

## Original Article

# Association of serum microRNA-125b and HBV-related hepatocellular carcinoma in Chinese Han patients

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**Abstract:** Objective: microRNA-125b has been reported to be associated with microvascular invasion in patients with hepatocellular carcinoma. The aim of this study was to characterize the correlation between microRNA-125b and hepatocellular carcinoma (HCC) susceptibility and occurrence in patients with chronic Hepatitis B virus (HBV) infection in the Chinese Han population. Methods: Real-time quantitative RT-PCR was used to quantify serum microRNA-125b levels in 100 patients with HCC, 100 with HBV related liver cirrhosis (LC), 100 with chronic hepatitis (CHB), and 100 healthy controls (HC). Mann-Whitney U test was utilized to detect differences in serum microRNA-125b levels in patients with HCC vs. LC, HCC vs. CHB, and HCC vs. HC. The potential diagnostic significance of microRNA-125b was investigated using receiver-operator characteristic (ROC) curves. Results: We found that HCC patients had significantly lower levels of serum microRNA-125b than patients with CHB ( $P < 0.05$ ) and LC ( $P < 0.05$ ) as well as healthy controls. Receiver-operator characteristic (ROC) curve analyses implied that microRNA-125b might be a helpful marker for recognizing patients with HBV-related HCC from healthy controls (Area under curve (AUC) = 0.94), chronic hepatitis (AUC = 0.80), and HBV-related liver cirrhosis (AUC = 0.91). Conclusion: Serum microRNA-125b was elevated in Chinese Han patients with HBV-related HCC and could be a potential biomarker for HBV-related HCC diagnosis.

**Keywords:** microRNA-125b, HBV, hepatocellular carcinoma

## Introduction

Hepatitis B virus (HBV) infection has caused a serious public health problem. There have been about 100 million patients with chronic HBV infection in China which is strongly correlated with hepatocellular carcinoma (HCC), one of the most frequently seen cancers or the most frequent cause of cancer-related deaths around the world [1-3]. It is vital that prognostic assessment of HCC is improved through discovery of specific biomarkers for accurate stratification and classification.

Until now, diagnosis of HCC has mostly been based on imaging techniques such as Magnetic Resonance (MRI), Computed Tomography (CT) and ultrasonography, and laboratory examinations such as serum  $\alpha$ -feto protein and/or liver biopsy [4]. However, all of the previous diagnostic methods have obvious limitations such as higher costs, poor reproducibility, and availability [5]. Recently, microRNAs

(small single-stranded non-coding RNAs with 20-25 nucleotides) in tissue or serum, which could downregulate endogenous messenger RNA (mRNA) transcripts, have been developed as biomarkers [6, 7]. microRNAs in HCC tissue were reported to be correlated with survival of patients [8-10]. Lack of microRNA-122 could enhance HCC metastatic properties [8]. mRNA let-7g could cooperate with collagen type I  $\alpha$ 2 to inhibit HCC cell migration [9]. microRNA-29 could regulate HCC cells apoptosis [10].

microRNA-125b has been reported to be associated with microvascular invasion in hepatocellular carcinomas patients [11]. However, there are limited studies focusing on correlation between serum microRNA-125b and HBV-related HCC or its diagnostic significance. The aim of this study was to characterize the correlation between microRNA-125b and HCC susceptibility and development in patients with chronic HBV infection.

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**Table 1.** Characteristics of the study population

Variables	HC (n = 100)	HCC (n = 100)	LC (n = 100)	CHB (n = 100)	P-value		
					HCC vs. HC	HCC vs. LC	HCC vs. CHB
Male/Female	74/26	74/26	74/26	74/26	1	1	1
Age	54.1±13.8	54.9±13.1	55.9±12.0	55.6±11.7	0.96	0.88	0.96

Note: HC, healthy control; HCC, chronic HBV infection patients with hepatocellular carcinoma; LC, chronic HBV infection with cirrhosis; CHB, chronic hepatitis B without cirrhosis or HCC.

