

Original Article

Profiles of serum miR-99a, let-7c and miR-125b in hepatitis B virus (HBV)-associated chronic hepatitis, liver cirrhosis and hepatocellular carcinoma

Fang Xiong^{1*}, Hong Ma^{2*}, Yachao Qu¹, Fei Wen¹, Xuli Bao¹, Dazheng Han², Jun Lu¹

¹Hepatology and Cancer Biotherapy Ward, Beijing Youan Hospital, Capital Medical University, Beijing, People's Republic of China; ²The First Affiliated Hospital of Henan University, Kaifeng, People's Republic of China. *Equal contributors.

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Abstract: Serum miRNAs have been found to be potential biomarkers for multiple diseases, including hepatocellular carcinoma (HCC) and other types of cancers. The aim is to identify serum miRNAs as novel non-invasive markers for the early detection of HCC. The serum levels of miR-99a, let-7c and miR-125b were measured in 30 chronic hepatitis B (CHB), 30 hepatitis B virus (HBV)-associated liver cirrhosis (HBV-LC) and 32 HBV-associated hepatocellular carcinoma (HBV-HCC) patients as well as 30 healthy controls using probe-based stem-loop quantitative reverse-transcriptase PCR (qPCR). Serum miR-99a levels were significantly elevated in the CHB ($P<0.001$), HBV-LC ($P=0.014$) and HBV-HCC groups ($P<0.001$) compared with the control group. Serum miR-125b levels were also significantly elevated in the CHB ($P<0.001$), HBV-LC ($P=0.0028$) and HBV-HCC groups ($P<0.001$) compared with the control group. The serum level of miR-99a was significantly different between the HBV-LC and HBV-HCC groups ($P=0.008$). Receiver-operator characteristic (ROC) curve analyses suggested that these serum miRNAs might be useful markers for discriminating patients with HBV-HCC from healthy controls. MiR-99a could be used as a novel non-invasive biomarker of HBV-positive HCC at early stage.

Keywords: MiR-99a, microRNA, hepatitis B, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is the second leading cause of cancer mortality [1]. In China, hepatitis B virus (HBV) chronic infection and related cirrhosis are the most important risk factors for HCC. The 5-year survival rate of HCC is low (less than 10%) because HCC is often diagnosed in advanced stages [2]. Currently, the most commonly used circulating marker for HCC screening is alpha-fetoprotein (AFP), but its value in the diagnosis of this disease is very limited, with only 40% to 65% sensitivity and 76% to 96% specificity [3]. Therefore, there is an urgent need for the identification of novel non-invasive markers for the early detection of HCC to facilitate effective treatment and a good prognosis.

MicroRNAs (miRNA) are a class of small non-coding RNA molecules that regulate gene

expression by binding the 3'untranslated region (3'UTR) of the mRNAs of their target genes, resulting in mRNA cleavage or translational repression [4]. Approximately 30% of human genes are regulated by miRNAs, suggesting that miRNAs play pivotal roles in physiological and pathological processes, including human carcinogenesis. Aberrant miRNA expression is involved in a large variety of neoplasms, including HCC [5]. A significant number of miRNA genes are closely adjacent to each other in miRNA polycistrons. The NCBI/hg18 human genome assembly features 1873 miRNA gene sequences, of which 42% are organized into polycistrons of two or more genes [6]. Numerous studies suggest that the miRNA genes in miRNA clusters act together to achieve a regulatory net outcome on the cell [7]. The miR-99a/let-7c/miR125b-2 cluster is a phylogenetically highly conserved tricistron of miRNA genes on chromosome 21. The genes of the miR-99a/let-7c/miR125b-2 tricistron act together and their

Table 1. Summary of clinical characteristics of CHB, HBV-LC, and HBV-HCC patients and healthy subjects

