Differential expression of serum microRNAs in cirrhosis that evolve into hepatocellular carcinoma related to hepatitis B virus

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Abstract. Circulating microRNAs (miRNAs) exist stably in body fluids and are potential biomarkers for hepatocellular carcinoma (HCC). Twenty-five patients with cirrhosis that evolved into HCC, who were treated at The Third Hospital of Zhenjiang Affiliated to Jiangsu University between January 2005 and December 2012, were enrolled. In the discovery stage, 2 serum samples pooled from 3 cirrhosis and 3 HCC samples were subjected to deep sequencing. Subsequently, differential expression of miRNAs was validated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the serum samples from an independent cohort of 22 patients with cirrhosis and HCC. Twenty-two miRNAs showed a >2-fold upregulation (P<0.01), and 2 miRNAs showed a >2-fold downregulation (P<0.01) in the cirrhosis and HCC samples. Using the comparative Ct method, we calculated the $2^{-\Delta\Delta Ct}$ for 40 candidate miRNAs in the sample sets. Eight of the 40 miRNAs demonstrated significantly differential expression levels between the disease categories. The miRNAs exhibiting differential expression were hsa-miR-122-5p, hsa-miR-199a-5p, hsa-miR-486-5p, hsa-miR-193b-5p, hsa-miR-206, hsa-miR-141-3p, hsa-miR-192-5p and hsa-miR-26a-5p. We identified the miRNAs differentially expressed in cirrhosis that evolved into hepatitis B virus-related HCC.

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide, and is associated with persistent infection from hepatitis B virus (HBV) or hepatitis C virus (HCV). These viruses play key roles in hepatocarcinogenesis; and therefore, HCC is highly prevalent in China due to chronic HBV and HCV infection (1). HCC patients show the shortest survival time among cancer patients, with most patients dying within 12 months of HCC tumor development (2). Furthermore, only 30-40% of HCC patients are found eligible for potentially curative intervention (3) upon diagnosis, partially due to the lack of highly sensitive and specific early-detection measures. Therefore, the effective identification of new markers for HCC is urgently needed.

MicroRNAs (miRNAs) are a class of single-stranded non-coding small RNAs (19-24 nt) that regulate the gene expression network and are known to contribute to a diverse range of functions, including development, apoptosis, differentiation and oncogenesis by binding to specific target mRNAs (4). Circulating miRNAs exist stably in body fluids and were first reported as a newly identified family of miRNAs by Valadi *et al* in 2007 (5). It has become clear that miRNAs potentially regulate all aspects of cellular activity. Recent studies have provided clear evidence that miRNAs are abundant in the liver and modulate a diverse spectrum of liver functions, including differentiation and development, metabolism, apoptotic cell death, cell proliferation, viral infection and tumorigenesis (6,7). Deregulation of miRNA expression may be a key pathogenic mechanism in many liver diseases, such as HCC, viral hepatitis and polycystic liver disease (8-11).

Differential miRNA expression in HCC and non-tumor tissue has been reported in numerous studies (12-17). Several differentially expressed serum miRNAs, including miR-16, miR-122, miR-21, miR-223, miR-24, miR-27a, miR-375 and let-7f have been recently reported in patients with HCC, when compared with hepatitis B patients and healthy individuals (18,19). However, the differentially expressed miRNAs found in these studies varied only between different individuals, and these differences were either investigated in control vs. HCC patients or cirrhosis vs. HCC patients. In the present study, we investigated miRNA expression profiles in cirrhosis patients who went on to develop hepatitis B virus-related HCC.

Materials and methods

Ethics statement. The present study was approved by the Medical Ethics Committee of The Third Hospital of Zhenjiang Affiliated to Jiangsu University, Zhenjiang, China, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient prior to their participation in the present study.

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Table I. Demographics and clinical features of the patients.

