Clinical value evaluation of microRNA-324-3p and other available biomarkers in patients with HBV-infection-related hepatocellular carcinoma

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Abstract

Background: Patients with hepatitis B virus (HBV) infection are at high risk of hepatocellular carcinoma (HCC). This study aimed to evaluate the expression of microRNA-324-3p (miR-324-3p) in HBV-related HCC, and explore the clinical significance of serum miR-324-3p and other available biomarkers in the diagnosis and prognosis of HBV-related HCC.

Methods: Expression of miR-324-3p in HBV-infection-related cells and patients was estimated using quantitative real-time PCR. The receiver operating characteristic (ROC) curves were constructed to evaluate the diagnostic performance of serum miR-324-3p, AFP and PIVKA-II in the differentiation of HBV-related HCC from healthy controls and chronic hepatitis B (CHB). The relationship between serum miR-324-3p and patients’ clinical features was assessed using Chi-square test, and the value of miR-324-3p to predict overall survival prognosis was evaluated using Kaplan-Meier methods and Cox regression assay in patients with HBV-related HCC.

Results: HBV-related HCC cells had significantly increased miR-324-3p compared with normal and HBV-unrelated HCC cells, and serum miR-324-3p in HCC patients with HBV infection was also higher than that in healthy controls and CHB. Serum miR-324-3p had relatively high diagnostic accuracy for the screening of HCC case with HBV infection, and the combination of miR-324-3p, AFP and PIVKA-II showed the improved diagnostic performance. Additionally, high serum miR-324-2p in HBV-related HCC patients was associated with cirrhosis, tumor size, clinical stage and poor overall survival prognosis.

Conclusion: Serum increased miR-324-3p may be involved in the progression of HBV-related hepatitis to HCC, and may serve as a candidate biomarker for the diagnosis and prognosis of HBV-related HCC.

Keywords: MicroRNA-324-3p; hepatitis B virus; hepatocellular carcinoma; diagnosis; prognosis
Introduction

Global statistics show that there are about 240 million people infected with hepatitis B virus (HBV), and approximately 1 million HBV-infection-related deaths occur annually. HBV infection is widespread in the Asia-Pacific region, and China is a major prevalent country of HBV infection. Although advances in HBV vaccination and antiviral therapy, the morbidity and mortality of HBV-related diseases remain high. Patients with HBV infection are at high risk of adverse sequelae, mainly including decompensated liver diseases, liver cirrhosis and hepatocellular carcinoma (HCC). HCC is the third leading cause of global deaths due to malignant tumors, and nearly half of HCC patients and deaths occur in China, owing to the high prevalence of HBV infection. Thus, early diagnosis is critical to predict HCC in patients with HBV infection. So far, many serological biomarkers have been developed for HCC diagnosis, and alpha-fetoprotein (AFP) and protein induced by vitamin k absence/antagonist II (PIVKA-II) are widely used in clinical practices. However, many HCC patients had normal AFP levels, while high AFP levels (more than 200 ng/mL) could be found under non-HCC conditions. Although the diagnostic performance has been improved by the combined analysis of AFP and PIVKA-II, the sensitivity and specificity remains unsatisfying. Therefore, discovering potential biomarkers with better diagnostic performance to screen HBV-related HCC cases from HBV infection patients is urgently needed for HCC treatment.

MicroRNAs (miRNAs) are a series of small non-coding RNAs, and play important regulatory roles in many cellular processes, such as cell proliferation, migration and invasion. miRNAs have been demonstrated to act as regulators of many oncogenes and tumor suppressors by directly binding the 3’-untranslated region (3’-UTR) of target mRNAs. Thus, the aberrant expression of miRNAs have been indicated as pivotal biomarkers in various human cancers, including HCC. To improve the serological examination for cancer diagnosis, some serum miRNAs have been determined as potential biomarkers for HCC diagnosis, such as serum miR-106b, miR-638, miR-130b and miR-21. Tuo et al. has investigated the functional role of miR-324-3p HCC progression, and demonstrated that miR-324-3p could promote HCC tumorigenesis. Another study by Wen et al. reported that miR-324-3p was significantly elevated in HBV-positive HCC patients compared with the noncancerous controls, which suggests that miR-324-3p may play a potential role in HBV-infection-related HCC.

