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Plasma miRNAs as early biomarkers for detecting hepatocellular carcinoma

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The early detection of hepatocellular carcinoma (HCC) presents a challenge because of the lack of specific biomarkers. Serum/plasma microRNAs (miRNAs) can discriminate HCC patients from controls. We aimed to identify and evaluate HCC-associated plasma miRNAs originating from the liver as early biomarkers for detecting HCC. In this multicenter three-phase study, we first performed screening using both plasma (HCC before and after liver transplantation or liver hepatectomy) and tissue samples (HCC, para-carcinoma and cirrhotic tissues). Then, we evaluated the diagnostic potential of the miRNAs in two case–control studies (training and validation sets). Finally, we used two prospective cohorts to test the potential of the identified miRNAs for the early detection of HCC. During the screening phase, we identified ten miRNAs, eight of which (miR-20a-5p, miR-25-3p,

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miR-30a-5p, miR-92a-3p, miR-132-3p, miR-185-5p, miR-320a and miR-324-3p) were significantly overexpressed in the HBV-positive HCC patients compared with the HBV-positive cancer-free controls in both the training and validation sets, with a sensitivity of 0.866 and specificity of 0.646. Furthermore, we assessed the potential for early HCC detection of these eight newly identified miRNAs and three previously reported miRNAs (miR-192-5p, miR-21-5p and miR-375) in two prospective cohorts. Our meta-analysis revealed that four miRNAs (miR-20a-5p, miR-320a, miR-324-3p and miR-375) could be used as preclinical biomarkers ($p_{meta} < 0.05$) for HCC. The expression profile of the eight-miRNA panel can be used to discriminate HCC patients from cancer-free controls, and the four-miRNA panel (alone or combined with AFP) could be a blood-based early detection biomarker for HCC screening.

What's new?

Half of all deaths from hepatocellular carcinoma (HCC) occur in China. But while early detection of the disease could improve survival, preclinical diagnostic biomarkers are lacking. Using tissue and plasma samples from HCC patients and plasma samples from prospective cohorts, the authors of this study evaluated the diagnostic and predictive potential of miRNAs. A panel of eight miRNAs dysregulated during HCC development successfully distinguished HCC patients from controls, while a panel of four miRNAs was found to have preclinical potential. The newly described miRNA panels could aid in the detection of HCC before the disease is otherwise clinically apparent.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with more than 782,000 newly diagnosed cases per year. As the second most frequent cause of cancer deaths, HCC is an extremely lethal disease, causing 746,000 cancer deaths annually, half of which occur in China.[1,2] Although it is well known that HBV infection is the most important risk factor for HCC in China, a lack of sensitive and specific biomarkers for the early detection of this disease has greatly impeded the development and effectiveness of therapeutic strategies. Currently, the most commonly used circulating marker for HCC is alpha-fetoprotein (AFP), but its value in the early diagnosis of this disease is very limited. Therefore, there is an urgent need to identify novel biomarkers to improve the early detection of HCC before it becomes clinically detectable and has progressed to lethal, advanced stages.

MicroRNAs (miRNAs) are a family of endogenous RNAs that play important roles in regulating gene expression by degrading target mRNAs or blocking their translation.[3] A number of studies have reported that the altered expression of circulating miRNAs is associated with many diseases, including HCC.[4–6] Binding to argonaute proteins stabilizes circulating miRNAs in the plasma/serum; thus, they are potentially stable noninvasive biomarkers.[7,8] However, tissue specificity and early activation are important properties of specific biomarkers for the early detection of cancer. Our previously published prospective study, which used a limited number of incident patients, has suggested that circulating miRNAs could function as biomarkers for the early detection of lung cancer.[9] The discovery of deregulated miRNAs as biomarkers in the plasma/serum may represent a useful approach to segregate HCC patients from controls,[4,10] but whether they are liver-originated or could serve as biomarkers for early detection has not been systematically investigated.

Here, we performed a multicenter, three-phase study to screen liver-originated HCC-associated plasma miRNAs in both plasma and tissue samples. We then evaluated and validated the diagnostic potential and effectiveness of these miRNAs for the early detection of HCC. Early detection refers to the identification of individuals who have this disease before a clinical diagnosis is made in prospective cohort studies using plasma miRNAs.

Material and Methods

Study design

Figure 1 shows the overview of the study.

Phase 1 (screening set). The first phase was to screen liver-originated HCC-associated plasma miRNAs (Supporting Information Fig. S1), which were found to overlap (Table 1) between TaqMan low-density array (TLDA, human microRNA panel V2.0; Applied Biosystems, Foster City, CA) screens of plasma (Supporting Information Table S1) and tissue samples (Supporting Information Table S2). We selected candidate miRNAs that met the following criteria: (*i*) having C_T values of <35 (considered as "detected"); (ii) showing fourfold ($|\Delta\Delta C_T| > 2$) altered expression between any of the comparisons and (iii) having alterations of the same direction in plasma or tissue samples. The miRNAs from the plasma TLDA screening (Supporting Information Table S1) that overlapped between the "HCC-associated plasma miRNAs (Supporting Information Table S3)" and the "potentially liver-originated plasma miRNAs (Supporting Information Table S4)" were identified, and the "miRNAs upregulated after liver transplantation (Supporting Information Table S5)" were then removed. The "HCC-associated plasma miRNAs" were derived from comparisons of the plasma miRNA expression levels in four HCC patients (A1-I, A2-I,