No.	Gender	Age (years)	Smoking status	Alcohol consumption	Antiviral treatment	HBeAg	HBV DNA	Genotype
1	M	43	Yes	No	ETV	N	ND	B
2	M	35	No	Yes	LAM	N	ND	C
3	M	46	No	No	ETV	N	ND	C
4	F	44	Yes	No	ETV	P	ND	C
5	M	53	No	Yes	ETV	N	ND	B
6	M	57	Yes	No	ETV	N	ND	C
7	M	63	Yes	No	LAM	P	ND	C
8	M	37	Yes	No	ETV	P	ND	C
9	M	47	No	Yes	ETV	N	ND	C
10	M	53	No	No	ETV	N	ND	C
11	F	67	Yes	No	LAM	N	ND	B
12	M	64	Yes	No	ETV	N	ND	C
13	M	57	No	No	ETV	N	ND	C
14	M	54	No	No	ETV	P	ND	B
15	M	56	No	No	LAM+ADV	N	ND	B
16	M	57	No	No	ETV	N	ND	C
17	M	54	No	Yes	ETV	N	ND	C
18	M	47	Yes	No	ETV	N	ND	C
19	M	45	Yes	No	ETV	N	ND	B
20	M	44	No	No	LAM	P	ND	C
21	M	47	Yes	No	ETV	N	ND	C
22	M	37	No	No	ETV	N	ND	B
23	F	43	No	No	ETV	P	ND	B
24	M	48	Yes	No	ETV	N	ND	C
25	M	51	No	No	ETV	N	ND	C

M, male; F, female. LAM, lamivudine; ETV, entecavirus; ADV, adefovirus. N, negative; P, positive. ND, not determined.

Study design. A total of 25 patients with cirrhosis that evolved into HCC, who were treated at The Third Hospital of Zhenjiang Affiliated to Jiangsu University between January 2005 and December 2012, were enrolled in the present study. In the discovery stage, 2 serum pooled samples from 3 cirrhosis and 3 HCC status samples from the patients were subjected to deep sequencing using the Illumina HiSeq 2000 system (Illumina, Inc., San Diego, CA, USA) to identify statistically significant differential miRNA expression. Subsequently, differentially expressed miRNAs were validated by qRT-PCR in serum samples of an independent cohort that included 22 cirrhosis and HCC status samples from patients. All patients were positive for HBsAg, the surface antigen of HBV, for a period of at least 6 months and were not co-infected with other types of hepatitis viruses such as hepatitis A, C, D or E. Patients with any other liver disease, such as alcoholic, autoimmune or metabolic liver diseases were excluded. The diagnosis of HCC and cirrhosis was histopathologically confirmed. As this was a retrospective study, collection of clinical data from the medical records, pathology reports, and regular follow-up interviews with the subjects was utilized. Serum samples used in biochemical tests and then miRNA detection were from the same specimens.

Demographics and clinical features of the patients are listed in Table I. Biochemical characteristics of the patients with cirrhosis that evolved into HCC are listed in Table II.

Illumina sequencing and data analysis. Procedures and methods of sample collection, RNA isolation and Illumina sequencing were described in detail in our previous studies (20,21).

qRT-PCR validation study and data analysis. qRT-PCR-based relative quantification of miRNAs (300 μl of serum from each participant) was performed with SYBR® Premix Ex Taq (Takara, Kyoto, Japan) according to the manufacturer's instructions using a Rotor-Gene 3000 Real-Time PCR instrument (Corbett Life Science, Sydney, Australia). miR-24 has been reported to be consistently present in human serum (22,23). Moreover, our previous experience was that miR-24 maintained stable expression levels, therefore the level of miR-24 served as an internal control in the serum miRNA relative quantitative analysis (20). The specificity of each PCR product was validated by melt curve analysis at completion of the PCR amplification cycles. All samples were analyzed in triplicate, and the cycle threshold (Ct) value was defined as the number of cycles required for the fluorescent signal to reach

Table II. Biochemical characteristics of the patients with cirrhosis that evolved into HCC.