In this study, we first analyzed miR-324-3p expression and its biological function in HBV-related HCC cells. The differentially expressed serum miR-324-3p levels in HBV-related HCC patients were then analyzed to evaluate its diagnostic and prognostic value. Additionally, the clinical value of serum miR-324-3p was compared to the results of AFP and PIVKA-II. The findings of this study may provide a candidate biomarker for the diagnosis and prognosis of HBV-related HCC.

Materials and methods

Cell culture and transfection

A normal hepatic cell line L02, a HBV-unrelated HCC cell line Huh7 and a HBV-related cell line Hep3B were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Dulbecco’s modified Eagle’s medium (DMEM, Solarbio, Beijing, China) was used to culture cells, which was supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO2.
miR-324-3p mimic was synthesized from GenePharma (Shanghai, China) to upregulate the expression of miR-324-3p in HCC cell lines. The negative control of the mimic (mimic NC, GenePharma) was obtained as a control. The sequences of the vectors were as follows: miR-324-3p mimic: 5'-ACUGCCCAGGUGCUGGCU-3'; mimic NC: 5'-UUCUCCGAACGUGACGU-3'. miR-324-3p mimic and mimic NC were respectively transfected into Huh7 and Hep3B cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), and the transfection efficiency was evaluated using quantitative real-time PCR (qRT-PCR) at 48 h post-transfection.

**Study population and serum collection**

Blood samples were collected from 96 HBV-related HCC patients, 52 HBV-unrelated HCC patients, 72 chronic hepatitis B (CHB) patients, and 76 healthy controls, who admitted to Yidu Central Hospital of Weifang from March 2015 to March 2019. The patients with HBV-related diseases were positive for HBsAg, and none of the participants had any other liver disease types, such as alcoholic liver diseases, hepatitis C infection, metabolic liver diseases or autoimmune liver diseases. The diagnosis of HCC was determined by a histopathological examination. The healthy volunteers were individuals underwent routine physical examination without malignant tumor history or hepatitis virus infection. The demographic and clinical data of the participants were summarized in Table 1, and no statistical differences in age and gender between the groups. Serum samples were obtained from the blood samples by centrifugation at 4°C and were used for serological examination or stored at -80°C for RNA extraction. The HBV-related HCC patients received radical hepatectomies in Yidu Central Hospital of Weifang, and a follow-up survey was performed after the surgery by telephone communication or outpatient visits. All the patients were followed up for their survival conditions until the date of death or the last follow-up time (March 2020). The Ethics Committee of Yidu Central Hospital of Weifang approved the study protocols, and the participants or their legal guardians signed written informed consent before sampling.

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

Total RNA from cells and serum samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. The concentration and purification of RNA were spectrophotometrically confirmed by calculating the OD ratio (A260/280 close to 2.0) using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, DE, USA). The RNA was then reversely transcribed into cDNA using a miRNA cDNA synthesis kit (CWBiotech, Beijing, China) following the manufacturer’s protocols. The expression of miR-324-3p was determined using a miRNA qPCR assay kit (CWBiotech, Beijing, China) and measured on an Applied Biosystems 7900 Real-Time PCR system (CA, USA) with setting as follows: 95°C for 10 min, 40 cycles of 95°C for 20 s, 58°C for 15 s, 72°C for 20 s. The expression quantitation of miR-324-3p was done using the 2^−ΔΔCt method and normalized to the control cel-miR-39-3p.