		Group III: HBV-associated HCC (32)	Group II: Cirrhosis (30)	Group I; Chronic HBV (30)	Group IV: Normal (30)	P- value
Age (years)	Mean $\pm$ SD	49.69 $\pm$ 11.53	51.57 $\pm$ 9.72	34.2 $\pm$ 10.89	37.26 $\pm$ 10.79	<0.001
Sex (n)	Male	26 (81.3)	21 (70)	19 (63.7)	21 (70)	0.47
	Female	6 (18.7)	9 (30)	11 (36.3)	9 (30)	
Alcohol	Yes	10 (31.3)	11 (36.7)	4 (13.3)		0.103
	No	19 (59.4)	18 (60)	16 (53.3)		
	Unknown	3 (9.3)	1 (3.3)	10 (33.3)		
ALT (n)	Elevated	14 (43.7)	17 (56.7)	15 (50)		0.471
	Normal	18 (56.3)	12 (40)	13 (43.3)		
	Unknown	0	1 (3.3)	2 (6.7)		
Total bilirubin	Elevated	8 (25)	17 (56.7)	8 (26.7)		0.012
	Normal	24 (75)	12 (40)	19 (63.3)		
	Unknown	0	1 (3.3)	3 (10)		
Lg (1+viral load)	Median (min, max)	0 (0-6.21)	2.63 (0-7.62)	7 (0,8)		<0.001
AFP	Median (min, max)	116.29 (3.1, 17342)	5.69 (1.13, 222.3)	2.25 (0.66, 18.07)		<0.001
(S) Grade (n)	1			22 (73.3)		
	2			7 (23.3)		
	3					
	4					
	Unknown			1 (3.3)		
(G) Stage (n)	0			15 (50)		
	1			12 (40)		
	2			2 (6.7)		
	3					
	4					
	Unknown			1 (3.3)		
HBsAg+	Positive	32 (100)	30 (100)	30 (100)	0 (0)	
Tumor size (cm)	<5	24 (75)				
	$\geq$ 5	8 (25)				
Tumor differentiation	Well	4 (12.5)				
	Moderate	22 (68.7)				
	Poor	6 (18.8)				
Tumor s stage	Stage I	22 (68.7)				
	Stage II	4 (12.5)				
	Stage III-IV	6 (18.8)				
Tumor relapse	<1 year	11 (34)				
	>1 year	5 (16)				
	No relapse	16 (50)				

deregulation contribute to the development of prostate cancer and acute megakaryoblastic leukemia [8, 9]. MiR-99a expression is markedly decreased in HCC tissue, and this miRNA serves as a prognostic predictor [10]. let-7c miRNA is significantly reduced in HCC tissues compared with corresponding normal adjacent tumor tissues, and a correlation between the downregulation of let-7c and poor tissue differ-

entiation in HCC has also been shown [11]. miR-125b expression is suppressed in primary HCC tissues and associated with patient survival [12]. Considering all of these previous results, the miR-99a/let-7c/miR125b-2 tricistron is a tumor suppressor for HCC.

In 2008, an exciting discovery was made: human serum/plasma contains a large amount

of stable miRNAs and that unique expression profiles of serum miRNAs can serve as markers for various diseases [13]. Similarly, miRNAs in serum or plasma are considered promising novel biomarkers for cancer diagnosis and prognosis [14]. We hypothesized that the deregulation of the miR-99a/let-7c/miR125b-2 cluster in HCC could be identified in the serum of HCC patients and might serve as a diagnostic or prognostic marker.

The development of HCC is a multi-step process, and it is unknown whether serum miR-99a, let-7c or miR-125b could be used as markers to differentiate the diverse stages of HBV-associated liver disease from each other. Therefore, in the present study, we obtained serum miR-99a, let-7c and miR-125b expression profiles from the sera of HBV-associated chronic HBV (CHB), HBV-associated liver cirrhosis (HBV-LC) and HBV-HCC patients to determine the diagnostic potential of these markers.

