# Identification of miRNA and mRNA expression profiles by PCR microarray in hepatitis B virus-associated hepatocellular carcinoma

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**Abstract.** The present study aimed to identify differentially expressed microRNAs (miRNAs) and mRNAs in hepatitis B virus-associated hepatocellular carcinoma (HCC). A total of five HCC tissues and paired adjacent non-tumor tissues were screened to identify the differentially expressed miRNAs and target mRNAs using polymerase chain reaction microarrays. The interaction between differential miRNA and mRNA expression was concurrently analyzed using bioinformatics methods. A total of 32 differentially expressed miRNAs (four upregulated miRNAs and 28 downregulated miRNAs) and 16 differentially expressed mRNAs (11 upregulated mRNAs and five downregulated mRNAs) were identified. Among these, upregulated hsa-miRNA (miR)-96-5p and hsa-miR-18b-5p suppressed their target mRNAs forkhead box O1 and MET transcriptional regulator MACC1 (MACC1). Downregulation of hsa-miR-199a-5p led to upregulation of its target mRNAs, cyclin dependent kinase 4 and insulin like growth factor 2 (IGF2). The high-level expression of IGF2 mRNA and cyclin E1 mRNA was due to the low-level expression of hsa-miR-145-5p, hsa-miR-181a-5p, hsa-miR-199a-5p and hsa-miR-223a-3p, and hsa-miR-26a-5p and hsa-miR-26b-5p, respectively. The low-level expression of coronin 1A mRNA and MACC1 mRNA was due to overexpression of hsa-miR-517a-3p and hsa-miR-18a-5p, and hsa-miR-18b-5p, respectively. Numerous gene ontology terms were associated with oncogenesis. The most enriched pathways targeted by the dysregulated miRNAs and mRNAs were associated with cancer and oncogenesis pathways. The present data suggested that differential miRNA and mRNA expression is present in HCC. Thus, interactions between certain miRNAs and mRNAs may be involved in the pathogenesis of HCC.

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*Key words:* hepatocellular carcinoma, microRNA, mRNA, microarray, hepatitis B virus, biomarker

## Introduction

Hepatocellular carcinoma (HCC) is the sixth most common neoplasm and the third most frequent cause of cancer-associated mortality (1). The majority of patients with HCC suffering from hepatitis B virus (HBV) infection have cirrhosis secondary to chronic necroinflammation (2). HBV, an oncogenic virus, likely causes HCC via direct (integration of its DNA into the host genome) and indirect (necroinflammation and regeneration injury) pathways (3). The aberrant expression of genes, which involves numerous types of RNA, serves an important role in the occurrence and development of HCC. MicroRNAs (miRNAs) and mRNA have been reported to be involved in the pathogenesis of HCC. In addition, miRNAs have clinical value in the diagnosis of HCC, since they are present in the blood and thus may be used as diagnostic markers, and in turn as potential targets for specific systemic treatments (4).

Mature human miRNA is a class of single-stranded, small non-coding RNAs that are ~22 nucleotides in length (5). miRNAs serve a key regulatory role in gene expression at the posttranscriptional level. miRNAs act by binding to imperfectly complementary sites within the 3'-untranslated regions of their target mRNAs, inhibiting translation or initiating degradation of the target mRNA through the miRNA-associated RNA-induced silencing complex (miRISC). Recruitment of the miRISC may thus modulate the expression of targeted protein-coding genes (6). A single mRNA transcript may have a number of miRNA response elements for different miRNAs and, conversely, one miRNA may target as many as 100 different mRNAs in a gene regulatory network (7,8). miRNAs are involved in a series of crucial biological processes (9). The most notable alterations in miRNA expression are observed in cancer. Certain miRNAs may function as oncogenes or tumor suppressor genes by targeting their corresponding mRNAs, and certain dysregulated miRNAs are able to promote tumorigenesis and cancer progression (10,11). As has been widely indicated, the expression of miRNAs and their corresponding target genes is often inversely modulated in different backgrounds (12). Mounting evidence has suggested the importance of miRNAs in the modulation of gene expression, cellular proliferation, cellular mobility, cellular differentiation, apoptosis and tumorigenesis (13).

In recent years, miRNA and mRNA expression profile studies have identified a large number of genes with differential expression in HCC (14-25). Numerous differentially expressed miRNAs and mRNAs have been identified to be involved in the occurrence and development of HCC, and thus may be potential prognostic and diagnostic markers (17,26). Studying the interaction of miRNAs and mRNAs has become an important part of cancer research.

The microarray is a powerful tool for conveniently and quickly analyzing miRNAs relevant to cancer. More importantly, it also simultaneously profiles the mRNA targets of the miRNAs, thereby providing insights into the interaction between the cancer-associated miRNAs and their target mRNAs (27). Collectively, the predicted target genes, network diagrams, and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses may be useful for determining the mechanism of HCC tumorigenesis.

In the present study, the expression profiles of miRNAs and mRNAs in HBV-associated HCC were identified by polymerase chain reaction (PCR) microarray, and the interactions between the differentially expressed miRNAs and their targets were analyzed using bioinformatics methods.