Variables	Screening set			Validation set		
	Cirrhosis status (n=3)	HCC status (n=3)	P-value[a]	Cirrhosis status (n=22)	HCC status (n=22)	P-value[a]
TBIL	13.67±6.87	13.90±6.79	0.701	16.61±9.48	18.62±11.90	0.573
ALB	40.17±3.01	41.63±3.78	0.745	41.66±2.17	41.96±3.09	0.755
ALT	35.26±13.25	43.62±25.36	0.392	35.94±21.32	32.38±23.57	0.363
AST	43.27±21.76	47.25±12.64	0.778	35.77±14.53	45.59±26.45	0.277
ALP	81.31±31.71	136.73±93.25	0.392	95.94±31.39	132.88±57.56	0.017
GGT	74.88±38.26	151.16±65.74	0.249	65.67±45.46	141.68±67.46	<0.001
PTA	98.34±5.13	99.53±3.35	0.862	97.46±4.36	98.56±3.21	0.764
AFP	5.23±2.48	126.23±73.63	0.171	6.49±4.44	101.46 ±78.4	0.021
PLT	115.37±43.86	132.26±48.34	0.948	132.23±47.98	131.28±28.42	0.897

TBIL, total bilirubin; PLT, platelets; PTA, prothrombin activity; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase. The normal range of ALT and AST is 5-40 U/l, PLT is 100-300x10^9/l, ALB is 35-55 g/l. [a]paired samples test.

Table III. Overview of reads from raw data to cleaned sequences.

Library	Type	Cirrhosis				HCC			
		Total	(%) of total	Uniq	(%) of uniq	Total	(%) of total	Uniq	(%) of uniq
Raw reads	NA	9,846,382	100	958,733	100	9,342,644	100	906,960	100
3 ADT and length filter	Sequence type	863,846	8.77	332,485	34.68	848,736	9.08	302,543	33.36
Junk reads	Sequence type	6,583	0.76	4,634	0.48	5,346	0.06	2,143	0.24
Rfam	RNA class	447,513	4.54	59,567	6.21	512,456	5.49	55,354	6.10
mRNA	RNA class	342,646	3.48	47,547	4.96	235,433	2.52	46,575	5.14
Repeats	RNA class	76,869	0.78	11,086	1.16	98,574	1.06	8,756	0.97
rRNA	RNA class	432,646	4.39	32,574	3.40	242,436	2.59	32,546	3.59
tRNA	RNA class	123,247	1.25	18,644	1.94	115,364	1.23	15,364	1.69
snoRNA	RNA class	32,536	0.33	6,537	0.68	20,123	0.22	4,365	0.48
snRNA	RNA class	28,328	0.29	4,234	0.44	25,346	0.27	2,456	0.27
Other Rfam RNA	RNA class	175,685	1.78	15,763	1.64	17,563	0.19	10,745	1.18
Clean reads	Sequence type	7,248,031	73.61	425,662	44.40	7,221,267	77.29	426,113	46.98

HCC, hepatocellular carcinoma; NA, not available.

the threshold. Using the comparative Ct method, the relative expression levels of miRNAs in serum were calculated using the formula for $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = [Ct (target, test) - Ct (ref, test)] - [Ct (target, calibrator) - Ct (ref, calibrator)]. All primers used were obtained from Invitrogen (Carlsbad, CA, USA).

Statistical analysis. All Illumina sequencing data were $\log_2$ transformed. The differences between samples were calculated using Chi-square and Fisher's exact tests. Only the miRNAs with the fold-difference >2.0 and P<0.01 were considered significant. Quantitative variables were expressed as mean ± standard deviation (SD). Comparison of biochemical characteristics was conducted by paired-samples and the Mann-Whitney test was used to compare the fold-differences of candidate miRNAs upon qRT-PCR in the validation data set, between cirrhosis and HCC status. All statistical analyses were performed using SPSS software, version 21.0 (SPSS, Inc., Chicago, IL, USA). All statistical tests were two-sided and the results were considered to indicate a statistically significant result when P<0.05.

Results

Global analysis of miRNAs by deep sequencing. Illumina HiSeq 2000 sequencing of the small RNA library from the serum of the patients with cirrhosis and HCC produced