Early Detection and Diagnosis

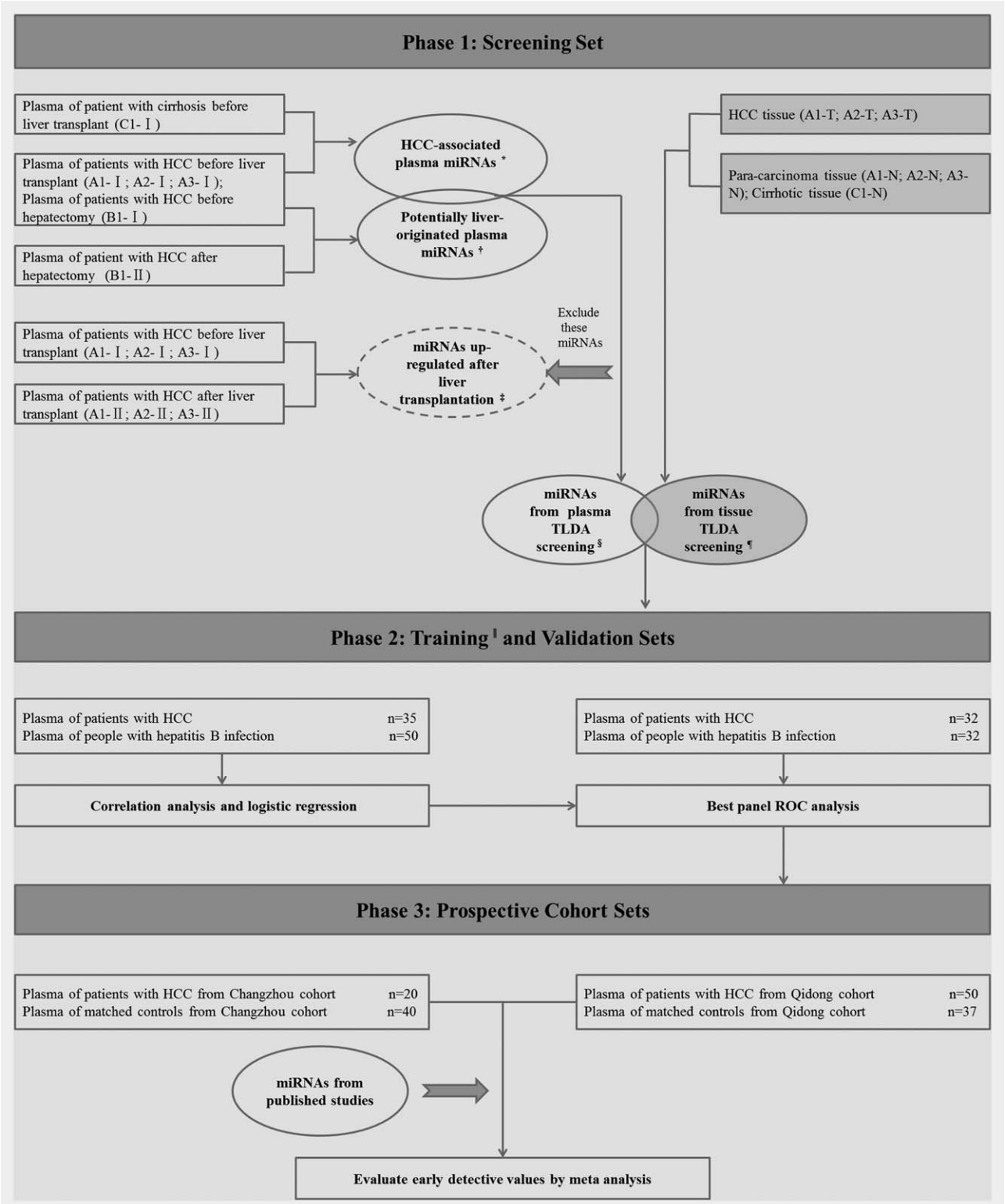

Figure 1. Overview of the study design. *HCC-associated plasma miRNAs were identified by comparisons of four HCC patients with one cirrhosis patient before liver transplantation or liver hepatectomy (Supporting Information Table S3). †Potentially liver-originated plasma miRNAs were detected from comparisons of four plasma samples from HCC patients before surgery with one plasma sample at 2 weeks after liver hepatectomy (Supporting Information Table S4). ‡miRNAs upregulated after liver transplantation are listed in Supporting Information Table S5. §miRNAs identified by the plasma TLDA screening (Supporting Information Table S1) were selected from the intersection of "HCC-associated plasma miRNAs (Supporting Information Table S3)" and "potentially liver-originated plasma miRNAs (Supporting Information Table S4)." Those that were "upregulated after liver transplantation (Supporting Information Table S5)" were excluded. ¶miRNAs identified by the tissue TLDA screening (Supporting Information Table S2) were selected from comparisons of the miRNA expression profiles of HCC tissues from three patients with their corresponding para-carcinoma tissues or with cirrhotic liver tissue from another patient. |The overlapping miRNAs identified in the plasma screening and tissue screening are listed in Table 1.

Early Detection and Diagnosis

Table 1. miRNAs upregulated by fourfold ($|\Delta\Delta C_T| > 2$) in HCC as shown by TLDA screening of both plasma and target tissues

	Plasma samples										Tissue samples					
	HCC-associated plasma miRNAs[1]						Potentially liver-originated plasma miRNAs[2]				miRNAs from tissue TLDA screening[3]					
miRNA	HCC before liver transplant or hepatectomy	ΔC_T	Cirrhosis before liver transplant	ΔC_T	$\Delta\Delta C_T$	Expression value	HCC at 2 weeks after liver transplant	ΔC_T	$\Delta\Delta C_T$	Expression value	HCC tissue	ΔC_T	Para-carcinoma tissue or cirrhotic tissue	ΔC_T	$\Delta\Delta C_T$	Expression value
miR-19a-3p	26.51	1.13	27.65	4.76	−3.63	0.0808	28.01	6.23		0.0292	28.44	2.97	32.64	6.63	6.63	0.0791
miR-19b-3p	20.52	−4.86	21.58	−1.31	−3.55	0.0854	22.28	0.50	−5.36	0.0243	25.35	−0.12	27.96	1.95	1.95	0.2382
miR-20a-5p	24.04	−1.33	24.83	1.94	−3.27	0.1037	25.48	3.70	−5.03	0.0306	27.04	1.57	30.01	4.01	4.01	0.1843
miR-25-3p	26.91	1.53	26.79	3.90	−2.36	0.1948	28.50	6.72	−5.19	0.0274	32.64	2.49	32.91	8.56	8.56	0.0149
miR-30a-5p	25.46	0.08	26.74	3.85	−3.77	0.0733	27.76	5.99	−5.91	0.0166	27.88	2.40	34.73	5.34	5.34	0.1303
miR-92a-3p	24.31	−1.07	23.90	1.00	−2.07	0.2382	25.71	3.93	−5.01	0.0310	29.70	3.50	30.43	6.30	6.30	0.1436
miR-132-3p	27.61	2.23	29.64	6.75	−4.52	0.0436	30.83	9.05	−6.82	0.0089	34.80	4.65	33.83	7.82	7.82	0.1111
miR-185-5p	27.71	2.33	28.81	5.91	−3.58	0.0836	28.89	7.11	−4.78	0.0364	33.03	2.87	34.11	8.10	8.10	0.0266
miR-320a	24.39	−0.99	24.49	1.60	−2.58	0.1672	25.96	4.18	−5.16	0.0280	26.50	1.03	32.75	3.37	3.37	0.1975
miR-324-3p	28.53	3.15	29.01	6.11	−2.96	0.1285	29.98	8.20	−5.05	0.0302	33.35	3.20	34.99	8.98	8.98	0.0181