**Cell proliferation analysis**

A cell counting kit-8 (CCK-8) assay was used to evaluate the proliferation of Huh7 and Hep3B cells. The cells with a concentration of 2 ×10^4 cells/well were seeded into 96-well culture plates and cultured at 37°C in a humidified incubator with 5% CO2. CCK-8 reagent (10 μL) was added into the wells at preset time points: 0, 24, 48 and 72 h. After 1 h of incubation at 37°C the optical density (OD) of cell cultures at 490 nm was measured using a microplate reader (Bio-Rad) to determine cell proliferation capacity. At least 3 repeated examinations were required.
Cell migration and invasion analysis

A Transwell assay was used to evaluate the migration and invasion abilities of Huh7 and Hep3B cells. Transwell chambers (24-well plates, 8 μm pore size; Corning, NY, USA) precoated with Matrigel (BD Biosciences, NJ, USA) were used for invasion assay, and those with no need of Matrigel were used for migration assay. The cells cultured with FBS-free DMEM medium were seeded into the upper chambers at a concentration of 2 ×10^5 cells/well. The lower chambers contained DMEM medium supplemented with 10% FBS as a chemotactic agent. After a 24 h of incubation of in a humidified incubator at 37°C the cells moved to the lower membrane surface were stained with 0.1 crystal violet for 15 min. Cell numbers in 5 random visual fields were counted using a light microscope.

Evaluation of serum levels of AFP and PIVKA-II

As serum biomarkers for HCC diagnosis, the levels of AFP and PIVKA-II were measured using a fully automated chemiluminescent microparticle immunoassay (CMIA) system (Abbott, CA, USA) according to the manufacturer’s protocols.

Statistical analysis

Each experiments were repeated at least 3 times, and the analysis data were analyzed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA). Data were expressed as median (interquartile range) or numbers. Differences in continuous variables were compared using Kruskal-Wallis H test or one-way ANOVA followed by Tukey’s test, and categorical variables were analyzed using Chi-square test. The expression of serum miR-324-3p was divided into low and high expression groups using its median expression value, and the relationship between miR-324-3p expression and patients’ clinicopathological characteristics was evaluated using Chi-square test. The receiver operating characteristic (ROC) curves were plotted based on serum miR-324-3p, AFP and PIVKA-II levels, the area under the curve (AUC) was calculated. The follow up survival information was analyzed using Kaplan-Meier method, and a log-rank test was used to compare the differences between survival curves. miR-324-3p levels and the other available markers of HCC were included in a Cox regression model to confirm the independency of miR-324-3p as a prognostic indicator in patients with HBV-related HCC. A P value of less than 0.05 indicated statistically significant.

Results

Expression and biological function of miR-324-3p in HBV-related HCC cells

The relative expression of miR-324-3p was compared between normal hepatic cells, HBV-related and –unrelated HCC cells. The results shown in Figure 1A indicated that both two HCC cell lines Huh7 and Hep3B had significantly higher miR-324-3p expression than normal cell line L02 (P < 0.01 for Huh7, P < 0.001 for Hep3B). In addition, the expression of miR-324-3p was clearly elevated in HBV-related HCC cell line Hep3B when compared to the HBV-unrelated HCC cell line Huh7 (P < 0.001).

To investigate the biological function of miR-324-3p in HBV-related HCC cells, the Hep3B cells with significant overexpression of miR-324-3p were obtained using the cell transfection with miR-324-3p mimic (P < 0.001, Figure 1B). The cell biological processes analysis results showed that the
overexpression of miR-324-3p could enhance Hep3B cell proliferation, migration and invasion (all $P < 0.01$, Figure 1C – E).

**Serum miR-324-3p levels are elevated in patients with HBV-related HCC**

Serum levels of miR-324-3p were measured in 76 healthy volunteers, 72 CHB patients, 52 HBV-unrelated HCC patients and 96 HBV-related HCC patients. The qRT-PCR results revealed that compared with healthy controls, serum miR-324-3p expression was significantly upregulated in CHB and HCC patients (all $P < 0.01$, Figure 2). Additionally, the significantly elevated miR-324-3p expression was observed in HBV-related HCC patients compared with CHB and HBV-unrelated HCC patients (both $P < 0.001$). However, no significant difference was found in miR-324-3p levels between CHB and HBV-unrelated HCC patients.