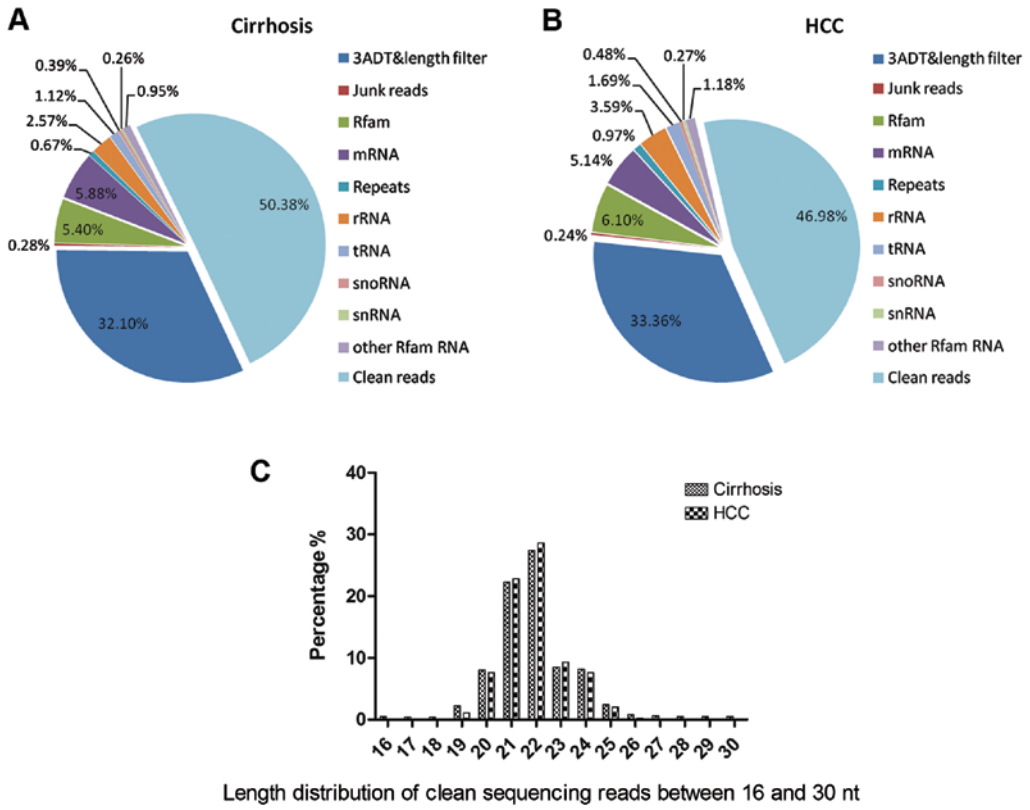

Figure 1. Distribution of all reads from 16 to 30 nt is presented in three groups.

9,846,382 and 9,342,644 raw reads, respectively. After extensive preprocessing and quality control, the raw reads were eventually removed, resulting in 425,662 and 426,113 clean reads, for cirrhosis and HCC status, respectively (Table III, Fig. 1A and B). The distribution of all reads between 16-30 nt is presented in Fig. 1C. In the present study, we found that the length of miRNAs was concentrated at 20 and 22 nt. A total of 1,653 unique reads were mapped to human miRNAs or pre-miRNAs from the iRbase database, and the pre-miRNAs could be further mapped to the human genome and expressed sequence tags (ESTs).

Identification of novel miRNAs. In total, 14 novel miRNA genes were identified in the two disease categories. The length of these candidate miRNAs ranged from 20 to 24 nt. The localization, sequence, structure and expression profile of these miRNAs are summarized in Table IV. However, several candidates among the predicted novel miRNAs were expressed at extremely low levels.

Analysis of differentially expressed miRNAs. When the cirrhosis and HCC status samples were compared, the differential expression levels of 127 miRNAs showed significant variability (Fig. 2). Among these, 22 miRNAs showed a >2-fold upregulation ($P<0.01$), and 2 miRNAs showed a >2-fold downregulation ($P<0.01$) in the cirrhosis and HCC patients (Table V).

Validation of the differentially expressed miRNAs. We used qRT-PCR to confirm the expression of 40 candidate miRNAs that were selected from the previous step in an independent cohort consisting of 22 serum samples. The threshold value for the miRNAs was determined as Ct <35 and the detection rate >75%. We then calculated the $2^{-\Delta\Delta Ct}$ of 40 candidate miRNAs in the 2 status types. Eight of the 40 miRNAs had significantly differential expression levels between the 2 statuses (Table VI). These miRNAs were hsa-miR-122-5p, hsa-miR-192-5p, hsa-miR-486-5p, hsa-miR-193b-5p, hsa-miR-206, hsa-miR-141-3p, hsa-miR-199a-5p and hsa-miR-26a-5p.

