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Original Article

Cluster of specified microRNAs in tissues and serum as biomarkers for early diagnosis of hepatocellular carcinoma

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Abstract: Objective: Hepatocellular carcinoma (HCC) is a global public health concern that lacks efficient methods for early diagnosis. Accumulated evidence has revealed great potential using microRNAs (miRNAs) as noninvasive biomarkers in HCC detection. Methods: Serum miRNAs (miR21, miR122, miR-214, miR15b, and let-7f) were detected in 75 patients with HCC and 80 healthy controls (HC). In addition, 75 HCC tissues and paired normal adjacent tissues were also analyzed for comparison. Quantified by real-time quantitative RT-PCR was used to evaluate the expression of miRNAs. Results: We discovered significant up-regulation of miR-21 ($P < 0.001$) and miR-15b ($P < 0.001$) as well as a down-regulation of miR-122 ($P = 0.01$) in HCC tissues compared to adjacent non-tumor tissues. Additionally, miR-21 ($P < 0.001$) and miR-15b ($P < 0.001$) levels were upregulated in serum and miR-214 ($P = 0.01$) was decreased in HCC patients compared to that in healthy controls. Multivariate logistic regression analysis showed that serum miR-21 (OR = 1.68, P = 0.012) and miR-15b (OR = 1.736, *P* = 0.012) were independently associated with HCC whereas miR-214 (OR = 0.631, *P* = 0.006) was associated with a decreased risk of HCC. When we employed miR-(21 + 122 + 15b) classifier as biomarkers, we could discriminate HCC tissues from adjacent non-tumor tissues with an AUC of 0.885 (specificity: 89.7%; sensitivity: 73.1%). In serum, the cluster of miR-(21 + 214 + 15b) classifier (AUC = 0.887) had a sensitivity of 80.3% and a specificity of 87.0% for HCC diagnosis. Conclusion: Our results suggest that these serum miRNAs may be useful markers for discriminating HCC patients from healthy controls. Combined determination of circulating miR-21, miR-122, miR-214, and miR-15b has great potential to serve as an accurate and noninvasive biomarker for the early HCC preliminary screening.

Keywords: Hepatocellular carcinoma, miRNAs, biomarkers, diagnosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth and seventh most frequently diagnosed cancer in males and females respectively. It is the second leading cause of cancer-related death in men and the sixth in women. HCC accounts for more than 90% of primary liver cancer (PLC) in China [1, 2]. The number of newly diagnosed cases of liver cancer is proximately 750000 each year. Among those cases, 82% occur in developing countries. The morbidity rate of liver cancer in most of the developed countries is less than 5/100000, whereas more than 35.5/100000 in China. Chronic infections with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) resulting in hepatic fibrosis and cirrhosis are major risk factors for HCC [3, 4]. Other risk factors include aflatoxin and chlorethylene exposure, hereditary hemochromatosis primary biliary cirrhosis, alcohol, diabetes mellitus, non-alcoholic fatty liver disease (NAFLD) and HIV infection. In China, HBV infection is the major risk factor for HCC [3, 5].

Recently, liver ultrasound (US), computed tomography (CT) and laboratory examination for alpha-fetoprotein (AFP) are the main methods for HCC screening and monitoring [6]. AFP is widely used as a tumor marker for the HCC

Table 1. Baseline characteristics of studied HCC patients

Baseline variable	Hepatocellular carcinoma patients, n (%)
Age	52.8 ± 9.7
Sex	
Male	71 (94.7)
Female	4 (5.3)
BCLC Stages	
A	21 (28)
B	36 (48)
C	11 (14.7)
D	7 (9.3)
Child-Pugh score	
A	62 (82.7)
B	12 (16)
C	1 (1.3)
Pathological differentiation	
Low	17 (22.7)
Middle	53 (70.7)
High	5 (6.6)
HBV-DNA	
Positive	72 (96)
Negative	3 (4)
Tumor numbers	
Single	66 (88)
Multiple	9 (12)

[7], while HCC guidelines in American Association for the Study of Liver Diseases (AASLD) suggest that AFP should no longer be an indicator for routine screening in 2010 [8]. The predictive value of imaging tests is only 14% for early diagnosis because of the multiple interfering factors [9]. The specificity for its early diagnosis is limited for failing to accurately differentiate tumors less than 2 cm with other benign occupying lesions [5, 10, 11]. Currently, the overall prognosis of HCC is poor, with an over-all 5-year survival rate of only 5%~9%. Routine surveillance and screening in high risk populations with HCC allows early diagnosis and treatment of HCC [7], thus increasing survival time and post-operative survival rate.