Materials and methods

Study subjects and clinical characteristics

The study protocol was approved by the Ethics Committee of Beijing YouAn Hospital, Capital Medical University and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from the participants for the use of their blood, tissue samples and clinical records in this study. Between January 2012 and October 2012, we gathered a total of 122 serum samples consisted of 32 HBV-HCC patients, 30 HBV-LC patients, 30 CHB patients and 30 healthy subjects. The patient characteristics are summarized in **Table 1**. All patients were positive for hepatitis B surface antigen (HBsAg) and did not have any other liver diseases, including chronic hepatitis C (CHC), alcoholic liver disease, autoimmune liver disease or metabolic liver disease, based on clinical reports. HBV-LC and HBV-HCC was diagnosed histopathologically. The degree of inflammation for all patients was between G1 and G2. The fibrosis stages of the CHB patients were between S0 and S2. The fibrosis stages of the HBV-LC and HBV-HCC patients were between S3 and S4. The blood samples from the HBV-HCC patients were obtained before surgical resection was performed. All data for all subjects were obtained from medical records,

pathology reports and personal interviews with the subjects. The collected data included age, gender, serum albumin (ALB) level, total bilirubin (T-Bil) level, alanine aminotransferase (ALT) level, prothrombin time (PT), HBV DNA viral load, AFP level, tumor number and size, tumor differentiation, tumor stage, relapse time and time of death. The clinical stage of HBV-HCC was evaluated based on the 7th edition of AJCC TNM (tumor, node, and metastasis) classification system. Child-Pugh scoring was performed to categorize the LC and HCC patients as Child-Pugh grade A, B or C.

Serum preparation

Ten milliliters of peripheral blood was directly collected into serum tubes from each individual at the time of liver biopsy or before surgery. Blood was processed for plasma isolation within two hours of collection using the protocol described. The tubes were initially centrifuged at 1500 g for 10 min. The serum was then aliquoted and centrifuged again at 13,000 g for 15 min at 4°C to completely remove cell debris and any remaining cells. The supernatant was then transferred to a new 2.0-ml tube without disturbing the pelleted debris. Any samples with signs of hemolysis were excluded from the study. The serum samples were then stored at -80°C until further use.

RNA extraction and reverse transcription

Total RNA was extracted from 200 µl of serum using a QuantoBio Total RNA Isolation Kit (QuantoBio Co., Beijing, China) following the manufacturer's protocol. Briefly, 200 µl of lysis buffer and 40 µl of lysis enhancer solution were added to each sample, and the samples were vortexed for 15 sec. For the normalization of sample-to-sample variation during RNA isolation and as an internal control, 25 fmol of synthetic *Caenorhabditis elegans* miRNA-39 (cel-miR-39-3p) was added to each lysed sample. This step was followed by the addition of 440 µl of acidified phenol: chloroform and vigorous shaking for 30 sec. The samples were centrifuged at 16,000 g in a microcentrifuge at 4°C for 10 min to separate the aqueous phase (upper phase) from the organic phase (lower phase). The aqueous phase was then transferred to a new RNase-free microcentrifuge DNA LoBind tube (Eppendorf, UK). Ethanol was added to the aqueous phase to a final concen-

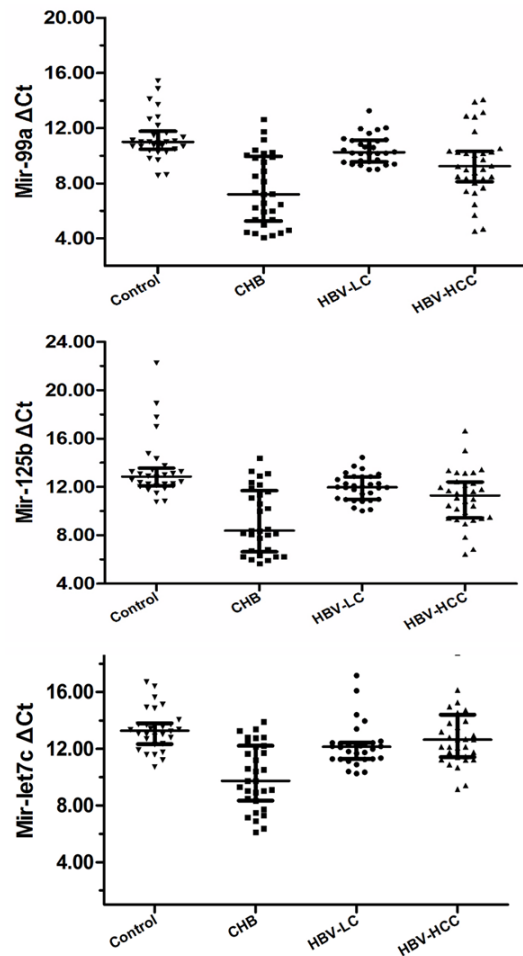


Figure 1. Serum miR-99a, let-7c and miR-125b levels in healthy subjects, patients with CHB, HBV-LC and HBV-HCC. The lines represent the range, median and quartiles of relative miRNA expressions (ΔC_t values) measured by qPCR.