Discussion

Since the discovery of circulating miRNAs, several studies have been conducted to investigate their potential as novel biomarkers in body fluid. Circulating miRNAs have already been shown to be relevant biomarkers for cancer detection, applicable in non-invasive diagnostic testing and have demonstrated several other successful applications (24-27). To date, three methods have mainly been used for the analysis of the expression profiles of circulating miRNAs in serum: qRT-PCR, microarray and next-generation sequencing technology (28). Although qRT-PCR has been widely employed for miRNA quantification, it is only capable of detecting a limited number of miRNAs at any one time. Microarray analysis, a high-throughput method, is capable of detecting only known fragments and is not suitable for detection of low-abundant miRNAs or for distinguishing between miRNAs with single nucleic acid polymorphisms. Compared to these techniques, next-generation sequencing technology appears to be more suitable for miRNA profiling. Thus, the Roche 454 Genome Sequencer, the Illumina Genome Analyzer and the ABI

Table IV. Novel miRNA candidates represented in the library.

Novel miR_name	miR_seq	Genome ID	Strand	Start	End	MFE	Cirrhosis count	HCC count	P-value
PC-5p-1700_1443	TGTAGGCAAGGGAAGTCGGC	gi|224514641|ref|NT_167214.1|	+	116070	116319	0.6	1,613	4,551	5.61E-42
PC-3p-8816_210	AGGACGGTGGTCATGGAAGTC	gi|224589820|ref|NC_000008.10|	-	109304597	109304710	0.6	859	3,508	6.89E-30
PC-3p-6084_321	ACGGGCTTGGCAGAATCAGC	gi|224589807|ref|NC_000016.9|	-	81902013	81902146	0.5	228	835	3.41E-13
PC-5p-7578_250	AATTTCATCGTGATGGGGA	gi|224589811|ref|NC_000002.11|	-	210764476	210764537	0.6	168	605	2.02E-11
hsa-mir-7641-2-p3	TCGGGCCTGGTTAGTACTTGGA	224589805	+	76070552	76070604	0.5	456	559	3.07E-11
hsa-mir-6240-p5_1ss17GT	TGCCCAGTGCTCTGAATGTC	gi|224589802|ref|NC_000011.9|	+	77597474	77597582	0.6	300	534	6.26E-11
PC-5p-250006_9	ATGTGATGCATCGCTTCTGT	gi|224589811|ref|NC_000002.11|	-	170685375	170685445	0.9	28	340	3.91E-08
hsa-mir-4459-p5_1ss4TC	AGGCGGAGGTTGCAGTGAGC	224589817	-	53371348	53371413	0.6	25	177	1.38E-06
PC-5p-156137_14	GCCGACTACAACATCCAGAA	gi|224589820|ref|NC_000008.10|	+	106730487	106730585	0.8	21	155	2.81E-06
PC-3p-1704_1400	AGGGCTGGGTCGGTCGGGCTGG	gi|224514641|ref|NT_167214.1|	+	116070	116319	0.6	2,580	140	1.14E-06
hsa-mir-566-p3_1ss6AG	AGGCGGAGGTTGCAGTGAGC	224589815	+	50210759	50210852	0.5	31	60	4.93E-04
PC-5p-32366_69	GTGGAAATCATGTGGGCTTT	gi|224589804|ref|NC_000013.10|	+	61836053	61836109	0.6	34	49	8.14E-04
PC-5p-42534_51	AGAATGAAACTCAAAGGAAT	gi|224589804|ref|NC_000013.10|	-	32584468	32584537	0.6	31	47	6.86E-04
hsa-mir-451a-p3	TTTAGTAATGGTAATGGTTCT	224589808	-	27188387	27188458	1.5	80	25	1.15E-02

Constitutively expressed novel miRNAs discovered by deep sequencing. Control count is the miRNA read count of cirrhosis and HCC patient count is the miRNA read count of carrier; location is the location of novel miRNA in genome (ID, start; end, strand); MFE, minimal free energy.