[1]HCC-associated plasma miRNAs were identified by comparisons of four HCC patients with one cirrhosis patient before liver transplant or liver hepatectomy (only the largest differences among the four comparisons are listed; ref. Supporting Information Table S3). miRNAs that met the fourfold change criterion with alterations that were in different directions among the four comparisons were excluded.

[2]Potentially liver-originated plasma miRNAs were identified by comparisons of four plasma samples from HCC patients before surgery with one plasma sample from a patient at 2 weeks after liver hepatectomy (only the largest differences among the four comparisons are listed; ref. Supporting Information Table S4). miRNAs that met the fourfold change criterion with alterations that were in different directions among the four comparisons were excluded.

[3]miRNAs identified by the tissue TLDA screening (only the largest differences are listed among the six comparisons; ref. Supporting Information Table S2). miRNAs that met the fourfold change criterion but with alterations that were in different directions among the six comparisons were excluded. $\Delta C_T = C_{T\ sample} - C_{T\ cel\text{-}miR\text{-}39}$, $\Delta C_{T\ for\ tissue} = C_{T\ sample} - C_{T\ U6}$, $\Delta\Delta C_T = \Delta C_{T\ 1} - \Delta C_{T\ 2}$ and expression value $= 2^{-\Delta C_T}$.

A3-I and B1-I) and one cancer-free cirrhosis patient (C1-I), whereas the "potentially liver-originated plasma miRNAs" were identified from comparisons between four HCC patients before liver transplantation/hepatectomy (A1-I, A2-I, A3-I and B1-I) and one HCC patient at 2 weeks after hepatectomy (B1-II). The "miRNAs upregulated after liver transplantation" were differentially expressed plasma miRNAs detected in three HCC patients before liver transplantation (A1-I, A2-I and A3-I) with similar expression after transplantation (A1-II, A2-II and A3-II) (Supporting Information Table S5). Twenty-nine miRNAs were excluded from further analyses.

The miRNAs obtained from the tissue screening (Supporting Information Table S2) were differentially expressed miRNAs that were derived from comparisons of three patients' HCC tissues (A1-T, A2-T and A3-T) with their corresponding para-carcinoma tissues (A1-N, A2-N and A3-N) or another independent cirrhotic liver tissue sample (C1-N) from a patient who had received a liver transplant.

Phase 2 (training and validation sets). The liver-originated plasma miRNAs from phase 1 were then tested in the phase 2 samples to evaluate their diagnostic potentials using individual TaqMan probe-based quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays. In the training set, ten candidate miRNAs (miR-19a-3p, miR-19b-3p, miR-20a-5p, miR-25-3p, miR-30a-5p, miR-92a-3p, miR-132-3p, miR-185-5p, miR-320a and miR-324-3p) were evaluated in a case–control study, of which four in the oncogenic miR-17–92 cluster (miR-19a-3p, miR-19b-3p, miR-20a-5p and miR-92a-3p) were overexpressed, and two (miR-19a-3p and miR-19b-3p) were highly correlated with miR-20a-5p (Spearman correlation value > 0.8 and p values < 0.05, Supporting Information Table S6). We chose miR-20a-5p and miR-92a-3p for further validation by excluding miR-19a-3p and miR-19b-3p. In the validation set, the eight miRNAs were further evaluated in an independent set of 64 subjects.

Phase 3 (prospective cohort sets). For phase 3, two prospective cohorts (the Changzhou cohort and Qidong cohort) were used to evaluate the potential of these miRNAs for the early detection of HCC. During this phase, we also included miRNAs from previously published studies. A review of published literature revealed two studies of HCC based on genome-wide serum/plasma miRNA screening and validation.[4,10] The miRNAs reported in these previously published studies and those identified in our own TLDA screening were selected for evaluation. For example, miR-192-5p, miR-21-5p and miR-801 are significantly upregulated in patients with HBV-related HCC, whereas miR-122-5p, miR-223-3p, miR-26a-5p and miR-27a-3p are downregulated in these patients.[10] Li *et al.* have demonstrated that miR-1-3p, miR-25-3p, miR-92a-3p, miR-206, miR-375 and let-7f-5p can be used to distinguish HCC cases from controls and that all of these miRNAs are upregulated in HCC patients.[4] We excluded miR-801, miR-1-3p, miR-206 and let-7f-5p because they were undetectable in some of the samples used in our screening. Considering our screening results and selection criteria, we included miR-25-3p, miR-92a-3p, miR-192-5p, miR-21-5p and miR-375 from previously published studies. We conducted two nested case–control studies and performed qRT-PCR on the plasma samples that were collected at baseline from these cohorts. The average leading time from clinical diagnosis was 34.5 months for the Changzhou cohort and 50.5 months for the Qidong cohort.