## Patients and methods

*Patients and clinical specimens*. A total of five HCC tissues and paired adjacent non-tumor tissues (NTs) were collected as surgical specimens between June 2012 and December 2013 at Beijing Youan Hospital, Capital Medical University (Beijing, China). All tissue specimens were immediately preserved in RNAfixer reagent (Bioteke, Beijing, China) and stored at -80˚C until use. The NTs were taken 5 cm from the edge of the cancer and contained no obvious tumor cells, as evaluated by an experienced pathologist. The five HCC patients were diagnosed with HBV infection; serum positive for hepatitis B surface antigen identified by ELISA method (ELISA kit Cat. no. 185982, Roche Diagnostics, Basel, Switzerland) was defined as HBV infection. Tumors were staged according to the tumor-node-metastasis staging system (28) and Barcelona Clinic Liver Cancer system (29) (Table I). No radiotherapy, chemotherapy or targeted therapy was administered prior to the isolation of tissue specimens. The protocol was approved by the appropriate ethics committees in Beijing Youan Hospital and was conducted in compliance with the Declaration of Helsinki. Written consent was obtained from all participants.

*Total RNA extraction and quality control*. Total tissue RNA was extracted from the HCC tissues and paired adjacent NTs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's protocol. Contaminating DNA was removed from the RNA preparations using DNase I. The RNA was purified using an RNeasy® MinElute™ Cleanup kit (Qiagen GmbH, Hilden, Germany). Subsequently, the RNA concentration of the samples was determined via the absorbance at 260 nm using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The integrity of the RNA was assessed by electrophoresis on a denaturing agarose gel.

*cDNA synthesis and quantitative (q)PCR*. cDNA was synthesized using a miRNA First-Strand cDNA Synthesis kit (Arraystar, Inc., MD, USA) and operated according manufacturer's protocol. Briefly, total RNA was ligated with the 3'-ligation adapter by RNA ligase. The cRNA template was obtained by a reverse transcriptase reaction at 42˚C for 60 min, then inactivated at 85˚C for 5 min. The reaction system included 10 $\mu$l ligated product, 1 $\mu$l Moloney murine leukemia virus reverse transcriptase, 8.5 $\mu$l RT Reaction Master Mix (contain dNTP) and 0.5 $\mu$l RNase inhibitor. qPCR was performed using miRStar™ Hu man Cancer Focus miRNA & Target mRNA PCR Array (Arraystar, Inc., Rockville, MD, USA) on an ABI PRISM7900 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). This array contained 184 critical tumor-associated miRNAs and 178 well-defined mRNA targets of these miRNAs. The PCR reaction system included diluted cDNA template, PCR primer mix and SYBR® Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 1 min. Following qPCR, the amplification products were optically measured referred to the instruction manual and the resulting melting curves were analyzed.

*Data analysis and statistical analysis*. The initial data analysis was performed using the software (version SDS 2.4) supplied with the qPCR instrument to obtain raw Cq values. Normalization and further data analysis was performed using GenEx qPCR analysis software (version 6.1, www.exiqon.com/mirna-pcranalysis). qPCR reactions results were calculated using the $2^{-\Delta\Delta Cq}$ method (30). The resulting data were analyzed by two-tailed Student's t-test. Fold change (FC) ≥2 and $P<0.05$ was considered to indicate a statistically significant difference.

*miRNA target prediction, GO and KEGG pathway analyses*. miRNA target prediction was performed using miRWalk software (version 7.0, http://mirwalk.umm.uni-heidelberg.de). A total of five algorithms were used for miRNA target gene prediction, namely miRWalk (version 7.0, mirwalk.umm.uni-heidelberg.de), miRanda (www.microrna.org/microrna/home.do), miRDB (Last modified: May 03, 2016, http://mirdb.org/mirdb/), RNA22 (version 2, cm.jefferson.edu/rna22/Interactive/l) and Targetscan (release 7.1, www.targetscan.org). Only when one gene was confirmed by all five algorithms was the gene identified as a target gene, in order to increase the accuracy of the target gene prediction. GO functional enrichment and KEGG pathway enrichment analyses for differentially expressed genes were performed using the cluster profiler package of R software (version 3.3.3, https://www.r-project.org) based on the GO database (www.geneontology.org) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID; version 6.7, https://david.ncifcrf.gov). Fisher's exact test was used to identify whether there was more overlap between the differentially expressed gene list and the GO annotation list compared with that which would be expected by chance.

## Results

*Identification of differentially expressed miRNAs and mRNAs*. In our study, 32 differentially expressed miRNAs (four upregulated

Table I. Clinical details of the five patients with hepatocellular carcinoma.

| Case ID | Sex | Age, years | HBV-DNA load, IU/ml | Liver cirrhosis | Differentiation | Stage, TNM | Stage, BCLC |
|---|---|---|---|---|---|---|---|
| Case 1 | M | 44 | <100 | None | Moderate | T1N0M0 | A |
| Case 2 | M | 63 | $4.01x10^6$ | Yes | Moderate | T1N0M0 | A |
| Case 3 | M | 44 | $9.85x10^2$ | Yes | Poor | T3aN0M0 | B |
| Case 4 | F | 45 | $1.98x10^3$ | Yes | Moderate | T1N0M0 | A |
| Case 5 | M | 59 | $1.60x10^6$ | Yes | Poor | T1N0M0 | A |

HBV, hepatitis B virus; TNM, tumor, node, metastasis; BCLC, Barcelona Clinic Liver Cancer; M, male; F, female.