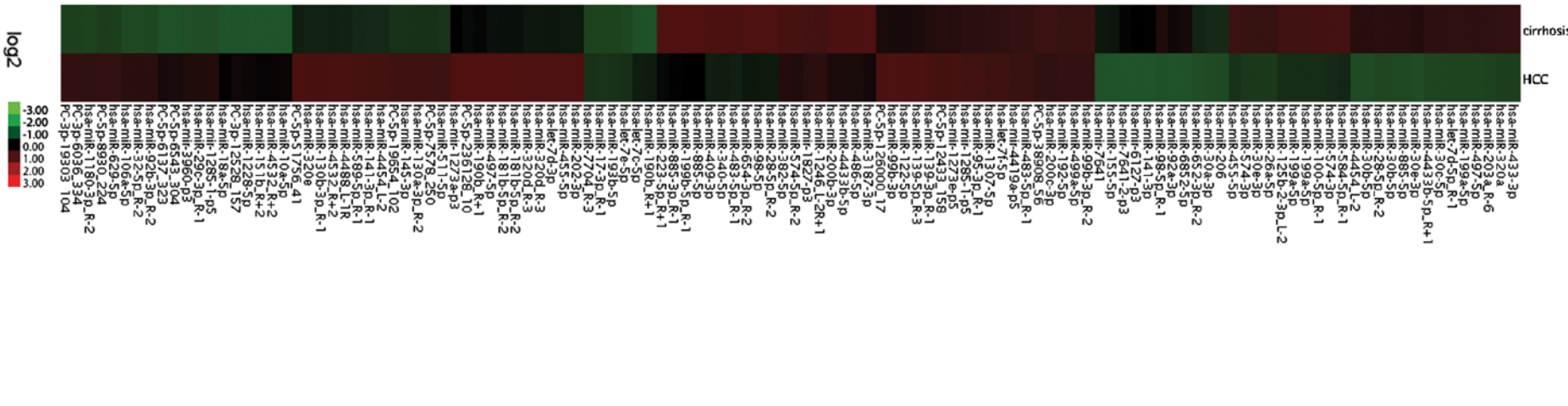

Figure 2. Heatmap of significantly differential expression levels of 127 miRNAs in the two disease caterories.

Table V. Differential expression of miRNAs between HCC and cirrhosis.

