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Microarray Expression Profiling of microRNAs Reveals Potential Biomarkers for Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) remains a major health problem for delayed diagnosis, inefficient surveillance and poor prognosis. Recent studies have indicated that non-coding RNAs contribute to the development of new strategies for diagnosis and treatment of HCC. In the present study, we employed 18 pairs of HCC and matched non-tumor tissues for the identification of differentially expressed microRNAs (miRNAs) in HCC, among which 7 paired specimens were selected randomly for microarray detection. Totally, twenty-three miRNAs were screened out to have statistically significant differences with the threshold of $P < 0.01$ and fold-change ≥ 2.0 or ≤ 0.5 using miRNA microarray. In the validation stage, two miRNAs exhibited higher expression levels in the HCC tissues compared with those in the matched non-tumor tissues, whereas the expression levels of ten miRNAs were lower in the HCC tissues than those in the matched non-tumor tissues. In further analysis, eight miRNAs, including miR-4270, miR-125b-5p, miR-199a-3p, miR-10a-5p, miR-424-5p, miR-195-5p, miR-106b-5p and miR-3651, were retained, when another constraint about the signal intensity of microarray probes was established. Among these miRNAs, our study was the first to show the higher expression level of miR-3651 and the lower expression level of miR-4270 in HCC. The areas under the receiver-operating-characteristic curve values of miR-3651 and miR-4270 were 0.730 and 0.967, respectively, indicating their potential diagnostic values. Our results may help provide the context for expanded interpretations of miRNA studies involved in the progression of liver disease, potentially serving as a diagnostic tool of HCC.

Keywords: diagnosis, hepatitis virus, hepatocellular carcinoma, microRNA, receiver operating characteristic
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Introduction

Hepatocellular carcinoma (HCC), the most frequent type of liver cancer, has been reported to be the second leading cause of cancer-related death worldwide (Jemal et al. 2011). Hepatitis B and C viral infections, aflatoxin B1 exposure, excessive alcohol consumption, obesity and some inherited metabolic disorders are generally considered to be major risk factors in the development of HCC (Sia et al. 2017). Regardless of comprehensive understanding of these risk factors, delayed diagnosis and poor prognosis remain key challenges for patients with HCC. Currently, human transcript was expanded to include not only protein-coding genes, but proverbial "dark matter" of the genome, non-coding RNAs (ncRNAs).

It has been reported that human genome may be extensively regulated by non-coding parts in various diseases, including cancers (Shi et al. 2013). microRNAs (miRNAs) are a class of small endogenous non-coding molecules ranging from 18-24 nucleotides that control gene expression by targeting mRNAs, and that trigger either translation repression or RNA degradation by antisense base pairing with their 3' untranslated region (3'UTR) (Meister and Tuschl 2004). The two short transcripts of lin-4 were identified as the first recognized miRNAs by targeting the 3'UTR of lin-14 mRNA in *C. elegans* (Lee et al. 1993). With the development of new biological technologies, evidence has revealed that miRNAs play vital roles in the fragile X syndrome, diabetes and various cancers (Jin et al. 2004; Poy et al. 2004; Calin and Croce 2006). In the fol-

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lowing years, studies concerning the role of miRNAs in carcinogenesis were reported. Two studies demonstrated that genome deletions in 13q14 covering the genes encoding miR-15 and miR-16 had strong connections with the occurrence of chronic lymphocytic leukemia (Dohner et al. 2000; Bullrich et al. 2001). Moreover, miRNA expression profiles were obtained to figure out potential biomarkers and prognostic indicators in HCC. For example, a miRNA panel (including miR-122, miR-192, miR-21, miR-223 miR-26a, miR-27a and miR-801) identified the high diagnostic accuracy for HCC, with areas under the receiver-operating-characteristic curve (AUC) = 0.888 for validation stage (Zhou et al. 2011). Also, another study showed that overexpressed miR-216a/217 contribute to tumor recurrence by targeting PTEN (phosphatase and tensin homolog) and Smad in HCC (Xia et al. 2013). Although a larger number of researches have been performed to explore the importance of miRNAs in HCC; however, conflicting conclusions have been reached on miRNAs as yet.

In our study, Agilent microarray was firstly applied to profile differentially expressed miRNAs. Candidate miRNAs were then selected and validated in a small scale independent cohort of HCC patients. Receiver operating characteristic (ROC) curve and AUC were finally used to estimate the diagnostic value of the candidate miRNAs for HCC.

Materials and Methods

Study design

To uncover the role of miRNAs in HCC, a three-step approach was applied to identify HCC-specific miRNAs. Firstly, a total of 18 pairs of HCC specimens were collected for the whole study, in which 7 pairs of the specimens were selected randomly for miRNA microarray. Secondly, all candidate miRNAs screened out from the first step were validated by quantitative real-time PCR (qPCR) in eighteen paired HCC and matched adjacent tumor-free tissues. Finally, further screening of candidate miRNAs via signal intensity was achieved and the risk score analysis (including ROC curve and AUC) were performed to figure out diagnostic value of the candidate miRNAs in HCC.