tration of 30%, and this mixture was vigorously shaken for 15 sec. Then, the 650- μ l mixture was centrifuged at 10,000 g at room temperature for 30 sec to pellet the RNA. After the washing steps, the total RNA, including miRNAs, was resuspended in 50 μ l RNase-free water and stored at -80°C until further use. The concentration and purity of the total RNA was determined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

cDNA was reverse transcribed from total RNA via the polyadenylation method. Briefly, 1 ng of the total RNA template was mixed with 2 μ l $10\times$ buffer, 2 μ l dATP (10 mM), 0.5 μ l poly (A) polymerase (NEB), 0.5 μ l RNase inhibitor (Promega)

and RNase-free water (Promega) to a final volume of 20 μ l, and the solution was incubated at 37°C for 1 h. Then, 1 μ l of 0.5 $\mu\text{g}/\mu\text{l}$ RT primer was added to the solution. The reaction was incubated at 70°C for 5 min followed by immediate incubation on ice for at least 2 min to disrupt the secondary structures of the RNA and the primer. In the final step, the 20- μ l reaction mixture described above was mixed with 4 μ l $5\times$ buffer, 1 μ l dNTP (10 mM) (Sigma), 0.5 μ l M-MLV reverse transcriptase (Promega), 0.5 μ l RNase inhibitor (Promega), 10 μ l A-Plus reaction mix, and 4 μ l RNase free water (Promega) and was incubated at 42°C for 1 h. The undiluted cDNA samples were then stored at -20°C in 0.2-ml DNA LoBind microcentrifuge tubes (Eppendorf, UK) until further use.

Real-time quantitative PCR (qPCR)

Real-time PCR was performed in a total reaction volume of 20 μ l containing of 1 μ l of diluted cDNA (1:100), 10.4 μ l $2\times$ qPCR mix with ROX, 2 μ l $10\times$ universal reverse primers, 2 μ l $10\times$ microRNA-specific forward primers (QuantoBio, China) and 4.6 μ l PCR grade RNase-free water. The PCR reactions were run on an ABI 7900 HT real-time PCR system (Applied Biosystems, USA) with the following cycle conditions: 95°C for 5 min; 40 cycles of 95°C for 30 sec, 60°C for 1 min and a dissociation stage of 95°C for 15 sec; and a final step of 60°C for 1 min. All reactions were performed in duplicate. The cycle threshold (CT) was defined as the number of PCR cycles required for the fluorescent signal to cross the threshold. Ct values greater than 35 were defined as undetectable. DCt is the mean (Ct of target miRNA)-(Ct of internal control).

Statistical analysis

The statistical analysis was performed using the SPSS 20.0 software (SPSS, Inc.). Differences among groups were evaluated using the Pearson's Chi-squared and Kruskal-Wallis with post-hoc testing. Mann-Whitney U test was performed to compare differences in miRNA levels between patients and controls. Spearman's non-parametric rank test was used to test the correlations between the miRNA expression levels and the examined clinical parameters. Receivers operating characteristic curves (ROC) were generated to classify patients into different groups and for the evaluation of the