No.	miR_name	Fold-change	Fold-change (log2)	Up/down	miR_seq
1	hsa-miR-455-5p	28.37	4.83	Up	UAUGUGCCUUUGGACUACAUCG
2	hsa-let-7e-5p	26.25	4.71	Up	UGAGGUAGGAGGUUGUAUAGUU
3	hsa-miR-483-5p_R-1	22.60	4.50	Up	AAGACGGGAGGAAAGAAGGGAG
4	hsa-miR-190b_R+1	22.36	4.48	Up	UGAUAUGUUUGAUAUUGGGUUU
5	hsa-miR-30e-3p	20.01	4.32	Up	CUUUCAGUCGGAUGUUUACAGC
6	hsa-miR-486-5p	17.41	4.12	Up	TCCTGTACTGAGCTGCCCCGAG
7	hsa-miR-584-5p_R-1	11.08	3.47	Up	UUAUGGUUUGCCUGGGACUGG
8	hsa-miR-10a-5p	10.45	3.38	Up	UACCCUGUAGAUCCGAAUUUGUG
9	hsa-miR-574-3p	9.38	3.23	Up	UGAGUGUGUGUGUGUGAGUGUGU
10	hsa-miR-193b-5p	9.13	3.19	Up	CGGGGTTTTGAGGGCGAGATGA
11	hsa-miR-4532_R+2	8.33	3.06	Up	CCCCGGGGAGCCCGGCGCG
12	hsa-miR-206	7.69	2.94	Up	UGGAAUGUAAGGAAGUGUGUGG
13	hsa-miR-28-5p_R-2	7.43	2.89	Up	AAGGAGCUCACAGUCUUGAG
14	hsa-miR-433-3p	6.13	2.62	Up	AUCAUGAUGGGCUCCUCGGUGU
15	hsa-miR-3187-3p	6.04	2.59	Up	TTGGCCATGGGGCTGCGCGG
16	hsa-miR-98-5p	5.71	2.51	Up	UGAGGUAGUAAGUUGUAUUGUU
17	hsa-miR-4433b-5p	5.29	2.40	Up	AUGUCCCACCCCCACUCCUGU
18	hsa-miR-497-5p	4.94	2.30	Up	CAGCAGCACACTGTGGTTTGT
19	hsa-mir-1285-1-p5	4.60	2.20	Up	GAUCUCACUUUGUUGCCCAGG
20	hsa-miR-141-3p	3.29	1.72	Up	UAACACUGUCUGGUAAAGAUGG
21	hsa-miR-100-5p_R-1	2.99	1.58	Up	AACCCGUAGAUCCGAACUUGUG
22	hsa-miR-99b-3p_R-2	2.36	1.24	Up	CACCCGUAGAACCGACCUUG
32	hsa-miR-199a-5p	0.50	-0.99	Down	ACAGUAGUCUGCACAUUGGUUA
23	hsa-miR-1228-5p	0.49	-1.03	Down	GUGGGCGGGGGCAGGUGUGUG
24	hsa-miR-202-3p	0.48	-1.05	Down	AGAGGTATAGGGCATGGGAA
29	hsa-miR-92a-3p	0.46	-1.13	Down	TATTGCACTTGTCCCGGCCTGT
25	hsa-miR-6852-5p	0.45	-1.14	Down	CCCUGGGGUUCUGAGGACAUG
31	hsa-miR-30b-5p	0.45	-1.14	Down	UGUAAACAUCCUACACUCAGCU
33	hsa-miR-511-5p	0.44	-1.18	Down	GUGUCUUUUGCUCUGCAGUCA
27	hsa-miR-320a	0.37	-1.44	Down	AAAAGCTGGGTTGAGAGGGCGA
28	hsa-mir-6127-p3	0.36	-1.48	Down	UGAGGGAGUGGGUGGGAGG
26	hsa-miR-26a-5p	0.36	-1.48	Down	UUCAAGUAAUCCAGGAUAGGCU
30	hsa-miR-885-5p	0.35	-1.50	Down	TCCATTACACTACCCTGCCTCT
34	hsa-miR-30a-3p	0.32	-1.63	Down	UGUAAACAUCCUCGACUGGAAG
35	hsa-miR-4454_L-2	0.19	-2.39	Down	GGAUCCGAGUCACGGCACCA
36	hsa-let-7c-5p	0.08	-3.61	Down	UGAGGUAGUAGGUUGUAUGGUU
37	hsa-miR-30c-5p	0.06	-4.17	Down	UGUAAACAUCCUACACUCUCAGC
38	hsa-let-7f-5p	0.05	-4.23	Down	UGAGGUAGUAGAUUGUAUAGUU
39	hsa-miR-122-5p	0.03	-5.00	Down	UGGAGUGUGACAAUGGUGUUUG
40	hsa-miR-192-5p	0.03	-5.00	Down	CUGACCUAUGAAUUGACAGCC

HCC, hepatocellular carcinoma.

SOLiD System sequencing platforms have become widely available and used over the past few years.

Several studies have shown that many miRNAs are dysregulated in HCC (17,29,30) and have also considered the potential of circulating miRNA levels to affect HCC progression. The high stability of miRNAs in circulation suggests them for use as potentially ideal biomarkers, particularly for early-stage detection (4). Various studies have observed and explored the upregulation of circulating miR-21 (18,31), miR-222 (31) and miR-223 (32) in the serum/plasma of HBV- or HCV-associated HCC patients.

Downregulation of miRNAs is also a common finding in HBV-related HCC; in this case, these miRNAs act as tumor-suppressor genes. The pathological mechanisms of

Table VI. Expression profiles of candidate miRNAs upon qRT-PCR in the validation set.