Sample characteristics

The study included 18 pairs of HCC and matched tumor-free frozen tissue specimens. All patients were confirmed as HCC by preoperative imaging tests and postoperative pathological diagnosis in Zhongshan Hospital of Fudan University from 2008 to 2009. Seven pairs of the specimens were selected randomly for Agilent microarray detection (Table 1). The study has obtained approval from the Institution Ethics Committee of Zhongshan Hospital of Fudan University. The entire experiment procedure was performed in accordance with the guidelines of Declaration of Helsinki, and written informed consents were obtained from each participant.

RNA extraction and purification for microarray

Total RNAs of the seven paired samples were purified by using mirVana™ miRNA Isolation Kit with phenol (Ambion, Austin, TX, USA). The concentration of RNA was determined by NanoDrop™ 2000 Spectorphotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the RNA integrity number (RIN) representing quality of RNA was measured by Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA).

Microarray data analysis

The Agilent human miRNA microarray release 21.0 (8 × 60 K) was applied for detection of profiling of miRNA in 7 pairs of HCC and matched tumor-free tissues. Totally, the of microarray covered 2,549 miRNAs from the latest miRBase database. The slide was scanned by the Agilent Microarray Scanner (Cat# G2565CA, Agilent technologies, Santa Clara, CA, US) and the Feature Exaction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data from Scanner were normalized by Quantile algorithm, Gene Spring Software. All of the performances were according to the manufacturer's protocol. To screen out the significantly differentially expressed miRNAs, the threshold of $P < 0.01$ and fold change ≥ 2 or ≤ 0.5 was set up. The data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE108724 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108724).

Specific primers design and qPCR validation

Total RNAs were extracted from the 18 paired HCC specimens, including 7 paired specimens for microarray analysis and were reversely transcribed into cDNA by miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN Biotech, Beijing, China). During this procedure, the 3' terminal of miRNAs was polyadenylated and converted into cDNA by reverse transcriptase using oligo-dT and universal primers. The specific primers of miRNAs were designed by RiboBio Corporation (RiboBio, Guangzhou, China) for qPCR and all primer sequences were shown (Table 2). qPCR was conducted to validate expression level of candidate miRNAs by usage of SYBR® Fast qPCR Mix (Takara, Dalian, China). Hsa-U6 was used as the internal control. All experiments were performed in triplicate following the manufacturer's instructions.

Statistical analysis

All statistical analysis was conducted using SPSS 22.0 statisti-

Table 1. Clinical characteristics of selected seven pairs of HCC and matched non-tumor tissue specimens.

Case No.	Sample label	Gender	Age (year)	Pathological diagnosis	Pathological grade	Cirrhosis	HBV
5	5T/5P	Male	57	HCC	II	+	+
6	6T/6P	Female	67	HCC	II	+	+
9	9T/9P	Male	58	HCC	II-III	+	+
10	10T/10P	Male	52	HCC	II	+	+
11	11T/11P	Female	62	HCC	III	+	+
13	13T/13P	Male	54	HCC	III	–	+
19	19T/19P	Male	27	HCC	II	+	+

No., number; HCC, hepatocellular carcinoma; T, HCC tissues; P, matched non-tumor tissues.

Table 2. Specific forward primers for candidate miRNAs.

miRNA	Forward primers
hsa-miR-375	TTTGTTCGTTCGGCTCGCGTGA
hsa-miR-338-3p	TCCAGCATCAGTGATTTTGTTG
hsa-miR-214-3p	ACAGCAGGCACAGACAGGCAGT
hsa-miR-8089	CCTGGGGACAGGGGATTGGGGCAG
hsa-miR-199a-3p	ACAGTAGTCTGCACATTGGTTA
hsa-miR-486-5p	TCCTGTACTGAGCTGCCCCGAG
hsa-miR-7845-5p	AAGGGACAGGGAGGGTCGTGG
hsa-miR-4270	TCAGGGAGTCAGGGGAGGGC
hsa-miR-10a-5p	TACCCTGTAGATCCGAATTTGTG
hsa-miR-125b-5p	TCCCTGAGACCCTAACTTGTGA
hsa-miR-195-5p	TAGCAGCACAGAAATATTGGC
hsa-miR-424-5p	CAGCAGCAATTCATGTTTTGAA
hsa-miR-378d	ACTGGACTTGGAGTCAGAAA
hsa-miR-106b-5p	TAAAGTGCTGACAGTGCAGAT
hsa-miR-93-5p	CAAAGTGCTGTTCGTGCAGGTAG
hsa-miR-6090	GGGGAGCGAGGGGCGGGGC
hsa-miR-4485-5p	ACCGCCTGCCCAGTGA
hsa-miR-3651	CATAGCCCGGTCGCTGGTACATGA
hsa-miR-130b-3p	CAGTGCAATGATGAAAGGGCAT
hsa-miR-222-3p	AGCTACATCTGGCTACTGGGT
hsa-miR-532-3p	CCTCCCACACCCAAGGCTTGCA
hsa-miR-221-3p	AGCTACATTGTCTGCTGGGTTTC
hsa-miR-3188	AGAGGCTTTGTGCGGATACGGGG

cal software (SPSS, Chicago, IL, USA). The qPCR data was analyzed by paired Wilcoxon rank-sum (Mann-Whitney) test and presented using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).