miRNAs are usually located in small chromosomal alterations in tumors or in common chromosomal-breakpoints that are associated with the development of cancer [12-14]. In addition, miRNAs can be silenced by promoting DNA methylation and deacetylation. miRNAs are stable in many body fluids, such as serum, urine and pleural effusion, which can be applied in early diagnosis for different diseases in harsh conditions. Since the abnormal expression of micro-RNA genes miR-15a and miR-16 at 13q14 was first discovered in chronic lymphocytic leukemia [15], a series of studies via tissue, cell, and animal models demonstrate that the miRNAs participate in the generation, classification, development, and prognosis of various cancers like breast cancer, pancreatic cancer, and HCC. According to specific miRNAs expression profile, the diagnosis, classification, prognosis and treatment can be guided [16, 17]. Here, we explored the miRNAs function in the generation, development, transfer and prognosis of HCC, and established a sensitive and accuracy assay for HCC early diagnosis.

Materials and methods

Clinical specimens and ethics statement

A total of 75 HCC patients were recruited from Zhongnan Hospital of Wuhan University from June 2015 to February 2017. The blood samples were collected from HCC patients before operation. All patients were confirmed as HCC by pathology, tumor tissues and paired normal adjacent tissues (NATs) were collected during operation. At the same time, peripheral blood from 80 healthy controls was recruited from Zhongnan Medical Center. Informed consent was obtained from each participant, and the ethics committee of Zhongnan Hospital of Wuhan University.

miRNA isolation

The miRNeasy Serum/Plasma Kit (QIAGEN, German) was used to isolate the total RNA from the serum samples and total RNAs of tissue samples were extracted using the miRNeasy mini Kit (QIAGEN, German) according to the manufacturer's protocols. Quantification of isolated RNA was performed by NanoDrop 2000c (Thermo Scientific, American). The RNA samples were immediately stored at -80°C or converted into cDNA immediately.

miRNA quantification by qRT-PCR

Real-time PCR was performed in a total volume of 10 μL per reaction in triplicate. Briefly, 10

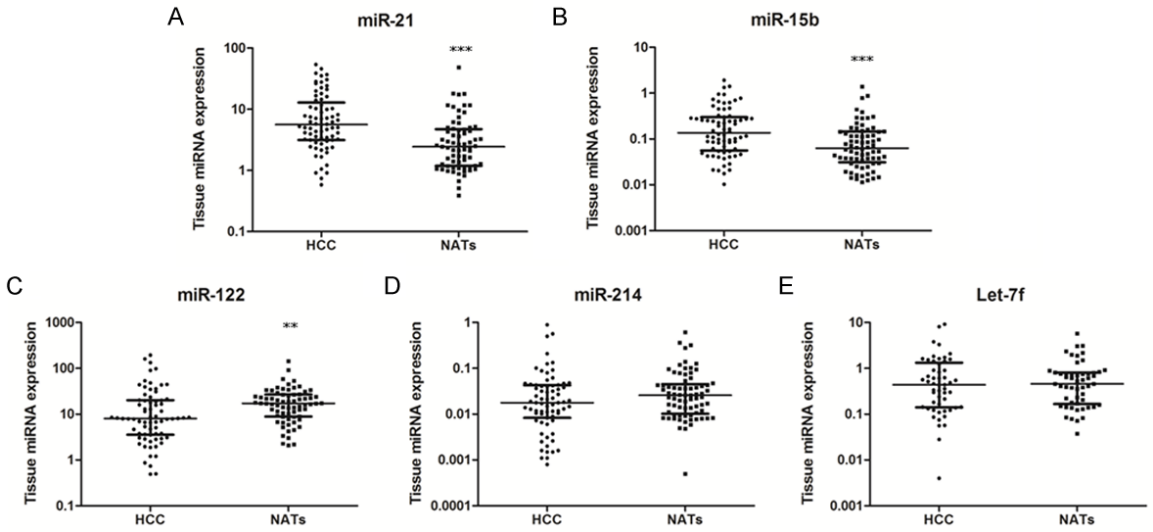

Figure 1. Tissue microRNA expression in HCC tumor tissues and paried NATs. A. MiR-21, HCC tumor tissues (n = 75) vs paried NATs (n = 75). B. MiR-15b, HCC tumor tissues (n = 75) vs paried NATs (n = 75). C. MiR-122, HCC tumor tissues (n = 75) vs paried NATs (n = 75). D. MiR-214, HCC tumor tissues (n = 75) vs paried NATs (n = 75). E. Let-7f, HCC tumor tissues (n = 53) vs NATs (n = 53). Data are shown as median (interquartile range). $^{**}P < 0.01$, $^{***}P < 0.001$.