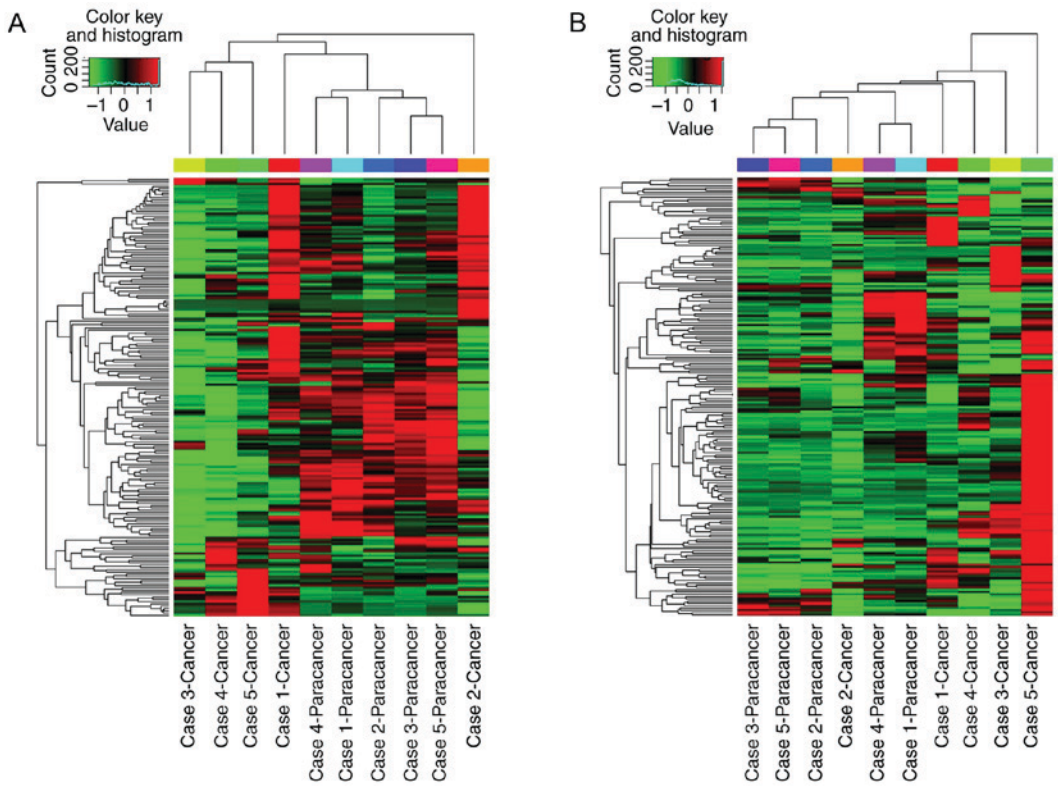


Figure 1. (A) miRNA and (B) mRNA expression hierarchical clustering plots. Unsupervised classification of hepatocellular carcinoma tissue samples and matched paracancerous tissue samples based on miRNA and mRNA expression profiles. The miRNA and mRNA expression data are depicted as a data matrix, with each row representing a probe and each column representing a sample. Expression levels are depicted according to the displayed color scale. Red and green indicate expression levels above and below the median, respectively. The magnitude of deviation from the median is represented by the color saturation. miRNA, microRNA.

miRNAs and 28 downregulated miRNAs) and 16 differentially expressed mRNAs (11 upregulated mRNAs and five downregulated mRNAs) were identified [fold change (FC)≥2 and P≤0.05]. The global miRNA and mRNA expression patterns were evaluated using a hierarchical clustering plot (Fig. 1). The results demonstrated that each differentially expressed miRNA was able to regulate one or more mRNAs. Similarly, one mRNA may be regulated by one or more miRNAs. The differentially expressed miRNAs and mRNAs with FC≥2 and P≤0.05 are presented in Tables II and III. In the HCC tissues, hsa-miR-96-5p and hsa-miR-18b-5p were the most significantly upregulated miRNAs, while hsa-miR-451a and hsa-miR-199a-5p were the most significantly downregulated miRNAs, compared with their levels in the NTs. In turn, the upregulated miRNAs were indicated to negatively regulate their target mRNAs; for instance, upregulated hsa-miR-96-5p and hsa-miR-18b-5p suppressed the mRNA expression of forkhead box O1 (FOXO1) and MET transcriptional regulator MACC1 (MACC1), respectively. The downregulated miRNAs were indicated to positively regulate their target mRNAs; downregulated hsa-miR-199a-5p facilitated an increase in the mRNA expression of cyclin dependent kinase 4 (CDK4) and insulin like growth factor 2 (IGF2).

IGF2 and cyclin E1 (CCNE1) were the most significantly upregulated mRNAs, while coronin 1A (CORO1A) and MACC1 were the most significantly downregulated mRNAs. The high-level expression of IGF2 and CCNE1 was due to the downregulation of hsa-miR-145-5p, hsa-miR-181a-5p, hsa-miR-199a-5p and hsa-miR-223-3p, and hsa-miR-26a-5p

Table II. Concurrent analysis of differential miRNA and relevant mRNA expression.