Risk score analysis including ROC and AUC were performed to figure out diagnostic value of the candidate miRNAs in HCC. All statistical calculations were considered to have significantly statistical differences when $P < 0.05$.

Results

Profiling of differentially expressed miRNAs

Total RNAs were extracted from 7 pairs of HCC and matched tumor-free tissues using the dedicated kit. These RNAs were qualified following the baseline value of RIN ≥ 6.0 and 28S/18S ≥ 0.7. After normalization of raw data by Gene spring software, Box-Whisker plot was generated to visualize the distribution of the intensity values (log2 transferred) in 7 pairs of HCC tissues (Fig. 1A). Meanwhile, scatter plot was performed to represent the expression variation of miRNAs. The findings demonstrated that fold change of quite a few of miRNAs were more than two times between the two group (Fig. 1B). Further, volcano plot showed miRNAs with significantly statistical differences (fold-change ≥ 2 or ≤ 0.5 and $P < 0.01$; Fig. 1C).

On basis of screening criteria (fold-change ≥ 2 or ≤ 0.5 and $P < 0.01$), only ten miRNAs in higher expression level (including miR-106b-5p, miR-93-5p, miR-6090, miR-4485-5p, miR-3651, miR-130b-3p, miR-222-3p, miR-532-3p, miR-221-3p and miR-3188) and thirteen miRNAs in lower expression level (including miR-375, miR-338-3p, miR-214-3p, miR-8089, miR-199a-3p, miR-486-5p, miR-7845-5p, miR-4270, miR-10a-5p, miR-125b-5p, miR-195-5p, miR-424-5p, miR-378d) were identified to have strong significantly statistical differences between HCC and matched tumor-free tissues (Table 3). Hierarchical clustering was then applied to figure out the expression pattern of these differentially expressed miRNAs (Fig. 1D).

Validation of differentially expressed miRNAs

All 23 miRNAs that had fold-change ≥ 2 or ≤ 0.5 and $P < 0.01$ were detected by qPCR to confirm the results of microarray analysis. In the validation phase, twelve miRNAs (including miR-4270, miR-375, miR-125b-5p, miR-199a-3p, miR-10a-5p, miR-7845-5p, miR-424-5p, miR-378d, miR-195-5p, miR-214-3p, miR-106b-5p and miR-3651) were identified as significantly differentially expressed miRNAs in 18 pairs of HCC compared with matched tumor-free tissues (Fig. 2A-L), while the remaining eleven miRNAs having no statistical differences (Fig. 3A-K). Also, the results of qPCR were consistent with the statistical analysis of microarray data (Fig. 4).

Confirmation of specificity and sensitivity of candidate miRNAs

To verify 12 candidate miRNAs that are suitable to serve as accurate biomarkers for HCC, another constraint was established that the signal intensity values of microarray probes were detectable and defined as “Present” instead of “Absent” or “Marginal”. In this stage, four miRNAs (including miR-375, miR-7845-5p, miR-378d and miR-214-3p) were excluded for unmet eligibility criteria. Thus, two miRNAs in higher expression level (including miR-106b-5p and miR-3651) and six miRNAs in lower expression level (including miR-4270, miR-125b-5p, miR-199a-3p, miR-10a-5p, miR-424-5p and miR-195-5p) were retained for further test of ROC curve and AUC calculations. All of these eight dysregulated miRNAs had remarkably statistical differences in AUC, whose values were