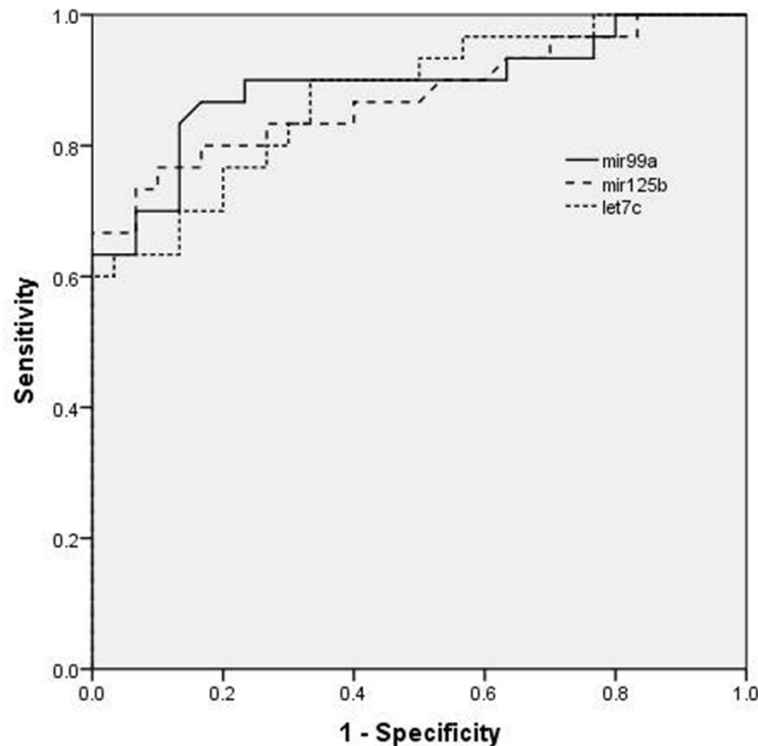


Figure 2. ROC curve analysis of serum miR-99a, let-7c and miR-125b levels for discriminating HBV-HCC patients from healthy subjects.

diagnostic potential of serum miRNAs via calculation of the area under the ROC curve (AUC), which was used to determine sensitivity and specificity according to standard formulas. *P*-values were two-sided. *P*-values less than 0.05 were considered statistically significant.

Results

Characteristics of study subjects

The selected characteristics of the study subjects are summarized in **Table 1**. No significant differences in sex distribution were noted among the four study groups. However, although the age distribution did not significantly differ between the HBV-HCC and HBV-LC groups, chronic hepatitis patients and healthy controls were younger than the HBV-HCC and HBV-LC patients (both $P < 0.001$). All patients were HBsAg positive. The other listed data include serum ALB levels, T-Bil levels, ALT levels, HBV DNA viral load, AFP levels, tumor number and size, tumor differentiation, tumor stage and tumor relapse (data not shown).

Expression profile of serum miR-99a, let-7c and miR-125b

MiR-99a, let-7c and miR-125b levels were measured in the sera of patients and healthy controls using qPCR. All data are summarized in **Figure 1**. The DCt value was negatively correlated with the serum level of the target miRNAs. MiR-99a serum levels were significantly increased in the CHB ($P < 0.001$), HBV-LC ($P = 0.014$) and HBV-HCC groups ($P < 0.001$) compared with the control group. Furthermore, the serum level of miR-99a was significantly increased in the CHB group compared with the HBV-LC ($P < 0.001$) and HBV-HCC groups ($P = 0.009$). The serum level of miR-99a was significantly different between the HBV-LC and HBV-HCC groups ($P = 0.008$). Similarly, the miR-125b serum levels

were significantly increased in the CHB ($P < 0.001$), HBV-LC ($P = 0.003$) and HBV-HCC groups ($P < 0.001$) compared with the control group. MiR-125b serum levels in the CHB group were significantly increased compared with the HBV-LC ($P < 0.001$) and HBV-HCC groups ($P = 0.006$). The serum level of miR-125b was almost significantly different between the HBV-LC and HBV-HCC groups ($P = 0.07$). However, the differences in the expression of let-7c among the four groups differed from those of the other miRNAs. No difference in let-7c expression was noted among the HBV-HCC and control groups ($P = 0.165$). Let-7c expression was significantly increased in the CHB ($P < 0.001$) and HBV-LC groups ($P = 0.001$) compared with the control group. Let-7c serum levels in the CHB group were significantly increased compared with the HBV-LC group ($P < 0.001$).