Study population

Phase 1 (screening set). The nine plasma samples and seven corresponding tissue specimens used in the screening set were obtained from patients recruited from the First Affiliated Hospital of Nanjing Medical University (Nanjing, Jiangsu, China) between December 2010 and December 2011 (Supporting Information Table S7). Of them, four HCC patients and one cirrhosis patient donated their peripheral blood before the treatments. Two weeks after liver transplantation or hepatectomy, the four HCC patients donated their peripheral blood again. Seven tissue samples, including three HCC tissue samples paired with their adjacent noncancerous liver tissues and one cirrhosis liver tissue sample were collected from three HCC patients and one cirrhosis patient during liver transplantation.

Phase 2 (training and validation sets). HBV-positive HCC patients from both the training and validation sets were recruited from the Nantong Tumor Hospital (Nantong, Jiangsu, China) between January 2010 and December 2012, and the controls were HBV carriers from the same hospital (Supporting Information Table S8). The training set consisted of 35 HCC cases and 50 cancer-free HBV carriers who were frequency matched for age and sex, whereas the validation set consisted of 32 HCC cases and 32 matched cancer-free HBV carriers.

Phase 3 (prospective cohort sets). The Changzhou cohort was established from 2004 to 2005 with the aim of exploring the environmental and genetic risk factors for common chronic diseases in Wujin County (Changzhou, Jiangsu Province, China).[11] Briefly, 17,723 individuals (7,426 men and 10,297 women) aged 35 and above at baseline completed the baseline survey. The first follow-up was finished in October 2009, and the second was completed in November 2013. Twenty-seven individuals were diagnosed with HCC during the follow-up period (excluding those diagnosed within the first year after recruitment), of which 20 HBV-positive HCC patients with sufficient plasma and 40 age- and sex-matched normal controls recruited at the same time were included in this study. The Qidong cohort was established by the Qidong Liver Cancer Institute in October 1989. It was comprised of 60,000 men aged 30–59 living in 15 townships (Qidong, Jiangsu Province, China).[12] Of the 60,000 men, 36,381 provided blood samples. A total of 667 individuals had been diagnosed as HCC up to December 2003. The blood samples we used were obtained in April 1993 or September 1995. We

randomly selected 50 HCC patients and 37 cancer-free HBV carriers (Supporting Information Table S8).

For all cases, HCC diagnosis was confirmed by histopathological examination of surgically resected tumors and/or AFP elevation (>400 μg/l) combined with positive images on magnetic resonance imaging, ultrasonography or computed tomography. We excluded subjects with HCV infection or other types of liver disease, such as toxic hepatitis, autoimmune hepatitis or primary biliary cirrhosis. All of the participants were genetically unrelated ethnic Han Chinese individuals, and they each donated 3–5 ml of venous blood at the time of both recruitment and follow-up. This study was approved by the institutional review boards of all institutions involved.

Plasma/tissue preparation and RNA isolation

After sample collection, the plasma was separated from the venous blood within 4 hr for screening, training and validation and within 24 hr for the cohort studies. We centrifuged the plasma samples at 12,000 rpm for 15 min to completely remove cell debris, and the supernatants were stored at −80 °C until analysis. Tissue samples were collected from three HCC patients and one cirrhosis patient during liver transplantation and frozen in liquid nitrogen before use.

RNA isolation was performed as described previously. Briefly, total RNA was extracted from plasma using Trizol LS Reagent (Invitrogen, Carlsbad, CA) to denature the plasma.[5] To control for variability in the extractions of RNA from the plasma samples, synthetic *Caenorhabditis elegans* miRNA cel-miR-39 (synthetic RNA oligonucleotides synthesized by Qiagen, Valencia, CA) was added to each denatured sample at a final concentration of 10^{-4} pmol/μl. RNA was collected and purified using a Qiagen miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. For the frozen tissues, RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's protocol. The miRNA expression levels in the tissue samples were normalized using U6.

TLDA chip assays and qRT-PCR

During phase 1, we used TLDA Chips to screen for differentially expressed miRNAs from nine plasma samples and seven tissue samples. Megaplex reverse transcriptase reactions were performed to evaluate both the plasma and tissue samples, and preamplification reactions were run for only the plasma samples, according to the manufacturer's protocol. Subsequently, PreAmp products were diluted with 75 μl of 0.1× TE, and 9 μl of diluted PreAmp product was used to carry out the RT-PCR reactions by dispensing 100 μl of the PCR reaction mix into each port of the TLDA chip. We used a standard PCR procedure and RQ manager software (Applied Biosystems) to analyze the data. The ΔC_T and $\Delta\Delta C_T$ were calculated using the following mathematical formulas: $\Delta C_T = C_{T\ \text{sample}} - C_{T\ \text{cel-miR-39}}$ for the plasma samples, $\Delta C_T = C_{T\ \text{sample}} - C_{T\ \text{U6}}$ for the tissue samples, and $\Delta\Delta C_T = \Delta C_{T\ \text{case}} - \Delta C_{T\ \text{control}}$.

To confirm the candidate miRNAs detected by the microarrays, qRT-PCR was performed using TaqMan microRNA probes (Applied Biosystems). Reverse transcriptase reactions were performed with a TaqMan miRNA RT Kit and stem-loop RT primers (Applied Biosystems) using an ABI 7900 Real-Time PCR System. The reactions were carried out in a 384-well optical plate at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. All reactions were carried out in triplicate, including the no-template controls, and the C_T values were determined using the fixed threshold settings. To calculate the relative expression levels of the target miRNAs, cel-miR-39 was used as a control miRNA for the plasma samples, and the relative expression levels of the target miRNAs were calculated according to the equation $2^{-\Delta C_T}$. Equal numbers of HCC patient and control samples were distributed in a 384-well plate, and the expression levels of the target and control miRNAs (cel-miR-39) were measured simultaneously.

Statistical analysis

A statistical comparison of the demographic and clinical characteristics between the HCC cases and controls was performed with the χ^2 test or the Mann–Whitney test, and a $p < 0.05$ was considered statistically significant.