| miRNA ID | Fold change | T-test P-value | mRNA ID | Fold change | T-test P-value |
|---|---|---|---|---|---|
| hsa-miR-96-5p | 3.23 | 0.015466 | FOXO1 | -3.06 | 0.003590 |
| hsa-miR-18b-5p | 3.30 | 0.028242 | MACC1 | -9.90 | 0.038804 |
| hsa-miR-18a-5p | 2.79 | 0.047523 | MACC1 | -9.90 | 0.038804 |
| hsa-miR-182-5p | 2.14 | 0.017416 | ZEB2 | -3.32 | 0.022872 |
| hsa-miR-142-5p | -2.95 | 0.006185 | RECK | 2.23 | 0.046041 |
| | | | MET | 2.30 | 0.007944 |
| hsa-let-7a-5p | -2.26 | 0.000101 | DMTF1 | 2.14 | 0.012181 |
| | | | CD34 | 3.06 | 0.026660 |
| hsa-miR-342-3p | -2.20 | 0.005877 | E2F1 | 3.70 | 0.013213 |
| hsa-miR-30e-3p | -2.07 | 0.009148 | CDK4 | 3.57 | 0.027208 |
| | | | PTK2 | 3.13 | 0.008627 |
| hsa-let-7b-5p | -2.53 | 0.000082 | DMTF1 | 2.14 | 0.012181 |
| | | | CD34 | 3.06 | 0.026660 |
| hsa-miR-145-5p | -3.92 | 0.008139 | IGF2 | 83.52 | 0.025926 |
| hsa-miR-101-3p | -3.05 | 0.001338 | EZH2 | 7.73 | 0.006231 |
| | | | MET | 2.30 | 0.007944 |
| hsa-miR-200b-3p | -3.53 | 0.043589 | RECK | 2.23 | 0.046041 |
| hsa-miR-130a-3p | -2.97 | 0.005732 | MET | 2.30 | 0.007944 |
| | | | CDK4 | 3.57 | 0.027208 |
| hsa-miR-422a | -2.64 | 0.000209 | SMO | 2.53 | 0.046150 |
| hsa-miR-130b-3p | -2.39 | 0.002171 | MET | 2.30 | 0.007944 |
| | | | CDK4 | 3.57 | 0.027208 |
| hsa-miR-150-5p | -2.57 | 0.046146 | CDK4 | 3.57 | 0.027208 |
| hsa-miR-199a-5p | -9.21 | 0.000657 | CDK4 | 3.57 | 0.027208 |
| | | | IGF2 | 83.52 | 0.025926 |
| hsa-let-7c-5p | -2.45 | 0.000184 | DMTF1 | 2.14 | 0.012181 |
| | | | CD34 | 3.06 | 0.026660 |
| hsa-let-7e-5p | -2.11 | 0.003287 | DMTF1 | 2.14 | 0.012181 |
| | | | CD34 | 3.06 | 0.026660 |
| hsa-miR-143-3p | -2.68 | 0.025475 | DMTF1 | 2.14 | 0.012181 |
| | | | SMO | 2.53 | 0.046150 |
| hsa-miR-26a-5p | -2.11 | 0.000546 | CCNE1 | 8.13 | 0.027245 |
| hsa-miR-181a-5p | -2.23 | 0.000035 | RECK | 2.23 | 0.046041 |
| | | | MET | 2.30 | 0.007944 |
| | | | IGF2 | 83.52 | 0.025926 |
| | | | SMO | 2.53 | 0.046150 |
| hsa-miR-122-5p | -2.68 | 0.015077 | MET | 2.30 | 0.007944 |
| hsa-miR-505-3p | -2.54 | 0.002885 | PTK2 | 3.13 | 0.008627 |
| | | | SMO | 2.53 | 0.046150 |
| | | | MET | 2.30 | 0.007944 |
| hsa-miR-26b-5p | -2.05 | 0.001286 | CCNE1 | 8.13 | 0.027245 |
| hsa-miR-125b-5p | -3.22 | 0.000950 | SMO | 2.53 | 0.046150 |
| hsa-miR-223-3p | -2.32 | 0.003535 | E2F1 | 3.70 | 0.013213 |
| | | | IGF2 | 83.52 | 0.025926 |
| hsa-miR-100-5p | -3.01 | 0.034014 | - | - | - |
| hsa-miR-188-5p | -2.48 | 0.003011 | - | - | - |
| hsa-miR-22-3p | -2.63 | 0.000404 | - | - | - |
| hsa-miR-451a | -10.55 | 0.000566 | - | - | - |
| hsa-miR-99a-5p | -3.23 | 0.007100 | - | - | - |

miRNA/miR, microRNA.

Table III. Concurrent analysis of differential mRNA and relevant miRNA expression.