## Materials and methods

### Study populations

We obtained blood samples from unrelated Han Chinese subjects in Huai'an First People's Hospital, China, between February 2013 and August 2015 for this retrospective study. This study used a total of 400 adults including 100 newly diagnosed HBV related hepatocellular carcinoma (HCC), 100 HBV related liver cirrhosis (LC), 100 patients with chronic hepatitis (CHB), and 100 healthy controls. Gender and age were matched among the four groups. Inclusion and exclusion criterions were in accordance with previous studies [12]. All of the HCC and cirrhosis patients were diagnosed by histopathology. All of the patients were found HBsAg-positive for more than 6 months. In the HC group, all individuals had negative HBV seromarkers, hepatitis, or other diseases. We excluded patients that had other hepatitis viruses except for HBV or human immunodeficiency virus infection, had other chronic liver diseases (such as autoimmune hepatitis, primary biliary cirrhosis etc.), or other diseases (such as systemic lupus erythematosus or tumors). The study protocol was approved by the Ethics Committee of Huai'an First People's Hospital. Written informed consent was obtained from every subject.

### RNA extraction

Serum was obtained from blood through centrifuging at 3000 g for 10 minutes at 4°C by discarding cellular components. According to the protocols of the manufacturer, TRIZOL Reagent (Invitrogen, Carlsbad, CA) was used to extract RNA from 100 mL serum. Each sample was added with 200-mL chloroform and 1.0-ml TRIZOL reagent and then vortexed for 20 seconds and stood at 25°C for 4 minutes. Then, it was centrifuged at 12000 g for 16 minutes at 4°C. We transferred the supernatant to a fresh

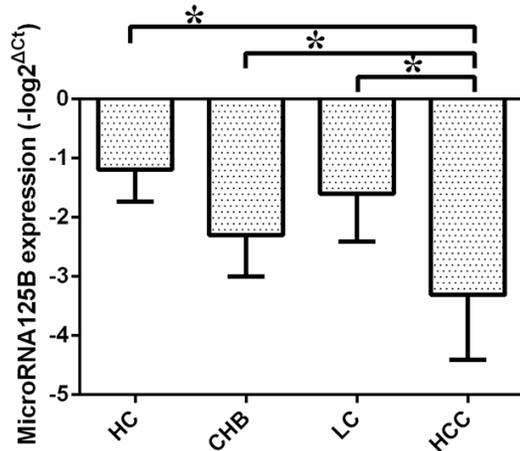
tube and added 500-mL isopropanol into the supernatant. It was located in 20°C for 20 minutes for incubation and centrifuged at 11 500g for 10 minutes at 4°C and we discarded the supernatant. At last, we used 75% ethanol to wash the RNA pellet and remove the ethanol by centrifuging at 7500 g for 5 minutes at 4°C. We dried the RNA in the air for 6 minutes and dissolved it in 40-mL RNase-free water. Nanodrop ND-1000 (Thermo Scientific, Worcester, MA) was used to determine the purity of the obtained RNA. High purity was defined as the value of the OD260/280 around 2.0.

### Reverse transcription

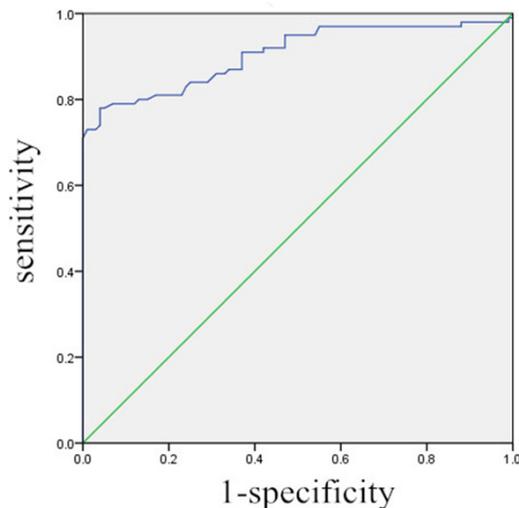
A total volume of 30 µL mixture including 10 µL RNA sample was reverse-transcribed to cDNA with polyadenylation polymerase (New England Biolabs, Beverly, MA) and First-Strand cDNA Synthesis Kit (Takara, Dalian, China) with related primers (mRNA-125B sense primer: GTCG-TATCCAGTGCAGGGTCCGAGGT; anti-sense primer: ATTCGACTGGATACGACTCAACAAG). We diluted the cDNA product with water and stored at -80°C for analysis.