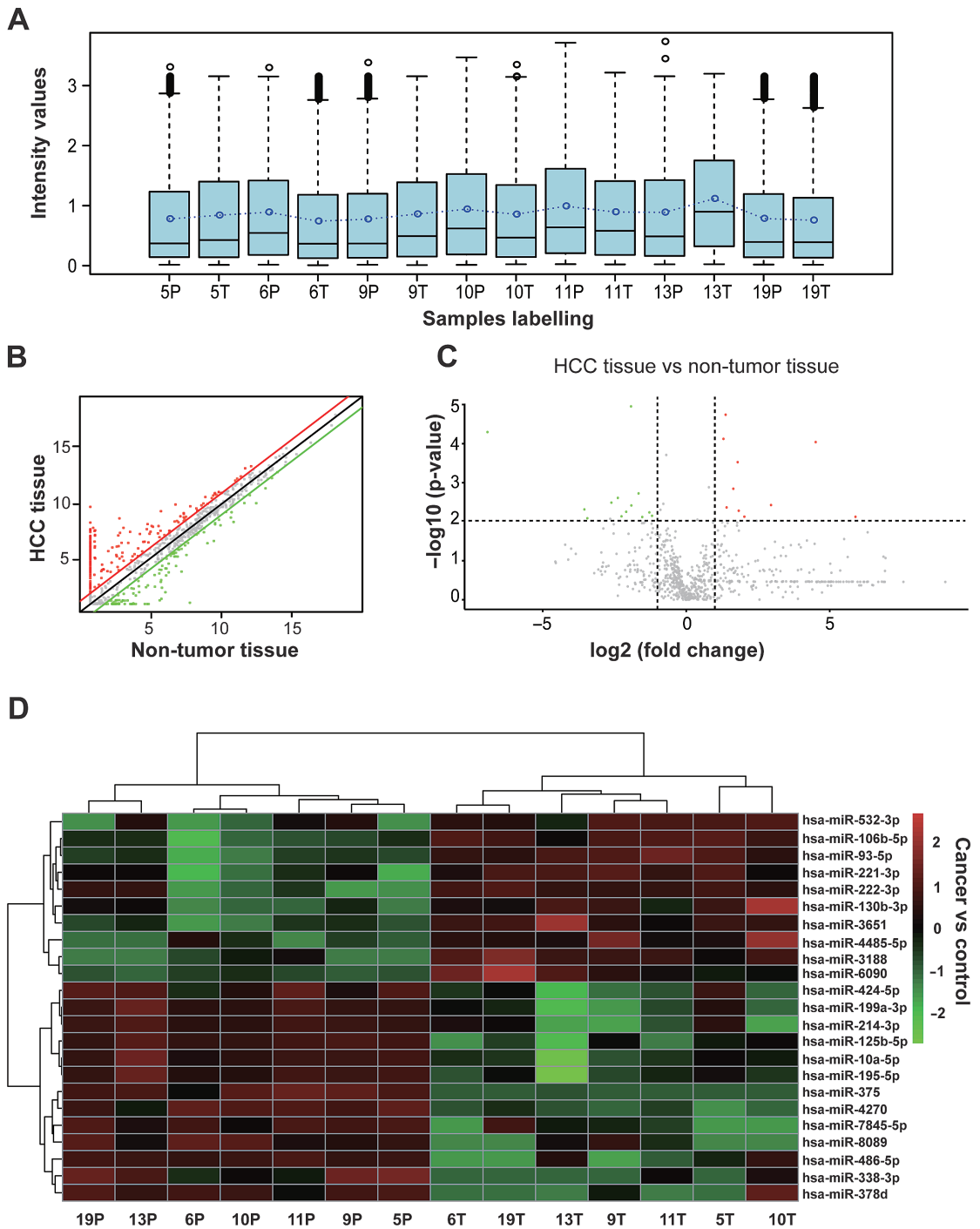

Fig. 1. Profiling of the microarray data.

(A) Box-Whisker plot is used to illustrate distribution of microarray data. The sample labels for 7 pairs of HCC tissues and matched non-tumor tissues line up in the X axis. Y axis stands for intensity values of miRNA microarray probes. (B) Scatter plot is performed to visualize the expression variation of differentially expressed miRNAs between HCC and matched non-tumor controls. The values of X and Y axis are log2 scaled intensity values of miRNA microarray probes. Red color implies miRNAs in higher expression level and green one signifies miRNAs in lower expression level. (C) Volcano plot is presented to show significantly differentially expressed miRNAs ($P < 0.01$). Fold change values are log2 transformed as the X axis while the log10 transformation of P values are set in the Y axis. (D) Hierarchical clustering is conducted to show the expression patterns of differentially expressed miRNAs in 14 samples. T stands for HCC and P stands for matched non-tumor tissue samples.

Table 3. Summary of differentially expressed miRNAs in HCC.

Expression level	miRNA	miBase accession No.	Chromosome	Fold change	*P* value
Low	hsa-miR-375	MIMAT0000728	2	0.008	5.05×10^{-05}
	hsa-miR-338-3p	MIMAT0000763	17	0.085	4.85×10^{-03}
	hsa-miR-214-3p	MIMAT0000271	1	0.160	8.47×10^{-03}
	hsa-miR-8089	MIMAT0031016	5	0.164	3.25×10^{-03}
	hsa-miR-199a-3p	MIMAT0000232	19	0.192	2.46×10^{-03}
	hsa-miR-486-5p	MIMAT0002177	8	0.210	7.29×10^{-03}
	hsa-miR-7845-5p	MIMAT0030420	2	0.235	5.57×10^{-03}
	hsa-miR-4270	MIMAT0016900	3	0.264	1.11×10^{-05}
	hsa-miR-10a-5p	MIMAT0000253	17	0.266	3.84×10^{-03}
	hsa-miR-125b-5p	MIMAT0000423	21	0.316	1.89×10^{-03}
	hsa-miR-195-5p	MIMAT0000461	17	0.346	7.59×10^{-03}
	hsa-miR-424-5p	MIMAT0001341	X	0.411	5.79×10^{-03}
	hsa-miR-378d	MIMAT0018926	8	0.438	7.06×10^{-03}
High	hsa-miR-106b-5p	MIMAT0000680	7	2.460	7.55×10^{-05}
	hsa-miR-93-5p	MIMAT0000093	7	2.592	1.82×10^{-05}
	hsa-miR-6090	MIMAT0023715	11	2.656	4.32×10^{-03}
	hsa-miR-4485-5p	MIMAT0032116	11	3.121	1.43×10^{-03}
	hsa-miR-3651	MIMAT0018071	9	3.467	2.98×10^{-04}
	hsa-miR-130b-3p	MIMAT0000691	22	3.557	5.25×10^{-03}
	hsa-miR-222-3p	MIMAT0000279	X	4.076	7.45×10^{-03}
	hsa-miR-532-3p	MIMAT0004780	X	5.487	9.52×10^{-03}
	hsa-miR-221-3p	MIMAT0000278	X	7.760	3.75×10^{-03}
	hsa-miR-3188	MIMAT0015070	19	22.820	9.13×10^{-05}

No., number.