| mRNA ID | Fold change | T-test P-value | miRNA ID | Fold change | T-test P-value |
|---|---|---|---|---|---|
| CCNE1 | 8.13 | 0.027245 | hsa-miR-26a-5p | -2.11 | 0.000546 |
| | | | hsa-miR-26b-5p | -2.05 | 0.001286 |
| IGF2 | 83.52 | 0.025926 | hsa-miR-145-5p | -3.92 | 0.008139 |
| | | | hsa-miR-181a-5p | -2.23 | 0.000035 |
| | | | hsa-miR-199a-5p | -9.21 | 0.000657 |
| | | | hsa-miR-223-3p | -2.32 | 0.003535 |
| CDK4 | 3.57 | 0.027208 | hsa-miR-130a-3p | -2.97 | 0.005732 |
| | | | hsa-miR-130b-3p | -2.39 | 0.002171 |
| | | | hsa-miR-150-5p | -2.57 | 0.046146 |
| | | | hsa-miR-199a-5p | -9.21 | 0.000657 |
| | | | hsa-miR-30e-3p | -2.07 | 0.009148 |
| E2F1 | 3.70 | 0.013213 | hsa-miR-223-3p | -2.32 | 0.003535 |
| | | | hsa-miR-342-3p | -2.20 | 0.005877 |
| PTK2 | 3.13 | 0.008627 | hsa-miR-30e-3p | -2.07 | 0.009148 |
| | | | hsa-miR-505-3p | -2.54 | 0.002885 |
| CD34 | 3.06 | 0.026660 | hsa-let-7a-5p | -2.26 | 0.000101 |
| | | | hsa-let-7b-5p | -2.53 | 0.000082 |
| | | | hsa-let-7c-5p | -2.45 | 0.000184 |
| | | | hsa-let-7e-5p | -2.11 | 0.003287 |
| EZH2 | 7.73 | 0.006231 | hsa-miR-101-3p | -3.05 | 0.001338 |
| SMO | 2.53 | 0.046150 | hsa-miR-125b-5p | -3.22 | 0.000950 |
| | | | hsa-miR-143-3p | -2.68 | 0.025475 |
| | | | hsa-miR-181a-5p | -2.23 | 0.000035 |
| | | | hsa-miR-422a | -2.64 | 0.000209 |
| | | | hsa-miR-505-3p | -2.54 | 0.002885 |
| MET | 2.30 | 0.007944 | hsa-miR-101-3p | -3.05 | 0.001338 |
| | | | hsa-miR-122-5p | -2.68 | 0.015077 |
| | | | hsa-miR-130a-3p | -2.97 | 0.005732 |
| | | | hsa-miR-130b-3p | -2.39 | 0.002171 |
| | | | hsa-miR-142-5p | -2.95 | 0.006185 |
| | | | hsa-miR-181a-5p | -2.23 | 0.000035 |
| | | | hsa-miR-505-3p | -2.54 | 0.002885 |
| DMTF1 | 2.14 | 0.012181 | hsa-let-7b-5p | -2.53 | 0.000082 |
| | | | hsa-let-7c-5p | -2.45 | 0.000184 |
| | | | hsa-let-7e-5p | -2.11 | 0.003287 |
| | | | hsa-miR-143-3p | -2.68 | 0.025475 |
| RECK | 2.23 | 0.046041 | hsa-miR-142-5p | -2.95 | 0.006185 |
| | | | hsa-miR-181a-5p | -2.23 | 0.000035 |
| | | | hsa-miR-200b-3p | -3.53 | 0.043589 |
| CORO1A | -14.43 | 0.014401 | hsa-miR-517a-3p | 1.60 | 0.357626 |
| MACC1 | -9.90 | 0.038804 | hsa-miR-18a-5p | 2.79 | 0.047523 |
| | | | hsa-miR-18b-5p | 3.30 | 0.028242 |
| CCL4 | -4.72 | 0.002124 | hsa-miR-183-5p | 1.86 | 0.037847 |
| ZEB2 | -3.32 | 0.022872 | hsa-miR-182-5p | 2.14 | 0.017416 |
| FOXO1 | -3.06 | 0.003590 | hsa-miR-96-5p | 3.23 | 0.015466 |

miRNA/miR, microRNA.

and hsa-miR-26b-5p, respectively. The low-level expression of CORO1A and MACC1 was due to the upregulation of hsa-miR-517a-3p, and hsa-miR-18a-5p and hsa-miR-18b-5p, respectively.

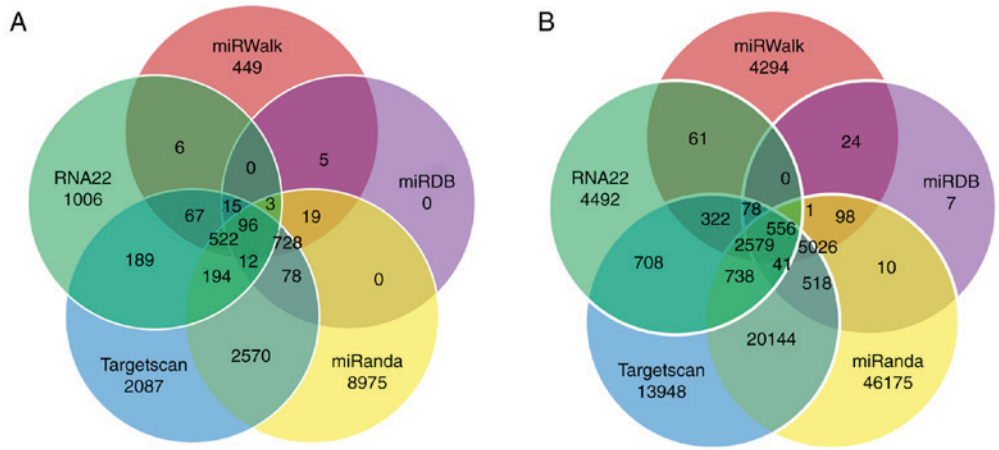


Figure 2. Up- and downregulated miRNA prediction. (A) Target prediction for the four upregulated miRNAs. (B) Target prediction for the 21 downregulated miRNAs. The Venn diagrams illustrate the mRNAs targeted by up-and downregulated miRNAs, and the overlap between the five databases. The four upregulated miRNAs targeted 96 targeted genes, and the 21 downregulated miRNAs targeted 556 genes. miRNA, microRNA.