μL reaction mixture consisted of 5 μL 2 × QuantiTect SYBR Green PCR Master Mix, 1 μL 10 × miScript Primer Assay, 1 μL 10 × miScript Universal Primer, 1 μL cDNA samples and 2 μL RNase-free Water. Quantitative Real time PCR was carried out on BIO-RAD C1000 (America) with a thermal profile beginning at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 30 s, and ending at 4°C. RNU6 was assessed as the reference control for tissue and cell studies and miR-39 was for serum studies. The sequence of miRNAs was obtained from miRBase online and the sequence of U6 was obtained from Genebank database (Gene ID: 26826).

Statistical analysis

Student's t test and Mann-Whitney U test were used to analyze the mean difference for normally distributed data and skewed data, respectively, and the results were presented as mean (SEM) or median (interquartile range). Correlation analysis and multivariable stepwise linear regression analysis was performed to analyze the relationship between the miRNAs and different clinicopathological features. Multivariate logistic regression was applied to analyze the correlation between miRNAs and HCC. The area under the receiver operating characteristic curve (AUC) was used to evaluate the sensitivities and specificities of miRNAs. All the data were analyzed with IBM SPSS Statistics version 18 (IBM, Armonk, USA), and $P < 0.05$ was considered statistically significant.

Results

Patient's characteristics

Among the enrolled HCC patients in this study, the mean age of 75 HCC patients was 52.8 years, and the male:female ratio was 71:4. In enrolled HCC patients, 21 cases had stage A disease, 36 cases had stage B disease, 11 cases had stage C disease and 7 cases had stage D disease based on the Barcelona clinic liver cancer (BCLC) staging system. Of 75 HCC patients, 62 patients had Child-Pugh A liver function, 12 patients had Child-Pugh B liver function and one patients had Child-Pugh C liver function. Patients demographic and clinicopathological information is presented in **Table 1**.

miRNAs expression in the tissue samples of HCC and NATs

Preliminary experiments were performed to examine the expression levels of 5 miRNAs (Let-7f, miR-15b, miR-21, miR-122, and miR-214) in HCC and the paired NATs (**Figure 1**). As shown in **Figure 1A**, miR-21 was significantly overex-

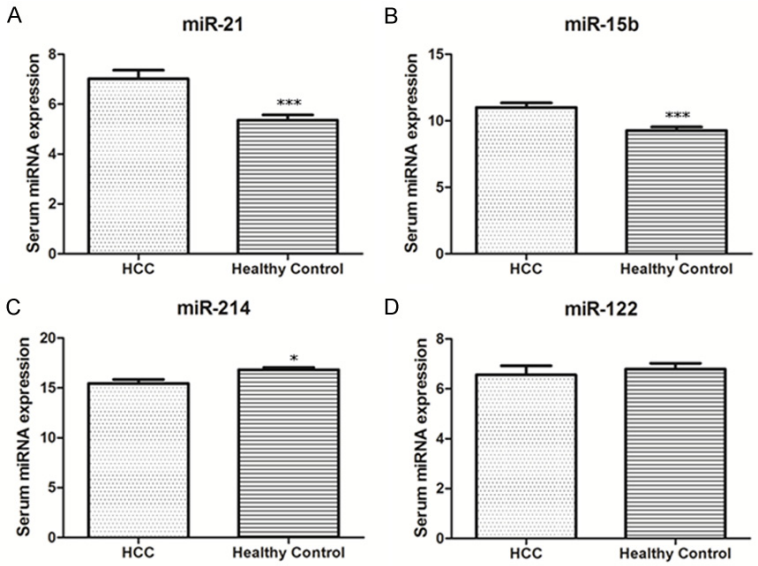

Figure 2. Serum microRNA expression in HCC patients and healthy controls. A. MiR-21, HCC patients (n = 75) vs healthy controls (n = 80). B. MiR-15b, HCC patients (n = 75) vs healthy controls (n = 80). C. MiR-214, HCC patients (n = 75) vs healthy controls (n = 80). D. MiR-122, HCC patients (n = 75) vs healthy controls (n = 80). Data are shown as mean (SE). **P* < 0.05, ****P* < 0.001.