### Real-time quantitative PCR analysis of serum microRNA-125b

For normalization, microRNA-U6 (sense primer: CTCGCTTCGG-CAGCACATATACT; anti-sense primer: ACGCTTACGAATTTGCG-TGTC) was used as an internal control. We used ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) to carry out real-time quantitative PCR (qPCR) quantification of serum microRNA-125b with SYBR Green PCR Master Mixture (Takara). The specificity of PCR product was validated by melting curve analysis when PCR cycles ended. For quality control of qPCR, each sample was in duplicate and a negative control. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in



**Figure 1.** Expression of serum microRNA-125b in HC, CHB, LC, and HCC. Each figure was made by  $-\log_2^{\Delta Ct}$  value,  $\Delta Ct = Ct^{\text{microRNA-125b}} - Ct^{\text{microRNA-U6}}$ . Data were shown as mean  $\pm$  SD. \*,  $P < 0.05$ . HC, healthy control; CHB, chronic hepatitis B; LC, HBV-related liver cirrhosis; HCC, HBV-related HCC.



**Figure 2.** Receiver-operator characteristic curves of the microRNA-125b in HCC vs. LC. AUC = 0.91.

qPCR. Levels of serum microRNA-125B was calculated by the formula  $2^{\Delta Ct}$ .  $\Delta Ct = Ct^{\text{microRNA-125b}} - Ct^{\text{microRNA-U6}}$ .

*Statistical analysis*

Differences in age and gender were compared using t-test or  $X^2$  test. We used Mann-Whitney test to compare the different levels of serum microRNA-125b in HCC vs. LC, HCC vs. CHB, and HCC vs. HC as data of the four groups were not in homogeneity of variance. Evaluation of

potential diagnostic markers-microRNA-125b was performed by receiver-operator characteristic (ROC) curves. Statistical analysis was carried out with SPSS 21.0 software (SPSS, Inc., Chicago, IL).  $P < 0.05$  was considered statistically significant and all tests were two sided.

**Results**

*Characteristics of the study population*

**Table 1** shows the characteristics of the study population. Compared to HCC group, the LC, CHB, and HC groups showed no difference in age distribution ( $P = 0.88$ ,  $P = 0.96$  and  $P = 0.96$  respectively). No gender difference was found among the groups.

*Correlation of serum microRNA-125b with HCC risk*

Correlations of serum microRNA-125b with HCC risk are shown in **Figure 1**. Compared to HC group, HCC patients had significantly lower level of microRNA-125b ( $P < 0.05$ ). Meanwhile, chronic HBV infection patients with lower levels of microRNA-125b had more HCC risk ( $P < 0.05$ ). We also found that lower levels of serum microRNA-125b could elevate risk of HCC from HBV related cirrhosis patients ( $P < 0.05$ ).

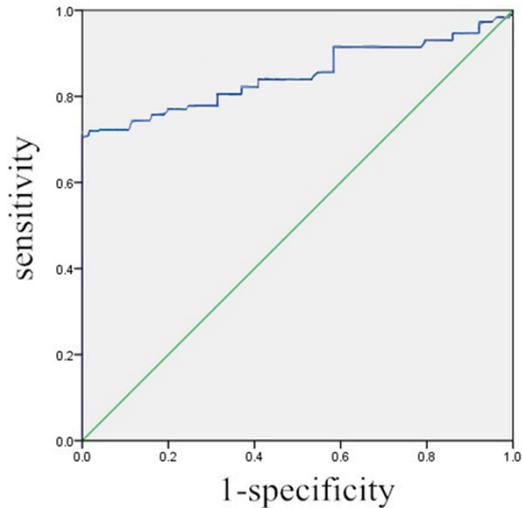
*Evaluation of potential diagnostic markers of microRNA-125B*

We used ROC curve analyses to evaluate the potential of serum miRNA-125b as a diagnostic marker. We found that levels of serum microRNA-125b could serve as potential markers for recognizing HCC from LC, CHB or HC, with ROC curve areas of 0.91 (95% CI: 0.86-0.95), 0.80 (95% CI: 0.73-0.87), and 0.94 (95% CI: 0.91-0.98), respectively (**Figures 2-4**). At the cut-off values of 2.91 (for LC), 2.66 (for CHB), and 2.45 (for HC), the sensitivity and specificity were 78% and 96%, 81% and 87%, and 83% and 96% for LC, CHB and HC, respectively. See **Figures 2-4**.