*miRNA target prediction*. The miRNA target prediction results demonstrated that a single miRNA may regulate one or more target mRNAs. Collectively, the four upregulated miRNAs targeted 96 targeted genes, and the 21 downregulated miRNAs targeted 556 genes. The differentially expressed miRNA-targeted genes that overlapped between the five databases (miRanda, miRDB, miRWalk, RNA22 and Targetscan) are presented in Fig. 2.

*GO and KEGG pathway analyses of differentially expressed miRNAs and mRNAs*. GO terms cover three domains: Biological process (BP), cellular component (CC) and molecular function (MF). In the present study, numerous GO terms were associated with oncogenesis, including: 'Positive regulation of cellular process', 'positive regulation of biological process' and 'positive regulation of metabolic process' for BP; 'intracellular part', 'intracellular' and 'nuclear lumen' for CC; and 'protein binding', 'receptor binding', 'kinase regulator activity', 'protein kinase regulator activity', 'transcription factor activity' and 'nucleic acid binding transcription factor activity' for MF. The top 10 most significantly enriched terms for each category are presented in Fig. 3.

Pathway analysis is a type of functional analysis that maps genes to KEGG pathways. As signal transduction may be involved in HCC, the associated pathways were analyzed, according to the functions and interactions of the differential genes. A total of 10 pathways associated with the most dysregulated miRNAs and mRNAs, and with the lowest P-values, were determined. The most enriched pathways targeted by the dysregulated miRNAs and mRNAs were associated with cancer, including 'small cell lung cancer', 'pancreatic cancer', 'glioma', 'prostate cancer', 'melanoma' and 'bladder cancer', in addition to oncogenesis pathways, including 'TGF-beta signaling pathway', 'cGMP-PKG signaling pathway', 'calcium signaling pathway', 'PI3K-Akt signaling pathway' and 'pathways in cancer' (Fig. 4). This suggested that the dysregulated miRNAs and mRNAs identified in the present study may regulate the oncogenesis of HCC through these pathways.

## Discussion

The present study identified four upregulated and 28 downregulated miRNAs, and 11 upregulated and five downregulated mRNAs in HCC tissues compared with their expression levels in NTs. A single differentially expressed miRNA may regulate one or more mRNAs. Similarly, a single differentially expressed mRNA may be regulated by one or more miRNAs. Upregulated miRNAs suppress target mRNAs, while downregulated miRNAs are matched by an increase in their target mRNAs. Furthermore, numerous GO terms and enriched pathways associated with dysregulated miRNAs/mRNAs are associated with oncogenesis.

In the present study, through coexpression analysis, it was observed that upregulated hsa-miR-96-5p suppressed the mRNA expression of FOXO1. Acting as an oncomiR, miR-96 has previously been identified to be upregulated in HCC and is associated with metastatic features, including the presence of microvascular invasion, advanced tumor differentiation and shorter recurrence-free survival (31). Ectopic expression of miR-96-5p induces effects on insulin-like growth factor binding protein-3, IGF2 and insulin-like growth factor-1 receptor transcripts (32). Additionally, upregulated miR-96 promotes the proliferation of human HCC cells. On the contrary, downregulated miR-96 led to reduced HCC cell proliferation and migration (33). However, the serum level of miR-96 in HCC patients is markedly higher compared with that in patients with liver cirrhosis or chronic hepatitis B infection and healthy controls, and may serve as a promising biomarker in patients with HCC patients and chronic HBV infection (34).

The upregulated expression of hsa-miR-18b-5p suppressed the mRNA expression of MACC1 in the present study. hsa-miR-18, which belongs to the oncomiR-1 or miR-17-92 cluster, is regarded as an oncogene that is closely associated with the occurrence and development of different types of cancer. miR-18 targets the expression of numerous genes, including heat shock factor 2, c-Myc, E2F transcription factor 1 (E2F1), phosphatase and tensin homolog (PTEN), Bim, tissue growth factor, SMAD family member 3, transforming