Table 2. Multivariate logistic regression analysis of the miRNAs correlated to HCC

Variables	OR	95% CI	*p*-value
miR-21	1.688	1.121-2.540	0.012
miR-214	0.631	0.453-0.879	0.006
miR-15b	1.736	1.129-2.670	0.012

pressed in HCC tissue samples compared with that in paired NATs (P < 0.001, **Figure 1A**). miR-15b was upregulated in HCC tissues as well (*P* < 0.001, **Figure 1B**). Whereas, miR-122 was significantly down expressed in HCC (*P* = 0.01, **Figure 1C**). However, no significant difference was found between HCC and NATs tissues for the expression of miR-214 (*P* = 0.24, **Figure 1D**) and let-7f (*P* = 0.94, **Figure 1E**). However, the expression of 5 miRNAs in liver tissues had no significant difference among different Barcelona Clinic Liver Cancer (BCLC) Stages, AFP levels or different ages.

miRNAs expression in serum samples of HCC and HC

The serum miRNA profile (miR-15b, miR-21, miR-122, miR-214) was detected in HCC patients and healthy controls. As shown in **Figure 2**, miR-21 and miR-15b were significantly upregulated in HCC serum samples (*P* < 0.001, **Figure 2A**, **2B**) compared with that in HC serum samples. Whereas, miR-214 was significantly downregulated in HCC (*P* = 0.02, **Figure 2C**). However, miR-122 expression had no evident changes between serum samples of HCC and HC (*P* = 0.567, **Figure 2D**).

After adjustment for age, gender, AFP, ALT, AST, HBV, GGT, and Child-Pugh score, we calculated the odds ratio (OR) with 95% confidence intervals (CI) to determine whether miRNAs in serum samples were independently associated with HCC. As no significant difference of miR-122 expression was found between HCC and HC (*P* = 0.568), the other 3 miRNAs (miR-21, miR-214, miR-15b) in the previous study were included for the multivariable logistic regression analysis after multicollinearity diagnosis. We discovered that miR-21 (OR = 1.68, 95% CI: 1.121-2.540, *P* = 0.012), miR-214 (OR = 0.631, 95% CI: 0.453-0.879, *P* = 0.006) and miR-15b (OR = 1.736, 95% CI: 1.129-2.670, *P* = 0.012) were the 3 independent predictors for HCC (**Table 2**).

miRNAs can distinguish HCC from healthy controls

Receiver operating characteristic curve (ROC) analyses were carried out to evaluate the diagnostic value of tissue miRNAs. In our research, for single evaluation, the highest area under the ROC curve (AUC) value was miR-21 (0.738, 95% CI: 0.654-0.817, *P* < 0.001), with a sensitivity of 78.6% and a specificity of 62.1% (**Figure 3A**). The AUC for combined evaluation of miR-(122 + 15b) classifier was 0.863 (95% CI: 0.799-0.927, *P* < 0.001), with a sensitivity of 88.2% and a specificity of 77.5% (**Figure 3A**). In addition, the ROC curve for the combined tissue miR-(21 + 122 + 15b) classifier had a slightly higher AUC (0.885, 95% CI: 0.828-0.943, *P* < 0.001) with a sensitivity of 89.7% and a specificity of 73.1% (**Figure 3A**). The