The optimal cutoff intensity values of the corresponding miRNAs during the training phase, denoted as t, were set as the thresholds for classifying low and high expression, and these optimal cutoff values were applied directly for the validation samples and combined samples. The risk score for each miRNA was denoted as s and was calculated as follows:

$$s_{ij} = \begin{cases} 1, & | \quad r_{ij} > t_j \\ 0, & | \quad r_{ij} \le t_j \end{cases},$$

where i denotes the ith sample, j indicates the jth miRNA and r is the expression level of the corresponding miRNA. Each subject was assigned a risk score function (RSF) according to a linear combination of miRNA expression levels. The RSF for subject i was calculated as follows:

$$\text{RSF}_i = \sum_{j=1}^{k} W_j \cdot s_{ij},$$

where s_{ij} is the risk score for miRNA j for subject i, and W_j is the weight of the risk score of a given miRNA j. The regression coefficient of the risk score for the corresponding miRNA was estimated by a univariate logistic regression model (adjusting for age and sex), which was used for weighting to indicate the contribution of each miRNA to the RSF. We used the areas under the receiver operating characteristic (ROC) curve (AUC) to evaluate the diagnostic performances of the miRNAs.

We performed meta-analysis of the results obtained using two different cohorts. Heterogeneity between these two cohorts was measured by the χ^2-based Q test. A fixed-effects model with the Mantel–Haenszel method was applied to

Table 2. Individual qRT-PCR confirmation of ten miRNAs in training set and eight miRNAs in validation set for HCC detection

Stage	miRNA[1]	Case		Control		p^2
		Mean	SD	Mean	SD	
Training set (case = 35 and control = 50)	miR-19a-3p	0.0012	0.0013	0.0005	0.0008	<0.001
	miR-19b-3p	0.0226	0.0262	0.0082	0.0108	<0.001
	miR-20a-5p	0.0080	0.0086	0.0040	0.0059	0.001
	miR-25-3p	0.0049	0.0046	0.0026	0.0024	0.005
	miR-30a-5p	0.0050	0.0055	0.0027	0.0036	0.014
	miR-92a-3p	0.1162	0.1124	0.0527	0.0487	<0.001
	miR-132-3p	0.0002	0.0002	0.0001	0.0001	0.002
	miR-185-5p	0.0005	0.0005	0.0002	0.0003	<0.001
	miR-320a	0.1406	0.1327	0.0803	0.0786	0.009
	miR-324-3p	0.0003	0.0005	0.0002	0.0002	0.019
Validation set[3] (case = 32 and control = 32)	miR-20a-5p	0.0158	0.0147	0.0041	0.0045	<0.001
	miR-25-3p	0.0044	0.0038	0.0016	0.0015	<0.001
	miR-30a-5p	0.0084	0.0059	0.0050	0.0044	0.005
	miR-92a-3p	0.1291	0.0884	0.0496	0.0311	<0.001
	miR-132-3p	0.0003	0.0003	0.0001	0.0001	<0.001
	miR-185-5p	0.0012	0.0011	0.0002	0.0002	<0.001
	miR-320a	0.1166	0.0944	0.0688	0.0480	0.010
	miR-324-3p	0.0005	0.0005	0.0003	0.0003	0.041

[1]Normalized by cel-miRNA-39 ($\Delta C_T = C_{T\ sample} - C_{T\ cel\text{-}miR\text{-}39}$, and expression value = $2^{-\Delta C_T}$).
[2]p for the Mann–Whitney test.
[3]The validation set excluded miR-19a and miR-19b because they are in the same cluster with miR-20a and are highly correlated with miR-20a (ref. Supporting Information Table S6).

estimate the pooled ORs in the absence of heterogeneity ($p > 0.10$ and $I^2 < 50\%$ for the Q test). Otherwise, a random-effects model based on the DerSimonian and Laird method was used. The significance of the pooled ORs was evaluated by the Z test.

We used Statistical Analysis System version 9.1.3 (SAS Institute, Cary, NC) software packages, and the ROC curves were plotted in R language (version 3.03) using ROCR package. Meta-analysis of the two prospective cohorts was performed using Stata software (Version 11.0, Stata Corp, College Station, TX).

Results

Characteristics of study participants

The demographic characteristics of the HCC patients and controls are summarized in Supporting Information Table S8, which shows their comparable distributions of age and sex. The nested cases and controls from the two prospective cohorts were also well matched for age and sex.

Screening for candidate miRNAs

TLDA chip analysis performed according to the above-mentioned methods revealed that 114 miRNAs had C_T values of <35 in all nine plasma samples (Supporting Information Table S9), of which 19 (Supporting Information Table S1) were selected from the plasma sample screening and 21 (Supporting Information Table S2) were identified from the tissue sample screening, including ten overlapping miRNAs, which were selected as candidate differentially expressed liver-originated plasma miRNAs (Table 1).

Establishing and validating the diagnostic miRNA panel

We performed individual qRT-PCRs for the ten plasma miRNAs in the training set. All of them had significantly higher expression levels in the HCC patient samples than in the HBV-positive cancer-free control samples. The expression levels of the eight miRNAs (two were excluded, as explained in "Material and Methods") were all consistently and significantly higher in the HBV-positive HCC patients than in the cancer-free controls in the validation set (Table 2 and Supporting Information Fig. S2).

To evaluate the cumulative performances of the eight miRNAs in discriminating the HCC patients from the HBV carriers, a linear combination of the expression levels of the miRNAs weighted by the regression coefficient was used to construct a risk score for the eight-miRNA panel in the training set. The optimal cutoff value for each miRNA was determined according to the ROC obtained from the

Table 3. Diagnostic potential of eight miRNAs for HCC in training, validation and combination datasets