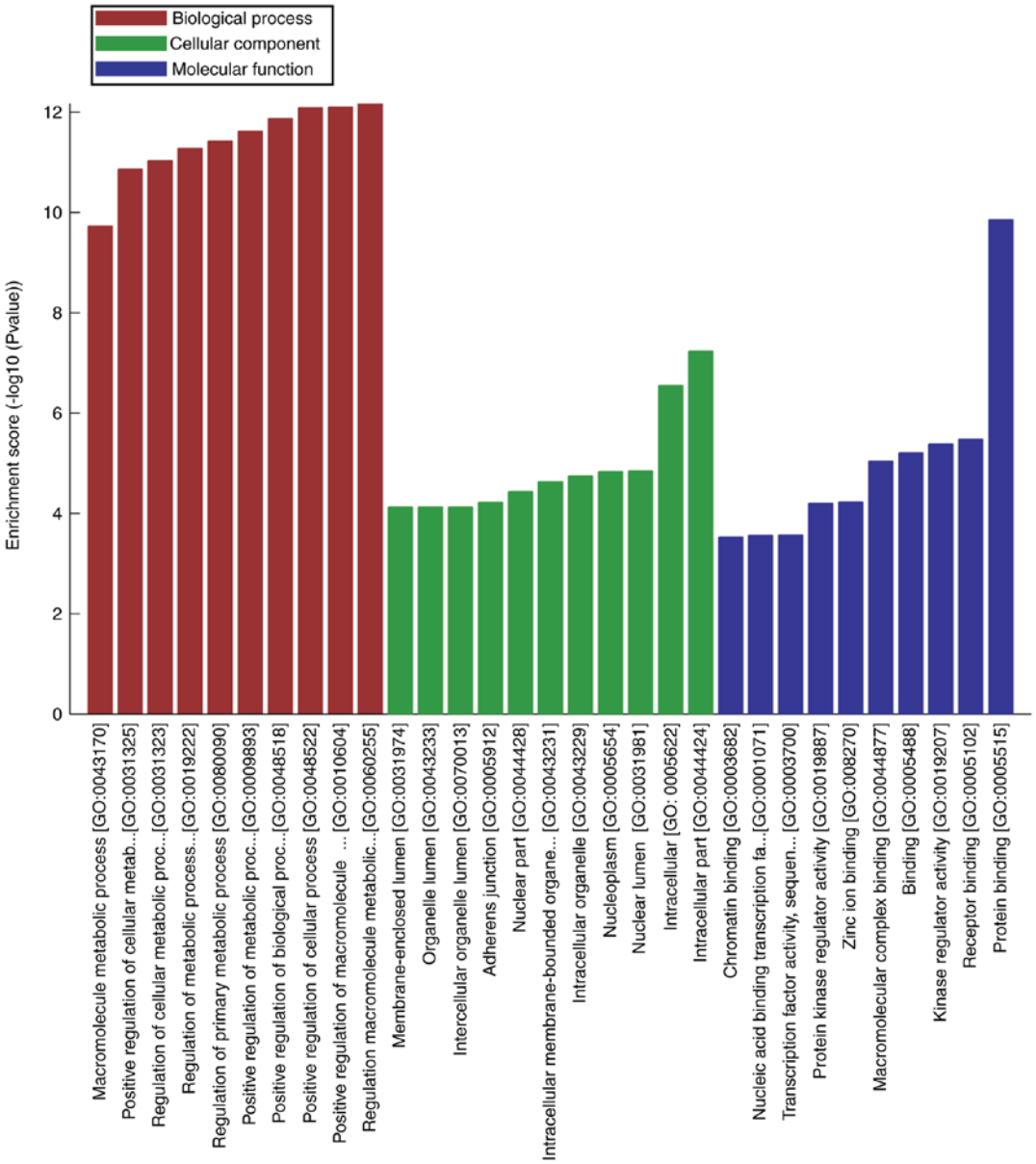


Figure 3. GO enrichment scores of differential expressed gene. X axis, GOID. Y axis, enrichment score values of the GOID [-log10 (P-value)]. GO, gene ontology.

growth factor (TGF)-β, trinucleotide repeat containing 6B, phosphoinositide 3-kinase (PI3K), cellular tumor antigen p53 and angiogenesis inhibitor thrombospondin-1, to thus regulate the associated signaling pathways (35). Overexpression of miRs in the miR-17-92 cluster has been identified during the development of cirrhosis, and also subsequently during the development of HCC, and may be regulated by c-Myc and E2F1 (36). miR-18b expression in poorly-differentiated HCC was reported to be significantly higher compared with well-differentiated HCC (36). Additionally, overexpression of miR-18b accelerates cell proliferation and the loss of cell adhesion ability, and following surgical resection, patients with HCC exhibiting high miR-18b expression have a significantly shorter relapse-free period compared with those with low expression (37). Overall, the concentration of miR-18a in the plasma/serum of patients with cancer is increased compared with that of controls, and miR-18 may be a potential diagnostic biomarker for numerous types of cancer (35,38).

hsa-miR-451a was demonstrated to be downregulated in HCC tissues in the present study. miR-451 has been demonstrated to be downregulated in a number of human malignancies and is correlated with tumor progression. miR-451 was observed to be downregulated in HCC tissues, and significantly correlated with advanced clinical stage, metastasis and poorer disease-free or overall survival (39). miR-451 has been indicated to inhibit cell growth, induce $G_0/G_1$ arrest and promote apoptosis in HCC cells by regulating the epithelial-mesenchymal transition process. The oncogene c-Myc is the direct and functional target of miR-451. miR-451 downregulation-induced c-Myc overexpression leads to the activation of extracellular signal regulated kinase (ERK)1/2 signaling (38). miR-451 may also function as a potential suppressor of tumor angiogenesis by targeting interleukin-6