0.715, 0.730, 0.967, 0.901, 0.958, 0.934, 0.877 and 0.784, respectively ($P < 0.05$; Fig. 5 and Table 4).

Discussion

Up to now, HCC is still one of the malignant tumors that are hardest to crack for delayed diagnosis and poor prognosis worldwide. Usually, α-fetoprotein (AFP) has been used as diagnosis biomarker for screening out HCC patients for decades (Zhang et al. 2004; Bai et al. 2017). However, studies indicate that AFP has poor sensitivity and specificity for HCC (Akeyama et al. 1972; Wang et al. 2016). Thus, either doctors or researchers kept craving more efficient biomarkers for HCC.

In recent decades, non-coding genes contributing to epigenetic regulations have been verified to associate with the occurrence and progression of HCC (Wahid et al. 2017). Dating back to ten years ago, researchers performed miRNA microarray assay to classify specific diagnostic miRNAs for HCC (Murakami et al. 2006). Thereafter, a report illustrated that down-regulated miR-29 associates with poor prognosis in HCC and becomes a potential therapeutic target for HCC (Xiong et al. 2010). In the following years, a large quantity of miRNAs was verified to play important roles in the prognosis and therapy of HCC.

In the present study, we performed Agilent microarray to validate the expression profiles of miRNAs in 7 pairs of HCC and matched tumor-free samples. Based on the criteria set as $P < 0.01$ and fold change ≥ 2 or ≤ 0.5, the investigation figured out that 23 differentially expressed miRNAs have significantly statistical differences. In the further validation phase, eight candidate miRNAs (including miR-106b-5p, miR-3651, miR-4270, miR-125b-5p, miR-199a-3p, miR-10a-5p, miR-424-5p and miR-195-5p) were presented to be underlying biomarkers for HCC.

Till now, there were no reports concerning the two differentially expressed miRNAs (including miR-3651 and miR-4270) in HCC. Abnormal expression of miR-3651 has been studied in other digestive cancers other than HCC, such as esophageal squamous cell cancer and colorectal cancer (Della Vittoria Scarpati et al. 2014; Wang et al. 2015). However, no further detailed investigations were reported for the two gastrointestinal cancers, and this is the first time, to our knowledge, that we present its role as a biomarker for HCC. Additionally, in myasthenia gravis, the miR-3651's putative target-CRISP3 (cysteine-rich secretory protein 3), plays an important role in innate immune response, which indicate it may be a vital regulatory factor in immunological process (Barzago et al. 2016).

Also, miR-4270 has never been reported to associate with HCC, even though the miRNA has been proven to have the best ROC diagnostic value in our study. A previous report showed that miR-4270 is down-regulated in stage IV of breast cancer than other early stages, while another study presented evidence that miR-4270 is considered to involve in the malignant ascites in gastric cancer (Tokuhisa et al. 2015; Hamam et al. 2016). Furthermore, miR-4270 was validated to modulate antigen presentation activity of macrophages by targeting CD300E during helicobacter pylori infection (Pagliari et al. 2017). These findings consistent with the potential role of miR-3651 in immune response, suggesting that miR-4270 may get involved in the immunological process in diseases. Still, in spite of the inconsistent with other cancers, the miR-4270, with an AUC value of 0.967, can be serve as a promising biomarker role in HCC.