Stage	miRNA[1]	β	AUC	Cutoff point[2]	Sensitivity	Specificity
Training set (case = 35 and control = 50)	miR-20a-5p	3.436	0.710	2.555×10^{-3}	0.800	0.620
	miR-25-3p	1.459	0.682	2.996×10^{-3}	0.600	0.740
	miR-30a-5p	1.349	0.657	3.203×10^{-3}	0.457	0.820
	miR-92a-3p	2.064	0.723	5.573×10^{-2}	0.714	0.700
	miR-132-3p	2.788	0.701	4.888×10^{-5}	0.886	0.500
	miR-185-5p	3.174	0.724	6.831×10^{-5}	0.886	0.500
	miR-320a	1.507	0.667	1.190×10^{-1}	0.429	0.860
	miR-324-3p	2.026	0.650	1.231×10^{-4}	0.657	0.660
	Eight-miRNA panel	4.431	0.823	10.802	0.829	0.740
Validation set (case = 32 and control = 32)	miR-20a-5p	3.436	0.846	2.555×10^{-3}	0.938	0.500
	miR-25-3p	1.459	0.782	2.996×10^{-3}	0.500	0.875
	miR-30a-5p	1.349	0.706	3.203×10^{-3}	0.844	0.469
	miR-92a-3p	2.064	0.817	5.573×10^{-2}	0.813	0.656
	miR-132-3p	2.788	0.774	4.888×10^{-5}	0.938	0.156
	miR-185-5p	3.174	0.874	6.831×10^{-5}	0.938	0.219
	miR-320a	1.507	0.687	1.190×10^{-1}	0.344	0.906
	miR-324-3p	2.026	0.648	1.231×10^{-4}	0.844	0.469
	Eight-miRNA panel	4.431	0.780	10.802	0.906	0.500
Combination set (case = 67 and control = 82)	miR-20a-5p	3.436	0.770	2.555×10^{-3}	0.866	0.573
	miR-25-3p	1.459	0.718	2.996×10^{-3}	0.553	0.793
	miR-30a-5p	1.349	0.681	3.203×10^{-3}	0.642	0.683
	miR-92a-3p	2.064	0.765	5.573×10^{-2}	0.761	0.683
	miR-132-3p	2.788	0.722	4.888×10^{-5}	0.910	0.366
	miR-185-5p	3.174	0.788	6.831×10^{-5}	0.910	0.390
	miR-320a	1.507	0.678	1.190×10^{-1}	0.388	0.878
	miR-324-3p	2.026	0.656	1.231×10^{-4}	0.746	0.500
	Eight-miRNA panel	4.431	0.802	10.802	0.866	0.646

[1]Normalized by cel-miRNA-39 ($\Delta C_T = C_{T\ sample} - C_{T\ cel\text{-}miR\text{-}39}$, and expression value = $2^{-\Delta C_T}$).
[2]The optimal cutoff miRNA expression values in the training set representing the thresholds for coding the expression values as 0 or 1.

training set and was further assessed in the validation set (Table 3). The diagnosis probability was based on the RSF of the eight-miRNA panel according to the following equation: RSF = 2.788 × miR-132-3p + 3.174 × miR-185-5p + 3.436 × miR-20a-5p + 1.459 × miR-25-3p + 1.349 × miR-30a-5p + 1.507 × miR-320a + 2.026 × miR-324-3p + 2.064 × miR-92a-3p. The RSF value for each individual was used to construct the ROC curve. The AUC for the miRNA panel was 0.823 for the training set [sensitivity = 82.9%, specificity = 74.0%, positive predictive value (PPV) = 69.0% and negative predictive value (NPV) = 86.0%].

Using the same logit model and parameters based on the training set, we calculated the diagnosis probability to construct an ROC curve. The AUC of the miRNA panel was 0.780 (sensitivity = 78.1%, specificity = 68.7%, PPV = 73.7% and NPV = 85.1%) for the validation set, and it was 0.802 when the training and validation samples were combined (sensitivity = 86.6%, specificity = 64.6%, PPV = 66.7% and NPV = 85.5%, Fig. 2). These results indicated that the eight-miRNA panel may have had a high potential for distinguishing the HCC patients from the controls.

Cohort evaluations and meta-analyses

Prediagnostic samples were obtained from two prospective cohorts. The expression levels of the eight miRNAs from our current screening and five miRNAs from previously published studies are listed in Table 4 (with two overlaps). Considering that these two cohorts were recruited from different centers

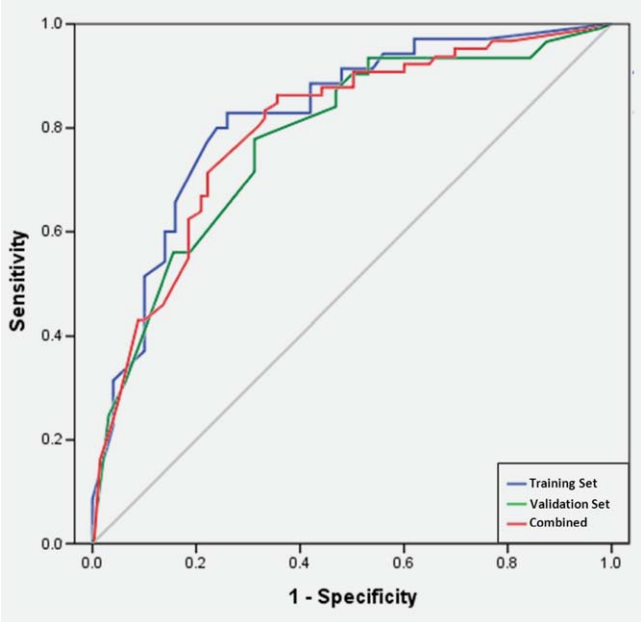

Figure 2. The discriminative ability of the eight-miRNA panel for the HBV-positive HCC patients *versus* the HBV-positive cancer-free controls as determined by receiver operating characteristic curve (ROC) analysis. Blue line, the training set (AUC = 0.823, sensitivity = 0.829 and specificity = 0.740); green line, the validation set (AUC = 0.780, sensitivity = 0.906 and specificity=0.500) and red line, the two sets combined (AUC = 0.802, sensitivity = 0.866 and specificity = 0.646). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

with varying processing/storage conditions and that the patients had differing HBV infection statuses, we performed meta-analysis of the results. Three miRNAs (miR-20a-5p, miR-320a and miR-324-3p) from our current screening and one (miR-375) from a previously published study were confirmed to be significantly overexpressed in the HCC patients compared with the controls at a $p_{meta} < 0.05$ (Table 4).