In the CHB group, no correlation was observed between miR-99a, let-7c or miR-125b levels and ALT, aspartate aminotransferase (AST), or T-Bil levels or viral DNA load. The correlations between serum miR-99a, let-7c and miR-125b levels and the clinicopathological features of

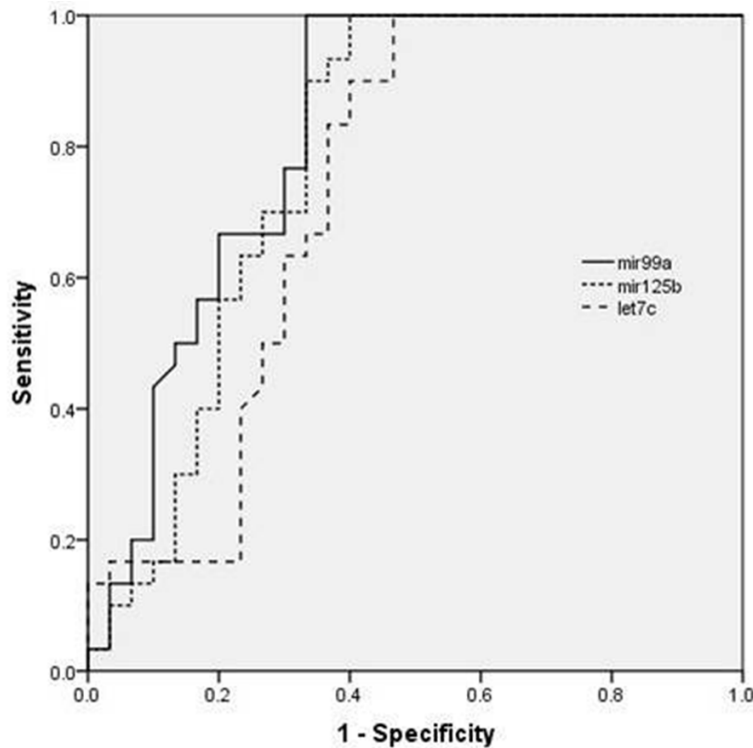


Figure 3. ROC curve analysis of serum miR-99a, let-7c and miR-125b levels for discriminating HBV-LC patients from HBV-HCC patients.

HCC patients were investigated. No correlation between miR-99a, let-7c or miR-125b expression levels and any of the examined clinicopathological features, including tumor size, grade, stage and relapse time, was noted.

The diagnostic potential of serum miR-99a, let-7c and miR-125b levels

To evaluate whether serum miR-99a, let-7c or miR-125b levels could be used as diagnostic markers for CHB, HBV-LC or HBV-HCC, ROC curve analyses were performed. As shown in **Figure 2**, miR-99a, let-7c and miR-125b serum levels were potential markers for discriminating CHB patients from healthy controls, with ROC curve areas of 0.892 (95% confidence interval [CI]: 0.805-0.979), 0.874 (95% CI: 0.788-0.961), and 0.876 (95% CI: 0.784-0.967), respectively. Using the cut-off values of 10.21 (for miR-99a), 11.98 (for let-7c), and 12.25 (for miR-125b), the sensitivities and specificities of these markers were 83.3% and 86.7%, 76.7% and 80.0%, and 80.0% and 83.3%, respectively. For the discrimination of HBV-LC patients from healthy controls, miR-99a, let-7c and miR-

125b exhibited AUC values of 0.684 (95% CI: 0.548-0.821), 0.746 (95% CI: 0.615-0.877) and 0.725 (95% CI: 0.597-0.853), respectively. For the discrimination of HBV-HCC patients from healthy controls, miR-99a and miR-125b exhibited AUC values of 0.774 (95% CI: 0.646-0.902) and 0.770 (95% CI: 0.648-0.892), respectively. Let-7c was unable to differentiate HCC patients from healthy controls.