The remaining six differentially expressed miRNAs

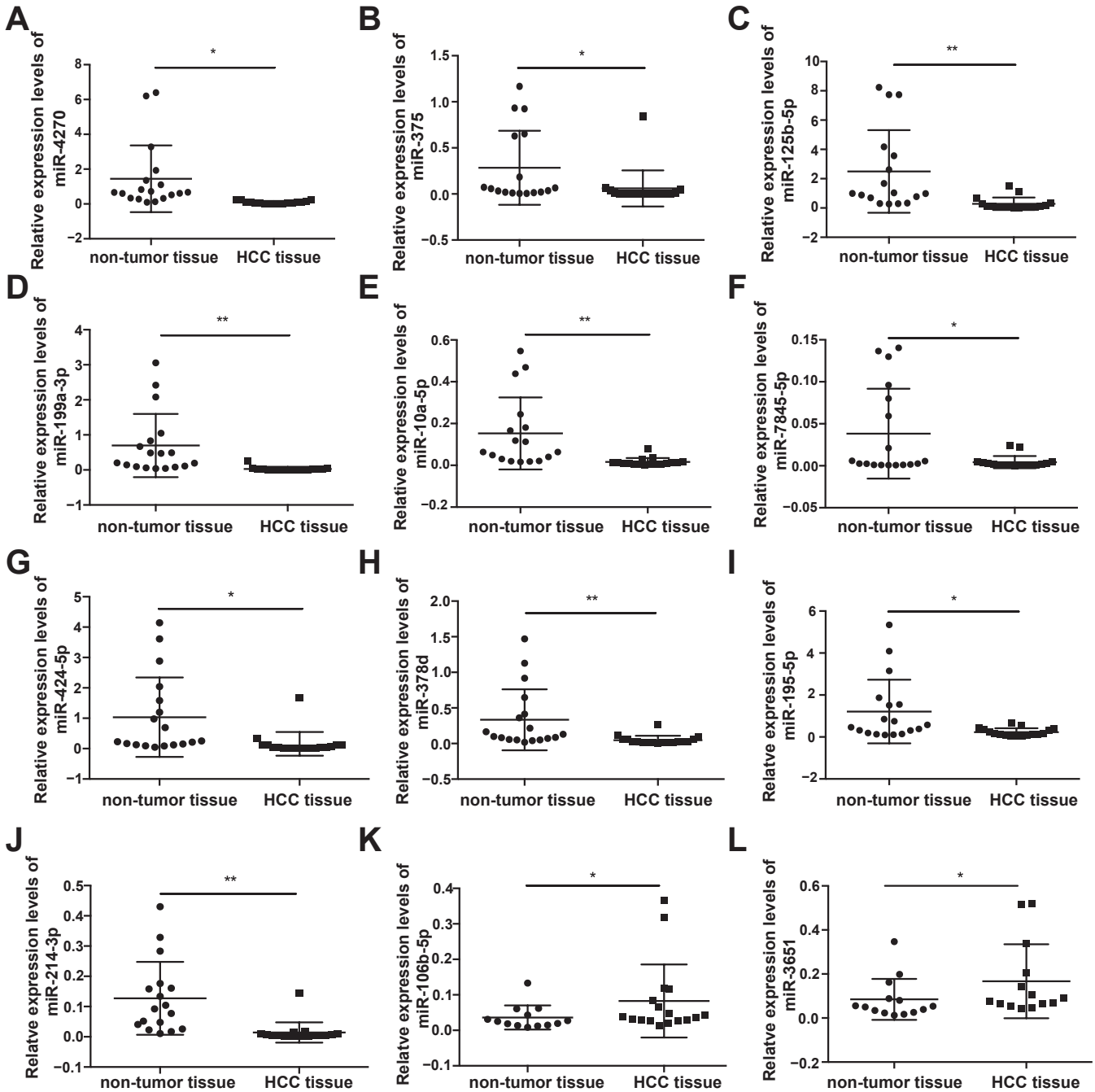

Fig. 2. Validation of candidate miRNAs in 18 pairs of HCC and adjacent non-tumor tissues by qPCR.
Relative expression of 12 candidate miRNAs, including miR-4270 (A), miR-375 (B), miR-125b-5p (C), miR-199a-3p (D), miR-10a-5p (E), miR-7845-5p (F), miR-424-5p (G), miR-378d (H), miR-195-5p (I), miR-214-3p (J), miR-106b-5p (K) and miR-3651 (L). Data are presented as the mean with standard deviation (SD).
*$P < 0.05$, **$P < 0.01$.

(including miR-106b-5p, miR-125b-5p, miR-199a-3p, miR-10a-5p, miR-424-5p and miR-195-5p) have been validated as important regulatory factors in HCC. Among these six miRNAs, miR-106b-5p has been reported to promote stem-like properties of HCC cells via targeting PTEN, consistent with our present study (Shi et al. 2018). However, the expression level of miR-125b-5p increased in the serum of HBV-positive HCC patients compared with matched controls, which is contradictory to our findings (Giray et al. 2014). The reason for the difference may be due to the different roles of miR-125b-5p in serum and tissue in patients with HCC. Furthermore, compared with our microarray data, conflicting data was also found with regards to miR-10a-5p expression. It indicated that sorafenib-resistant HCC cells was associated with an upregulated miR-10a-5p level, which implies that miR-10a-5p has its own spatio-