The ROC of the four-miRNA panel (miR-20a-5p, miR-320a, miR-324-3p and miR-375) and AFP were used to predict the HCC risk using the risk scores for the Changzhou cohort and Qidong cohort separately (Supporting Information Figs. S3 and S4). The optimal cutoff values for each miRNA and AFP level were determined according to the ROC curve for each cohort. For the Changzhou cohort, the AUC for the four-miRNA panel was 0.768 (sensitivity = 65.0%, specificity = 77.5%, PPV = 59.1% and NPV = 81.6%); that for AFP was 0.575 (sensitivity = 15.0%, specificity = 100.0%, PPV = 100.0% and NPV = 70.2%) and that for the combination of the four-miRNA panel and AFP was 0.789 (sensitivity = 70.0%, specificity = 77.5%, PPV = 60.9% and NPV = 83.8%). For the Qidong cohort, the AUC for the four-miRNA panel was 0.706 (sensitivity = 56.0%, specificity = 83.8%, PPV = 82.4% and NPV = 58.5%); that for AFP was 0.579 (sensitivity = 16.0%, specificity = 100.0%, PPV = 100.0% and NPV = 46.8%) and that for the combination of the four-miRNA panel and AFP was 0.767 (sensitivity = 64.0%, specificity = 83.8%, PPV = 84.2% and NPV = 63.3%).

Discussion

Emerging evidence suggests that circulating miRNAs are stable and noninvasive diagnostic biomarkers for cancer. A specified miRNA panel can be of great value in the mass screening for or early detection of cancer. In our study, we developed a novel strategy, combining plasma and target tissue samples from HCC patients and cancer-free controls to screen for genome-wide miRNA expression profiles. First, we designed a case–control study to select HCC-associated plasma miRNAs. The selection criterion was set as more than fourfold altered expression between the case and control groups, which has been used in our recent study to investigate the role of plasma miRNAs in detecting early-stage gastric cancer.[6] After we identified the HCC-associated plasma miRNAs, we added two additional conditions to screen for liver-originated HCC-associated plasma miRNAs in both plasma and tissue samples. One additional condition was that these miRNAs should originate in the liver, and the other was that they should also be differentially expressed in the tissue samples. In addition, we excluded the miRNAs that were upregulated after liver transplantation. As a result, our profile may be more specific for HCC detection.

Previously, Li *et al.*[4] compared the pooled serum samples of HBV infectors and normal controls and identified 13 differentially expressed miRNAs, of which six were upregulated in the HCC samples compared with the normal controls. In Zhou's study,[10] the discovery of deregulated miRNAs on the basis of pairwise comparisons of HCC *versus* control samples may represent a useful approach to segregate HCC patients from controls, but whether they are liver-originated or could serve as biomarkers for early detection has not been systematically investigated. Here, we developed a novel strategy that is more specific for HCC detection. In addition, during the prospective stage, we included miRNAs from Li's study and Zhou's study to evaluate their potentials for the early detection of this disease. Thus, our study combined these previous studies, revealing that four miRNAs (miR-20a-5p, miR-320a, miR-324-3p and miR-375) could be used as preclinical biomarkers for HCC.

Of the currently identified eight miRNAs, miR-25-3p and miR-92a-3p have also been reported as biomarkers for discriminating HCC patients from controls in our previously published study.[4] The upregulation of miR-25-3p in HCC cell lines and human HCC tumor samples has been consistently demonstrated by other research groups, and this miRNA belongs to the miR106b-25 cluster.[13–17] The miR-92a-3p, which is a member of the oncogenic miR-17-92 cluster, is not only highly expressed in HCC but also in colorectal cancer and epithelial ovarian cancer tissues, and their oncogenic effects likely occur *via* the regulation of angiogenesis and some other oncogenic characteristics.[4,18–20] Considering the high expression of *c-myc* in HCC, the miR-17-92 cluster together with *c-myc* overexpression may play a role in cancer development.[21,22] Four miRNAs (miR-19a-3p, miR-19b-3p, miR-20a-3p and miR-92a-3p) from this cluster were

Table 4. Results from two prospective cohorts obtained using eight novel miRNAs and those from previously published studies

miRNA[1]	Changzhou cohort (case = 20 and control = 40)				Qidong cohort (case = 50 and control = 37)				Meta analyses				
	Case Mean ± SD	Control Mean ± SD	OR (95% CI)	P^2	Case Mean ± SD	Control Mean ± SD	OR (95% CI)	p^2	Heterogeneity I^2 (%)	Heterogeneity p^3	Heterogeneity Model	OR (95% CI)	P^4
miR-20a-5p	0.0128 ± 0.0126	0.0062 ± 0.0070	4.714 (1.424–15.609)	**0.016**	0.0026 ± 0.0065	0.0023 ± 0.0054	2.182 (0.843–5.854)	0.425	1.9	0.313	Fixed	2.845 (1.390–5.825)	**0.004**
miR-25-3p	0.0019 ± 0.0016	0.0014 ± 0.0017	3.157 (1.006–9.906)	0.060	0.0003 ± 0.0007	0.0001 ± 0.0001	2.021 (0.644–6.347)	0.415	40.8	0.194	Fixed	1.803 (0.852–3.818)	0.123
miR-30a-5p	0.0130 ± 0.0123	0.0076 ± 0.0082	14.043 (1.709–115.410)	**0.011**	0.0037 ± 0.0106	0.0018 ± 0.0021	1.722 (0.728–4.075)	0.918	71.3	0.062	Random	3.968 (0.496–31.769)	0.194
miR-92a-3p	0.0901 ± 0.0999	0.0661 ± 0.1025	5.127 (1.296–20.285)	0.110	0.0182 ± 0.0374	0.0208 ± 0.0330	1.479 (0.617–3.547)	0.455	55.6	0.133	Random	2.450 (0.737–8.141)	0.144
miR-132-3p	0.0005 ± 0.0005	0.0006 ± 0.0005	1.519 (0.501–4.611)	0.683	0.0001 ± 0.0001	0.0001 ± 0.0001	4.267 (1.421–12.809)	0.198	51.3	0.152	Random	2.700 (0.788–9.258)	0.114
miR-185-5p	0.0017 ± 0.0018	0.0006 ± 0.0007	11.000 (2.247–53.841)	**0.002**	0.0003 ± 0.0010	0.0001 ± 0.0002	2.222 (0.843–5.854)	0.979	75.2	0.045	Random	3.892 (0.646–23.457)	0.138
miR-320a	0.0935 ± 0.0967	0.0611 ± 0.0725	4.636 (1.171–18.363)	0.071	0.0276 ± 0.0538	0.0194 ± 0.0130	2.222 (0.843–5.854)	0.738	0.0	0.391	Fixed	2.894 (1.322–6.336)	**0.008**
miR-324-3p	0.0006 ± 0.0007	0.0003 ± 0.0003	7.364 (1.504–36.042)	**0.011**	0.0003 ± 0.0005	0.0001 ± 0.0001	3.125 (1.282–7.617)	0.105	0.0	0.353	Fixed	4.015 (1.868–8.631)	**<0.001**
miR-21-5p	0.0092 ± 0.0133	0.0056 ± 0.0052	2.250 (0.752–6.730)	0.339	0.0046 ± 0.0136	0.0029 ± 0.0028	1.313 (0.558–3.085)	0.435	0.0	0.447	Fixed	1.610 (0.823–3.150)	0.165
miR-192-5p	0.0030 ± 0.0045	0.0007 ± 0.0014	10.524 (2.973–37.247)	**<0.001**	0.0148 ± 0.0428	0.0079 ± 0.0108	1.927 (0.703–5.285)	0.870	76.4	0.040	Random	4.308 (0.818–22.685)	0.085
miR-375	0.0004 ± 0.0004	0.0002 ± 0.0002	3.222 (1.050–9.890)	0.069	0.0002 ± 0.0005	0.0001 ± 0.0001	3.399 (1.390–8.313)	0.064	0.0	0.942	Fixed	3.331 (1.655–6.702)	**0.001**