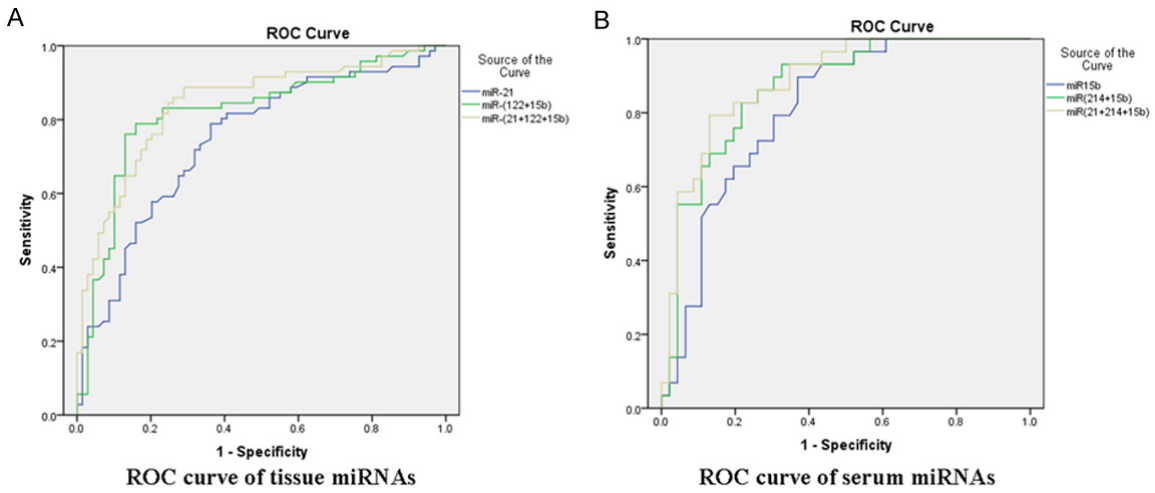

Figure 3. Differential microRNAs discriminated HCC samples from non-HCC samples. A. ROC curves of three different combined classifiers for discriminating HCC tissues (n = 75) from non-HCC tissues (n = 75). B. ROC curves of three different combined classifiers for discriminating HCC patients (n = 75) from healthy controls (n = 80).

Table 3. Respective and joint diagnostic value of 5 miRNAs

Items	AUC	95% CI	SE%	SP%
miR-21	0.738	(0.654, 0.817)***	78.6	62.1
miR-122	0.648	(0.556, 0.739)***	55.4	80.1
miR-15b	0.683	(0.596, 0.769)***	57.7	56.8
miR-(21 + 122)	0.860	(0.799, 0.921)***	78.5	77.1
miR-(21 + 15b)	0.735	(0.653, 0.817)***	64.8	74.5
miR-(122 + 15b)	0.863	(0.799, 0.927)***	88.2	77.5
miR-(21 + 122 + 15b)	0.885	(0.828, 0.943)***	89.7	73.1

Asymptotic test was used to analyze AUC. ***P < 0.001.

Table 4. Respective and joint diagnostic value of serum miRNAs

Items	AUC	95% CI	SE%	SP%
miR-21	0.771	(0.654, 0.888)***	69.1	78.3
miR-214	0.733	(0.610, 0.856)**	79.3	69.6
miR-15b	0.809	(0.712, 0.905)***	89.7	63.1
miR-(21 + 214)	0.828	(0.730, 0.925)***	93.1	59.7
miR-(21 + 15b)	0.830	(0.730, 0.929)***	72.4	87.0
miR-(214 + 15b)	0.868	(0.787, 0.949)***	89.1	87.0
miR-(21 + 214 + 15b)	0.887	(0.813, 0.961)***	80.3	87.0

Asymptotic test was used to analyze AUC. **P < 0.01; ***P < 0.001.

remaining ROC curve results of tissue microRNAs are shown in **Table 3**.

In addition, we discovered that the serum microRNAs had a higher efficacy in discriminating HCC patients from healthy controls. For the single microRNA, miR-15b had the highest AUC (0.809, 95% CI: 0.712-0.905, P < 0.001), with a sensitivity of 89.7% and specificity of 63.1% (**Figure 3B**). Notably, the ROC curve analysis for the combined serum miR-(214 + 15b) showed a higher AUC (0.868, 95% CI: 0.787-0.949, P < 0.001) with a sensitivity of 89.1% and a specificity of 73.1% (**Figure 3B**). Furthermore, the ROC curve for the combined serum miR-(21 + 214 + 15b) classifier resulted in a better AUC (0.887, 95% CI: 0.813-0.961, P < 0.001) with a sensitivity of 80.3% and a specificity of 87.0% (**Figure 3B**). The remaining ROC curve results of serum microRNAs are shown in **Table 4**.

Discussion

MicroRNAs play important ro-les in all biological processes via post-transcriptional regulation of protein-coding genes, which participate cell proliferation, differentiation, survival and carcinogenesis [18]. Accumulated evidence suggests that miR-21 could be novel potential biomarker for tumor diagnosis [19]. By comparing the statistics from 17 articles about mi-21 as tumor diagnosis marker, Wang Y found that the sensitivity and specificity of miR-21 on tumor diagnosis is 75.7% and 79.3% respectively [20]. Until now, aberrant miR-21 has be-

en found in various tumor cases including lung cancers, colorectal carcinomas, breast cancers, gastric carcinomas, pancreatic cancers, esophagus cancers, osteosarcoma and HCC. Over expression of SOCS6 could significantly decrease cell growth rate and invasion capability. miR-21 might play an important role in modulating cell growth and invasion of HCC cells by simultaneously targeting SOCS6 gene and modulating its expression at protein level [21].