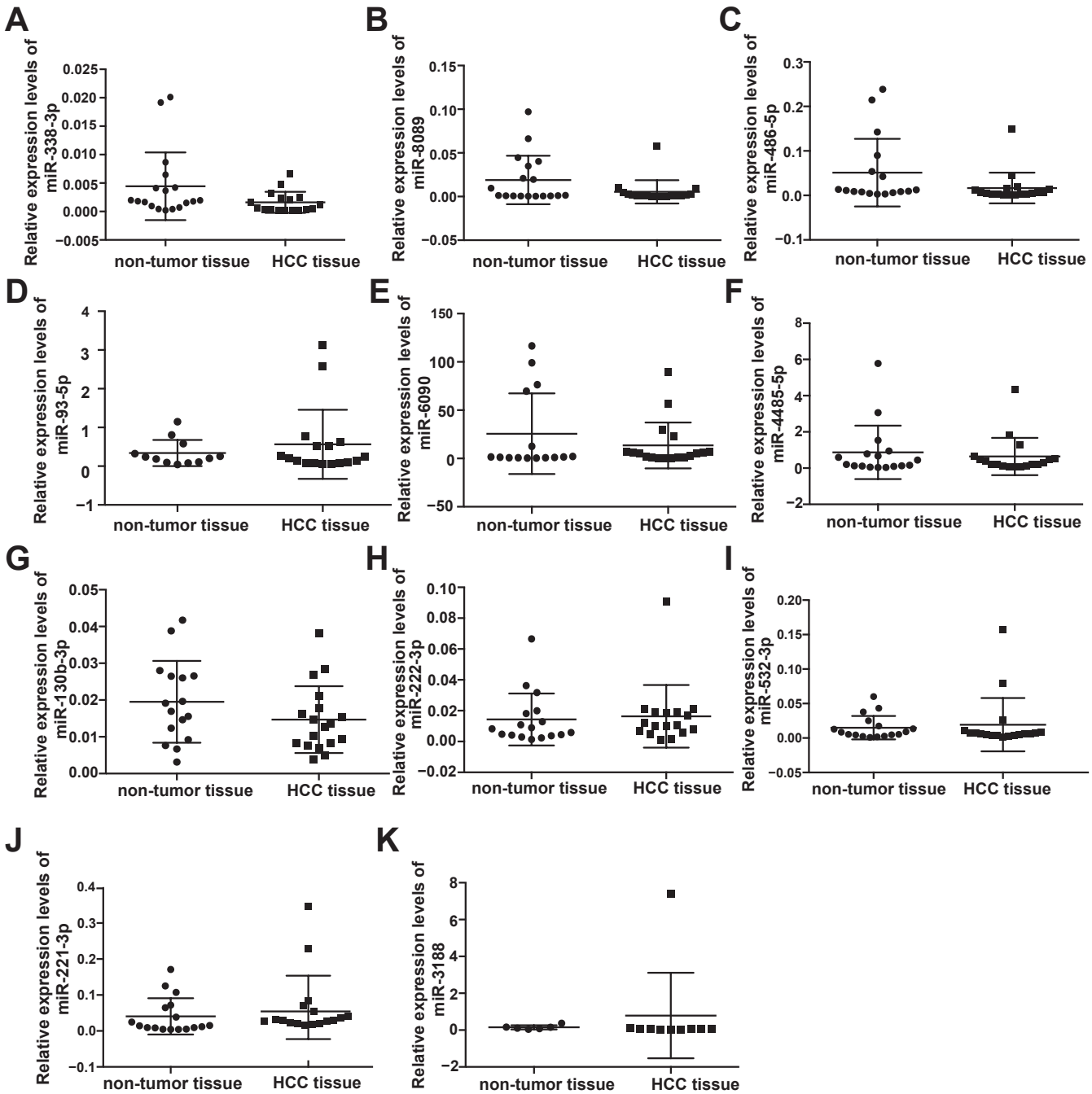

Fig. 3. Validation of miRNAs showing no statistical differences in 18 pairs of HCC and adjacent non-tumor tissues by qPCR.
Relative expression of 11 miRNAs, including miR-338-3p (A), miR-8089 (B), miR-486-5p (C), miR-93-5p (D), miR-6090 (E), miR-4485-5p (F), miR-130b-3p (G), miR-222-3p (H), miR-532-3p (I), miR-221-3p (J) and miR-3188 (K). Data are presented as scatter plot of the mean with standard deviation (SD).

temporal expression in HCC (Tang et al. 2016). The mechanism explorations of miR-199a-3p, miR-424-5p and miR-195-5p have demonstrated that they all act as tumor suppressors in HCC (Fornari et al. 2010; Zhang et al. 2014; Xu et al. 2015).

In summary, our study applied the Agilent microarray to determine expression profiles of the miRNAs in HCC specimens. Our results found two new miRNAs (miR-3651 and miR-4720) that have never been reported and provided new diagnostic and therapeutic targets for research in HCC. Actually, more researches using microarray profiling of miRNAs may be helpful to pave the most efficient and consolidated way for the molecular mechanisms in the pathogenesis of HCC in the future.

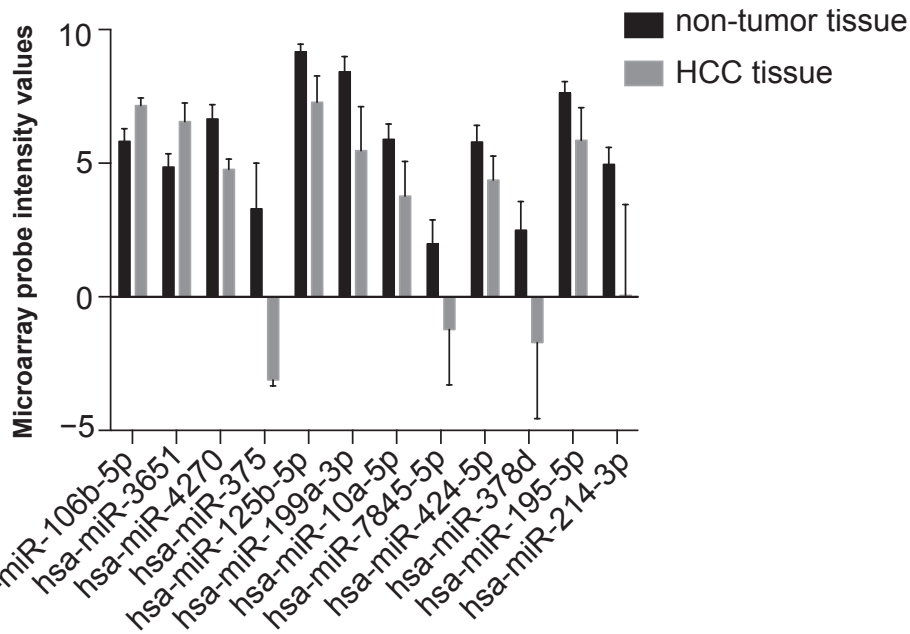

Fig. 4. Analysis of the microarray probe intensity values of 12 candidate miRNAs.
The microarray probe intensity values of 12 candidate miRNAs were analyzed and visualized to distinguish their expression level in microarray row data, which is consistent with the results from qPCR.

