designed regions into blocks (3 kb per block), and sought for peaks in each block. For a single block, if there were peaks in all cancerous hepatocytes while no peaks in all normal hepatocytes, we referred the block as HMR. In contrast, we regarded it as LMR.

Correlation analysis between DNA methylation and miRNA expression

We previously detected the miRNA expression profiles in 7 hepatoma cell lines (SK-HEP-1, HepG2, Hep3B, Huh7, MHCC97-H, MHCC97-L, and SMMC-7721) and 3 cases of normal primary human hepatocytes (PHHC-1, PHHC-2, and PHHC-3) and identified 37 dysregulated miRNAs in HCC.[5] Thus, we have three hepatoma cell lines (SK-HEP-1, HepG2 and MHCC97-H) with both miRNA expression data and DNA methylation data. For the 37 differentially expressed miRNAs, we screened out miRNAs with DMRs and then calculated their DNA methylation and expression changes between these 3 HCC cell lines and normal hepatocytes. Then, the correlation coefficient of DNA methylation and miRNA expression of these miRNAs was calculated using the Spearman rank correlation method in R package. Candidate miRNAs under potential regulation of DNA methylation were screened out as their correlation coefficients were lower than −0.5.

MiRNA target prediction and enrichment analysis

Target genes of miRNAs were obtained from the union dataset of three databases TarBase (May 2013),[45] miR2Disease (April 2013)[46] and miRTarBase (April 2013),[47] which are databases collecting experimentally validated or literature reported miRNA targets. Targets of TFs were extracted from a union of validated targets from Transfac (2012)[48] and predicated targets from the UCSC genome browser. For the UCSC predicted targets, we used a strict cutoff with *Z* score < 2.33 and limited them to be conserved among humans, mice and rats to reduce false-positives, which were the same as our previous study.[49] GO and KEGG pathway enrichment analyses of target genes were conducted using the latest GO and KEGG dataset by our in-house scripts.

Conflict of interest

None declared.

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