miR-122 is a highly abundant hepatocyte-specific miRNA which accounts for 70% of the total adult human liver miRNA population [22]. Recent studies show that miR-122 was a tumor suppressor in HBV-transformed HCC by inhibiting cell migration and proliferation via down-regulating the expression of multiple target genes which are associated with HCC pathogenesis. It has been demonstrated that decreased miR-122 levels are associated with poorer overall survival of HCC patients [23]. Other findings also suggest that miR-122 levels are closely linked to the metastasis, invasion, and prognosis of HCC. In our research, a significant down regulation of miR-122 levels was observed in HCC compared to normal liver tissue which was consistent with the result in the studies by Wang et al. [24] and Huang et al. [25]. A study on hepatitis B patients [26] suggested that cancer-induced hepatocyte damage could release the abundant intracellular miR-122 into the circulation. Also, other studies found that the release of circulating miR-122 possibly mirrors acute liver injury [27]. This might explain the results that there exists an up-regulation of circulating miR-122 despite the decreased levels of miR-122 in HCC tissues. Recently, an investigation shows that the inhibition of miR-122 can cause a small but significant increase in its complementary strand miR-122*, which is liver specific and acts as a tumor suppressor by targeting and inhibiting Mdm2, a prominent negative regulator of the tumor suppressor p53 thus causing the elevated p53 levels [28]. In addition, our research found that the miR-122 levels in HCC tissues are associated with the differentiation and pathological stages of the disease, which indicate that there might be a correlation between miR-122 and the disease prognosis.

Accumulated evidence suggests that there exists a significant down-regulation of miR-214 in various cancer tissues including breast cancer, colon cancer, pancreatic cancer, and prostate cancer [29, 30]. Also a specific change of miR-214 was found in serum and urine. Studies also found its multiple functions in tumor metastasis, invasion, proliferation, and apoptosis. All the researches indicate that miR-214 is often dysregulated in various cancers, which governs both tumorigenic and tumor suppressive functions. The stable existence of miR-214 in urine and serum samples indicates a relationship between miR-214 and tumor development. In our research, a significant reduction of miR-214 level in tissues and serum samples of HCC was observed. Moreover, serum miR-214 was found to be negatively correlated with HCC in our study, which demonstrates that miR-214 might be a negative predictor for HCC. Our multivariable analysis only detected a negative correlation between tissue miR-214 and cirrhosis. Altogether, the results imply that there might be a potential relationship between miR-214 and liver functions. However, the role of miR-214 on tumorigenesis and other surrogates in liver function needs more investigation [31].

The aberrant expression of miR-15b has been observed in various cancer including breast cancer, glioma, lung adenoma, colorectal cancer, and pancreatic cancer [32]. Some studies imply that miR-15b may exert its function as tumor suppressor in HCC cells targeting on associated genes. In our research, we observed an up-regulation of miR-15b in tumor tissues, also, a positive relationship was found between serum miR-15b and HCC development. The results indicated a positive correlation between miR-15b and HCC which was inconsistent with the conclusion that miR-15b might function as tumor suppressor gene in HCC patients [32, 33]. Moreover, we observed a significant correlation between miR-15b and HCC pathological stages. Multivariate analysis detected a negative correlation between tumor numbers and miR-15b levels in tissues. The role of miR-15b has not been explicit yet, while the detailed mechanism should be further studied.

The aberrant expression of Let-7f is widely found in breast cancer, medulloblastoma, aggressive papillary thyroid carcinoma, gastric cancer and has been demonstrated to correlate significantly with tumor invasion and me-

tastasis [34]. Enhanced expression of Let-7f has been assumed to suppress glioma cell proliferation. Some researchers report a significant down-regulation of let-7f in HCC tissues. However, in our study, no differential expression of let-7f was found in HCC tissues compared to NATs. Also, up-regulated serum let-7f was found in patients with a tumor more than 5 cm in diameter and with early recurrence [35] which indicates that the detailed mechanism of let-7f regulation should be further researched.

Similar with previous studies, we also showed that microRNAs could discriminate HCC patients from healthy controls, and promise to be an effective biomarker for HCC diagnosis. Thirteen articles on the early diagnosis by miRNA provide evidence that the mean data of the sensitivity, specificity, and AUC of miRNAs are 83.73%, 84.77% and 0.885 respectively. Also, great differences on specificity and sensitivity have been shown among various miRNAs.

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Disclosure of conflict of interest

None.

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