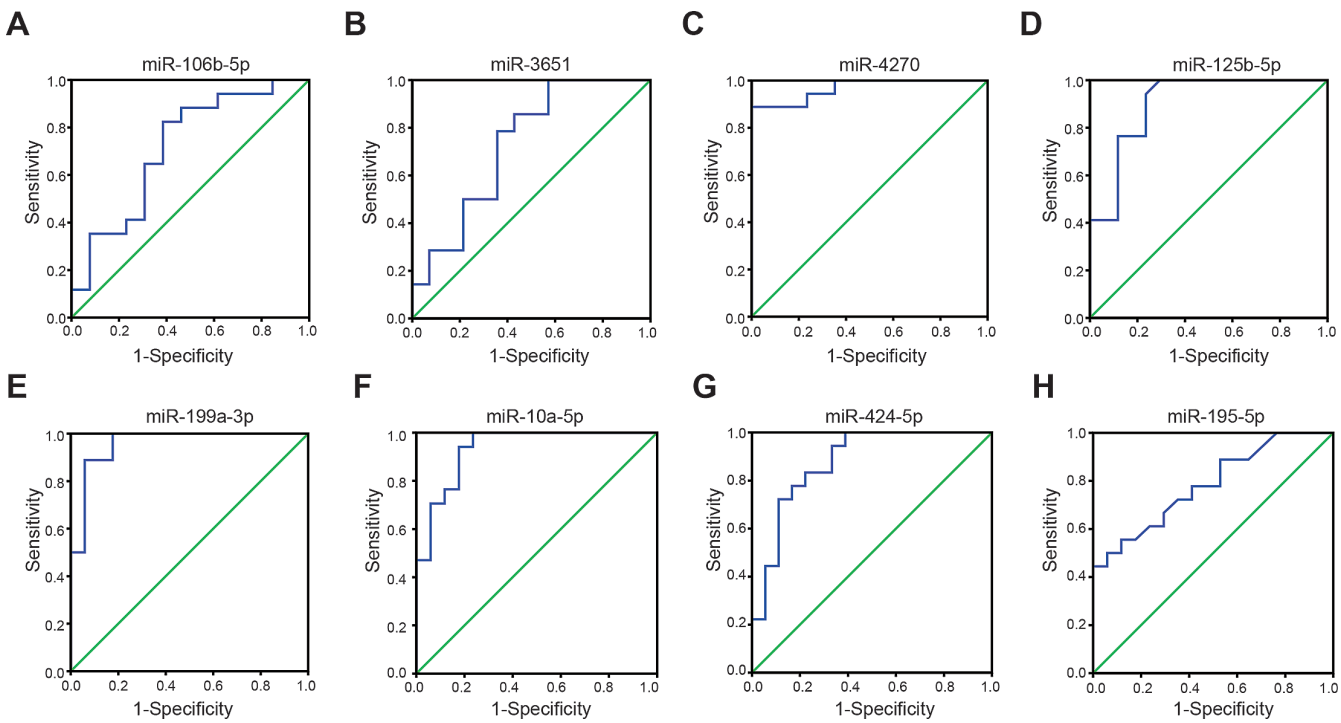

Fig. 5. Analysis of diagnostic capability of 8 candidate miRNAs in HCC.
ROC curve analysis of the 8 candidate miRNAs, including miR-106b-5p (A), miR-3651 (B), miR-4270 (C), miR-125b-5p (D), miR-199a-3p (E), miR-10a-5p (F), miR-424-5p (G) and miR-195-5p (H).

Table 4. Illustration of area under the curve of 8 candidate miRNAs.

miRNA	AUC	Std. Error	Asymptotic Significance	Asymptotic 95% confidence interval
hsa-miR-106b-5p	0.715	0.098	0.047	0.522-0.908
hsa-miR-3651	0.730	0.097	0.039	0.539-0.920
hsa-miR-4270	0.967	0.026	< 0.0001	0.916-1.000
hsa-miR-125b-5p	0.901	0.054	< 0.0001	0.796-1.000
hsa-miR-199a-3p	0.958	0.033	< 0.0001	0.893-1.000
hsa-miR-10a-5p	0.934	0.041	< 0.0001	0.854-1.000
hsa-miR-424-5p	0.877	0.059	< 0.0001	0.761-0.992
hsa-miR-195-5p	0.784	0.076	0.004	0.635-0.934

AUC, areas under the receiver-operating-characteristic curve; Std. Error, standard error.

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Conflict of Interest

The authors declare no conflict of interest.

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