No.	miR_name	HCC vs. cirrhosis	
		P-value	Fold-change
1	hsa-miR-455-5p	0.108	1.58
2	hsa-let-7e-5p	0.063	4.50
3	hsa-miR-483-5p_R-1	0.108	3.19
4	hsa-miR-190b_R+1	0.246	3.47
5	hsa-miR-30e-3p	ND	ND
6	**hsa-miR-486-5p**	0.006	2.42
7	hsa-miR-584-5p_R-1	0.108	3.25
8	hsa-miR-10a-5p	0.144	1.43
9	hsa-miR-574-3p	ND	ND
10	**hsa-miR-193b-5p**	0.008	2.64
11	hsa-miR-4532_R+2	0.023	1.23
12	**hsa-miR-206**	0.003	3.64
13	hsa-miR-28-5p_R-2	0.069	2.45
14	hsa-miR-433-3p	0.108	1.64
15	hsa-miR-3187-3p	ND	ND
16	hsa-miR-98-5p	0.723	1.63
17	hsa-miR-4433b-5p	ND	ND
18	hsa-miR-497-5p	<0.001	1.33
19	hsa-mir-1285-1-p5	0.012	1.64
20	**hsa-miR-141-3p**	0.001	2.44
21	hsa-miR-100-5p_R-1	0.289	1.54
22	hsa-miR-99b-3p_R-2	0.125	2.14
23	hsa-miR-1228-5p	0.268	2.54
24	hsa-miR-202-3p	ND	ND
25	hsa-miR-6852-5p	ND	ND
26	**hsa-miR-26a-5p**	0.002	0.64
27	hsa-miR-320a	0.056	0.53
28	hsa-mir-6127-p3	0.108	0.77
29	hsa-miR-92a-3p	0.256	0.87
30	hsa-miR-885-5p	0.442	0.65
31	hsa-miR-30b-5p	0.256	0.45
32	**hsa-miR-199a-5p**	<0.001	0.53
33	hsa-miR-511-5p	0.284	0.45
34	hsa-miR-30a-3p	0.084	0.54
35	hsa-miR-4454_L-2	ND	ND
36	hsa-let-7c-5p	0.073	0.65
37	hsa-miR-30c-5p	0.077	0.76
38	hsa-let-7f-5p	0.112	0.78
39	**hsa-miR-122-5p**	<0.001	0.12
40	**hsa-miR-192-5p**	<0.001	0.24

ND, not determined; miRNA Ct value >35 and detection rate <75%. HCC, hepatocellular carcinoma. Bold text, significant differences.

tumor-suppressive miRNAs is involved in cell cycle arrest, increased apoptosis and eventual reductions in tumor angiogenesis and metastasis by inhibiting migration and invasion. Among these downregulated miRNAs, miR-122 and miR-199 appear to be particularly important in HCC (33-35). In the present study, we also found that the miRNA downregulated in cirrhosis status that evolved into HCC was miR-122, a liver-specific miRNA that is abundant in the liver and plays an important role in regulating hepatocyte development and differentiation (36,37). The overexpression of miR-122 has been found to induce apoptosis and suppress proliferation in the human liver carcinoma cell lines HepG2 and Hep3B *in vitro* (38), and has been demonstrated *in vivo* directly by the generation of miR-122-knockout mice in liver cancer (39,40).

The present study revealed that serum hsa-miR-486-5p, hsa-miR-193b-5p, hsa-miR-206, hsa-miR-141-3p, hsa-miR-199a-5p, hsa-miR-122-5p, hsa-miR-192-5p and hsa-miR-26a-5p were potential circulating markers for HCC diagnosis, and 4 of these 8 miRNAs (miR-122, miR-199, miR-192 and miR-26a) in the present study have been previously reported to show differential expression (19,41,42).

At the circulating blood level, Xu *et al* (18) reported that miR-21, miR-122 and miR-223 could be utilized in discriminating HCC patients from a healthy group. Qu *et al* (43) found that miR-16 has moderate diagnostic accuracy in HCC. Li *et al* (14) reported an extraordinarily high diagnostic accuracy for serum miRNA profiles in the diagnosis of HCC [area under the curve (AUC) = 0.97-1.00] with miR-10a, miR-125b, miR-223, miR-23a, miR-23b, miR-342-3p, miR-375, miR-423, miR-92a and miR-99a. However, the need for different markers for different group comparisons with different critical values in their study (HCC vs. healthy, HCC vs. HBV, healthy vs. HBV, healthy vs. HCV and HBV vs. HCV) raised concerns about the robustness of these markers.

In our previous study (20), we established a logistic model of miRNAs for the diagnosis of HCC in a larger sample size and independent validation set. However, in the previous study, the cirrhosis and HCC patients were different individuals. While the present study was limited by the sample size, its innovation was the successful investigation of two phases of disease status in the same individuals.

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