Next, we investigated whether serum miR-99a, let-7c or miR-125b levels could differentiate among CHB, HBV-LC and HBV-HCC patients. For discrimination between CHB and HBV-LC patients, the ROC curve areas for miR-99a, let-7c and miR-125b were 0.822 (95% CI: 0.710-0.934), 0.733 (95% CI: 0.598-0.868), and 0.786 (95% CI: 0.662-0.909), respectively. For the discrimination

between CHB and HBV-HCC patients, the ROC curve areas for miR-99a, let-7c and miR-125b were 0.694 (95% CI: 0.561-0.827), 0.792 (95% CI: 0.680-0.903), and 0.703 (95% CI: 0.570-0.838), respectively. Using the cut-off values of 7.35 (for miR-99a), and 8.72 (for miR-125b), the sensitivities and specificities of these markers were 84.4% and 56.7%, and 90.6% and 56.7%, respectively. For the discrimination between HBV-LC and HBV-HCC patients (**Figure 3**), the ROC curve for miR-99a was 0.696 (95% CI: 0.559-0.834) with a sensitivity/specificity ratio of 96.7/56.3%, and miR-125b and let-7c were unable to differentiate between these two groups of patients. Then, the predicted probability of being diagnosed with HBV-HCC from a stepwise logistic regression model based on AFP and miR-99a was used to construct the ROC curve (**Figure 4**). The AFP alone yielded an AUC of 0.791 (95% CI: 0.681-0.901) with 53.1% sensitivity and 96.6% specificity when differentiating HBV-LC from HBV-HCC. The AUC of serum miR-99a combined with AFP was also calculated and found to be 0.780 (95% CI: 0.656-0.904) with a sensitivity/specificity ratio of 71.9/82.8%.

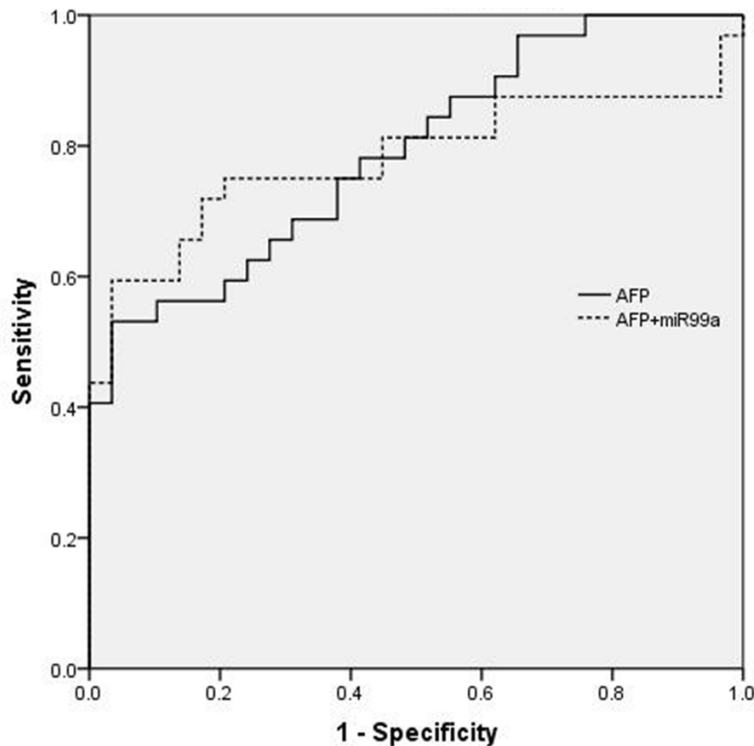


Figure 4. ROC curve analysis of AFP alone, the combination of serum miR-99a and AFP levels for discriminating HBV-LC patients from HBV-HCC patients.

Discussion

In the present study, serum miR-99a and miR-125b levels were significantly elevated in patients with HCC, patients with CHB and patients with HBV-HCC compared to healthy controls. Let-7c was significantly elevated in patients with chronic type B hepatitis and patients with HBV-LC compared with healthy controls. In addition, the serum levels of miR-99a, let-7c and miR-125b were significantly increased in patients with CHB compared with patients with HCC or HBV-LC. Serum miR-99a and miR-125b levels exhibited potential diagnostic value for HCC.