These miRNAs in bold type were identified from the screening set while others were from published literature.
The significance of bold values means p value < 0.05.
[1]Normalized by cel-miRNA-39
($\Delta C_T = C_{T\ sample} - C_{T\ cel\text{-}miR\text{-}39}$, and expression value $= 2^{-\Delta C_T}$).
[2]p for the Mann–Whitney test.
[3]p for the heterogeneity test.
[4]p values derived from the Z test.

overexpressed in our study, and the Spearman correlation values for miR-19a-3p, miR-19b-3p and miR-20a-5p were all above 0.8 ($p < 0.05$), indicating highly positive correlations. Functionally, miR-20a has been reported to repress *E2F1* directly by binding to its target sites, and the induction of apoptosis may also be related to *E2F1* due to the regulation of these miRNAs and the subsequently inadvertent cell cycle progression to S phase.[23,24] Previous data have indicated that miR-185-5p can directly interact with the *DNMT1* gene, which has a role in the establishment and regulation of methylated cytosine residues, and aberrant methylation patterns are known to be associated with human tumors.[25] miR-132-3p has been reported to inhibit *SirT1* expression,[26] which functions as a tumor suppressor as well as a tumor promoter.[27–29]

Furthermore, our results showed that three (miR-20a-5p, miR-320a and miR-324-3p) of the eight plasma markers could be used for the early detection of HCC. As a target of miR-320a/c/d in HCC cells, *GNAI1* participates in numerous physiological processes, such as proliferation, adhesion and differentiation.[30–33] *GNAI1*, which is a member of the Gα inhibitory family, is significantly downregulated in HCC and can inhibit the invasion of HCC cells and play roles in the cyclin D1 pathway and regulation of proliferation.[34,35] Although miR-375 met the criterion of a fourfold change in expression between the HCC and cirrhosis patients, it was excluded from the HCC-associated plasma miRNAs in our initial screening because of the different directions of the alterations among the four comparisons. Thus, some miRNAs could have been missed because of the limited sample size assessed during the screening stage and the individual comparisons. We therefore reviewed the published literature and added three additional miRNAs to the prospective cohort sets.

Adding the evaluation of the AFP level to the four-miRNA panel improved the AUC for the combination of miRNA biomarkers; thus, AFP could be useful for risk prediction. AFP is a widely used tumor marker for HCC detection; however, elevated levels have also been observed in some patients with chronic liver disease.[36] Although an elevation in AFP is not considered to be a preclinical diagnostic biomarker, the increased area under the ROC curve following the addition of the AFP level evaluation to the four-miRNA panel may be attributed to the presence of chronic liver disease, such as cirrhosis, in the patients. Another limitation of our study is that both cohorts were not originally established for this study design; therefore, the plasma samples were collected over a long period of time, in which different sampling conditions were used. However, despite these limitations, we performed an independent, well-controlled, nested case–control study of each cohort.

Our study used a multistage design, including a screening set, training and validation sets and prospective cohort sets. Because of individual heterogeneity, we combined miRNAs among different comparisons to select candidate miRNAs. Larger sample sizes normally lead to the increased capability to identify differentially expressed miRNAs. Because of the limited sample sizes of the training and validation sets, we still had enough power to identify the eight-miRNA panel (in fact, all of the initial results were validated later). However, the low specificity of 0.646 for distinguishing the HCC patients from the cancer-free controls may have been due to the limited sample size.

We only used exogenous spike-in cel-miR-39 to control efficiency of RNA extraction and the existence of putative PCR inhibitors co-purified with plasma RNA. But we could not normalize the potential variation introduced before RNA extraction. Further studies are required to validate our findings by using both exogenous and endogenous normalization controls.

In summary, we demonstrated that the expression profile of a miRNA panel in the plasma can serve as a biomarker for the early detection of HCC. Our work further indicated that the expression levels of the miRNAs in the four-miRNA panel in addition to the AFP level could be used as more effective blood-based early detection biomarkers for this disease. Further studies are warranted to confirm our findings, and additional functional studies should be conducted to elucidate the underlying mechanisms before the clinical use of the miRNA panel for HCC diagnosis.

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