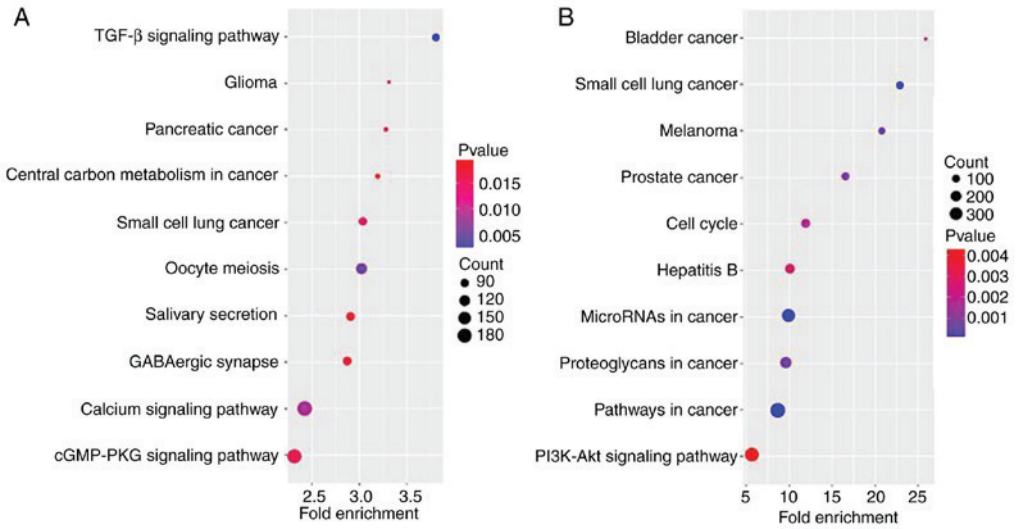


Figure 4. Dot plots of pathways. (A) Dot plot of dysregulated miRNA-target pathways. (B) Dot plot of dysregulated mRNA pathways. The dot plots illustrate the fold enrichment values of the top ten most significant pathways of dysregulated miRNAs-targets and mRNAs. The dot size represents the count of the pathway and the dot color represents the P-value of the pathway. X-axis, fold enrichment. miRNA, microRNA.

receptor-signal transducer and activator of transcription 3-vascular endothelial growth factor signaling in HCC (40).

Abnormal expression of miR-199a has been observed in various tumors, where it may influence the regulation of proliferation, metabolism, invasion, metastasis, angiogenesis and apoptosis of the tumor cells. The expression of miR-199a has been identified to be downregulated in non-small cell lung cancer, colorectal cancer (41), breast cancer (42), bladder cancer (43) and esophageal cancer (44); however, miR-199a is expressed at a high level in gastric cancer and positively regulates gastric cancer cell proliferation, migration and invasion (45). The different expression patterns of miR-199a in various tumor tissues suggests that miR-199a may serve as a tumor promoter or tumor suppressor. However, miR-199a/b-3p is consistently downregulated in HCC, and its reduction is significantly correlated with a poor survival rate in patients with HCC (18,46). miR-199a/b-3p may target tumor-promoting p21 (Rac1) activated kinase 4 (PAK4) to suppress HCC growth by inhibiting the PAK4/Raf/MEK/ERK pathway *in vitro* and *in vivo* (47). miR-199a-5p and let-7c cooperatively and efficiently inhibit HCC cell migration and invasion by targeting the metastasis promoter mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) and, consequently, MAP4K3-mediated drug sensitization (48). Decreased expression of miR-199a-5p contributes to increased cell invasion through functional deregulation of discoidin domain receptor tyrosine kinase 1 activity in HCC (46). miR-199a is significantly downregulated in the tissues and sera of patients with HCC. miR-199a may be used as a potential circulating biomarker for HCC (49,50). In the present study, downregulated miR-199a-5p elevated the expression of its targets CDK4 and IGF2.

GO enrichment and KEGG pathway analyses are widely used to determine the organization and functional annotation of molecular components (51,52). The development of HCC is associated with alterations in molecular functions and biological pathways (53,54). In the present study, various GO terms and KEGG pathways were indicated to be involved in HCC tumorigenesis. A number of BP terms were significantly enriched for cellular, biological and metabolic processes. Additionally, numerous MF terms were enriched for protein binding, receptor binding, protein kinase regulator activity, nucleic acid binding and transcription factor activity. Thus, through GO enrichment analysis, it was possible to determine the biological functions of the differentially expressed genes.

The most enriched pathways were observed to be associated with cancer (small cell lung cancer, pancreatic cancer, glioma, prostate cancer, melanoma and bladder cancer) and oncogenic pathways [TGF-β signaling pathway, cyclic guanosine monophosphate (cGMP)-protein kinase cGMP-dependent 1 signaling pathway, calcium signaling pathway, and PI3K-RAC-α serine/threonine-protein kinase signaling pathway]. Certain differentially expressed miRNAs and mRNAs have been reported to be associated with small cell lung cancer, glioma and pancreatic cancer. This three-way association ought to be the focus of future studies (18).

In conclusion, the present study identified four upregulated and 28 downregulated miRNAs, and 11 upregulated and five downregulated mRNAs by PCR microarray. Upregulated hsa-miR-96-5p and hsa-miR-18b-5p suppressed FOXO1 and MACC1 mRNA expression. Furthermore, downregulated hsa-miR-199a-5p increased CDK4 and IGF2 mRNA expression. The high-level expression of IGF2 and CCNE1 mRNAs was a result of hsa-miR-145-5p, hsa-miR-181a-5p and hsa-miR-199a-5p downregulation, and the low-level expression of CORO1A and MACC1 mRNAs was a result of upregulated hsa-miR-517a-3p and hsa-miR-18a-5p, and hsa-miR-18b-5p, respectively. Furthermore, various GO terms and KEGG pathways associated with the dysregulated miRNAs and mRNAs were likely involved in HCC tumorigenesis.

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**Availability of data and materials**

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

XWC and SCC was involved in the design of the experiment, drafted and revised the manuscript, collected and processed the specimens, was responsible for the collection and analysis of the data. ZLQ and CL were involved in the design of the experiment, collected and processed the specimens and provided final approval of the version to be published. All the authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the ethics committee of Beijing Youan Hospital, Capital Medical University (Beijing, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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