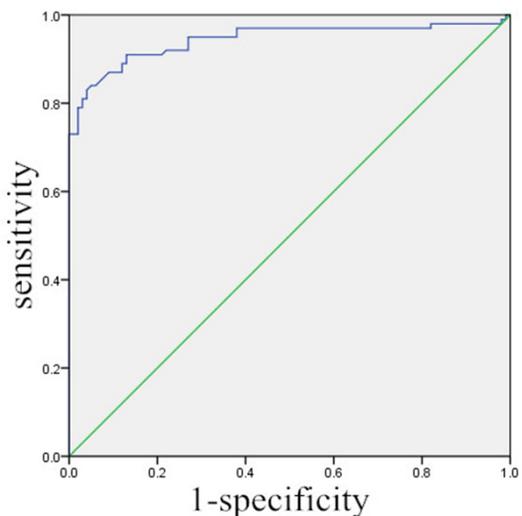
**Discussion**

HCC is recognized as one of the most aggressive human malignancies with an extremely poor prognosis. It is extremely important and valuable to diagnose HCC as early as possible, making the patients undergo surgical resection

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**Figure 3.** Receiver-operator characteristic (ROC) curves of the microRNA-125B in HCC vs. CHB. AUC = 0.80.



**Figure 4.** Receiver-operator characteristic (ROC) curves of microRNA-125b in HCC vs. HC. AUC = 0.94.

which could improve the survival rate of HCC. Alpha-fetoprotein ( $\alpha$ -AFP) is used for primary HCC diagnosis, currently. However, the sensitivity and specificity of  $\alpha$ -AFP are not enough for early diagnosis of HCC [13]. In this study, we validated the assumption that levels of serum microRNAs can be recognized as biomarkers for diagnosis of HBV-positive HCC from LC, CHB, and HC. We found that microRNA-125b was significantly downregulated in HCC patients compared to LC, CHB, and HC.

The microRNA125 family is one of the most important microRNA families and is associated

with a variety of cancers [14]. Members of microRNA125 family could recognize growth factors, matrix-metalloprotease, and some transcription factors [14-18]. Serum microRNA-125b has been used to predict microvascular invasion in hepatocellular carcinomas patients, which might provide a powerful tool in making an accurate diagnosis of microvascular invasion. Previous studies have found that microRNA-125b was downregulated in HCC tissue and acted as a tumor suppressor [19, 20]. These results were consistent with our findings.

We used ROC curve analyses to evaluate the values of miRNA125b serving as potential diagnostic markers. We found that levels of serum microRNA-125b could serve as potential markers for recognizing HCC from LC, CHB or HC, with ROC curve areas of larger than 0.8. Sensitivity and specificity were all > 70% in discriminating HBV-HCC from HC and LC. The results suggest that serum microRNA-125b could act as a potential diagnostic marker for detection of HBV-related HCC.

The exact mechanisms of microRNA-125b in HCC are not clear. Ectopic expression of microRNA-125b could reduce cell cycle and proliferation of HCC cells [19, 21]. Moreover, microRNA-125b could suppress invasion and migration of hepatoma cells through transcriptional co-activator with PDZ-binding motif and oncogene LIN28B [21, 22]. Meanwhile, microRNA-125b was found to reduce HBV replication and played an important role in HBV-related HCC [23]. We conclude that microRNA-125b has multiple functions in HBV-related HCC.

However, our study still has some limitations. First, we had a relatively small sample size. Second, it lacks of a validation assay. Third, the patients were not divided into different stages and followed up.

In conclusion, we find that level of serum microRNA-125b is significantly correlated with HCC risk compared to HBV related cirrhosis or hepatitis patients and healthy controls and could be used to be a diagnosis biomarker for HBV-related HCC. In the future, more prospective longitudinal studies with a larger sample size are needed to prove our findings.

### Disclosure of conflict of interest

None.

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