miR-99a is one of the abundantly expressed miRNAs in normal human liver [15], but it is deregulated in a high proportion of primary HCC tissues relative to their matched noncancerous tissues [10, 16, 17]. Furthermore, the downregulation of miR-99a was also observed in other types of cancers, such as oral cancer [18], prostate cancer [19] and lung adenocarcinoma [20], suggesting that miR-99a acts as a

tumor suppressor. In our study, serum miR-99a was significantly elevated in patients with HCC, patients with CHB and patients with HBV-LC compared with healthy controls. Furthermore, serum miR-99a was significantly elevated in patients with HCC compared with patients with HBV-LC. When using the combination of AFP and serum miR-99a for differentiating HBV-LC and HCC, the sensitivities and specificities are more satisfying. In addition, our finding of increased serum miR-99a levels in patients with CHB is consistent with the conclusion of Maurizia [21].

miR-125b is an interesting microRNA that has opposing roles in cancer [22]. In some tumor types, such as colon cancer, prostate cancer and hematopoietic tumors, miR-125b is upregulated and displays oncogenic potential; however, in other

tumor types, including mammary tumors, ovarian carcinoma and hepatocellular carcinoma, miR-125b is heavily downregulated and has a tumor suppressive effect. Several studies have consistently demonstrated that miR-125b expression is reduced in hepatocellular carcinoma and that miR-125b acts as a tumor suppressor in that tumor type [23-25]. In our study, serum miR-125b levels were significantly elevated in patients with HCC, patients with CHB and patients with HBV-LC compared with healthy controls, which was consistent with the findings of Burcu's study [26]. Furthermore, increased miR-125b expression in hepatocellular carcinoma tissues was previously correlated with good survival in HCC patients. However, we did not examine the correlation between serum miR-125b levels and postoperative relapse of HCC.

let-7c is a member of the let-7 family, and let-7 family members are downregulated in lung cancer, breast cancer, acute lymphoblastic leukemia, prostate cancer and HCC [27]. One stu-

dy found that let-7c levels were significantly reduced in HCC tissues compared with corresponding normal adjacent tumor tissues, and a correlation was noted between the downregulation of let-7c and poor tissue differentiation in HCC [11]. In our study, let-7c was significantly elevated in patients with CHB and patients with HBV-associated liver cirrhosis compared with healthy controls. No correlation between serum let-7c levels and HCC grade was noted.

The miR-99a/let-7c/miR125b cluster is potentially suppressive in hepatocellular carcinoma, as all three of these miRNAs were downregulated in HCC. MiR-99a and miR125b serum levels were significantly elevated in HCC patients; however, serum let-7c levels were not altered in HCC patients. This phenomenon suggests that serum miRNAs are not directly associated with changes that occur in tumor tissues. Serum miRNAs are not only released from tumor cells but are also directly released into the bloodstream by blood cells or tissue cells affected by disease [28]. MiR-99a, let-7c and miR-125b serum levels were significantly increased in CHB patients compared with HCC or HBV-LC patients as well as healthy controls. This finding supported the hypothesis that damage to hepatocytes caused by inflammation due to viral infection or cancer results in the release of significant amounts of miR-99a, let-7c and miR-125b into the circulation. However, miR-99a, let-7c and miR-125b serum levels were not correlated with ALT or AST levels or HBV DNA load. There is a plan to pursue other studies to explore the change of serum miR-125b and miR-99a in CHB, HBV-HCC patients after antiviral treatment or to observe the miRNA change during disease progression from CHB to HBV-HCC. Therefore, determining the underlying mechanisms of miR-99a, let-7c and miR-125b in HCC requires further exploration.

In conclusion, we identified two miRNAs, miR-99a and miR-125b, that can be used as non-invasive biomarkers of HCC during the early stages of liver disease. These miRNAs are potential early diagnostic biomarkers of HBV-related HCC, even during the chronic hepatitis B stage of liver disease. These two miRNAs should be explored in additional larger CHB, HBV-LB and HBV-HCC patient populations in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Lu, Hepatology and Cancer Biotherapy Ward, Beijing Youan Hospital, Capital Medical University, Beijing 100069, People's Republic of China. Tel: 86-10-63291028; Fax: 86-10-63291028; E-mail: lujun98@ccmu.edu.cn

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