**Diagnostic value of serum miR-324-3p, AFP and PIVKA-II in distinguishing patients with HBV-related HCC**

Serum levels of miR-324-3p, AFP and PIVKA-II in the study objects were used to plot ROC curves to perform a diagnosis value analysis. First, the differentially expressed miR-324-3p and the available biomarkers between HBV-related HCC patients and healthy controls was analyzed, and the ROC curves were exhibited in Figure 3A. In addition, a ROC curve of the synthetic role of serum miR-324-3p, AFP and PIVKA-II was also performed. The diagnostic performance results shown in Table 2 revealed that serum miR-324-3p had a high diagnostic accuracy with an AUC of 0.926, and the sensitivity and specificity were 77.08% and 93.42% at a cutoff value of 1.608. Comparatively, AFP and PIVKA-II had moderate diagnostic accuracy with AUC values of 0.761 for AFP and 0.884 for PIVKA-II. The combination of the 3 variables further enhanced the diagnostic accuracy with an AUC of 0.966, a sensitivity of 88.54% and a specificity of 96.05%.

Second, the diagnostic accuracy of the 3 biomarkers in distinguishing HBV-related HCC patients from CHB patients were also evaluated (Figure 3B and Table 2). The performance of miR-324-3p was also the best (AUC = 0.907, cutoff value = 1.690, sensitivity = 81.25%, specificity = 87.5%), followed by PIVKA-II (AUC = 0.829, cutoff value = 40.3, sensitivity = 83.33%, specificity = 73.61%). However, the diagnostic accuracy of serum AFP was low, and the AUC was only 0.653 with the sensitivity and specificity were 62.5% and 75.00%, respectively. By integrating the 3 biomarkers, the diagnostic performance was improved with an AUC of 0.941 and the sensitivity and specificity of 81.25% and 97.22%, respectively.

**Relationship between serum miR-324-3p and clinicopathological features in HBV-related HCC patients**

In HCC patients with HBV infection, the association of serum miR-324-3p with major clinicopathological characteristics was assessed. The data shown in Table 3 suggested that miR-324-3p was associated with liver cirrhosis ($P = 0.003$), tumor size ($P = 0.010$), Barcelona Clinic Liver Cancer (BCLC) stage ($P = 0.003$) and Tumor Node Metastasis (TNM) stage ($P = 0.005$). In other words, patients had liver cirrhosis, larger tumor size, BCLC B stage or advanced TNM stage had a higher probability with high serum miR-324-3p levels. No relationship was observed between serum miR-324-3p and age, gender and tumor differentiation (all $P > 0.05$).
Differentially expressed miR-324-3p between HCC patients with different status of liver cirrhosis

The association of miR-324-3p with liver cirrhosis was found in HBV-related HCC patients by the Chi-square test. In addition, the serum expression of miR-324-3p was found to be significantly elevated in positive liver cirrhosis patients compared with the negative cases ($P < 0.001$, Figure 4A). A ROC curve based serum miR-324-3p levels was plotted for HBV-related HCC patients, and the results shown in Figure 4B indicated that serum miR-324-3p had potential to distinguish positive liver cirrhosis patients from liver cirrhosis negative HCC patients ($AUC = 0.886$).

Prognostic value of serum miR-324-3p, AFP and PIVKA-II to predict the overall survival of HBV-related HCC

Based on the follow up survival information, the Kaplan-Meier survival curves for patients with HBV-related HCC were plotted (Figure 5). It is found that patients with high serum miR-324-3p had a significantly poor overall survival than those with low miR-324-3p levels (log-rank $P = 0.023$). In addition, the clinical data and the 3 proposed biomarkers were included in a Cox regression analysis (Table 4), the univariate analysis results showed that BCLC stage, TNM stage, PIVKA-II and miR-324-3p were related with patients’ survival (all $P < 0.05$). The subsequent multivariate analysis, which included the variables with significant results from the univariate analysis, indicated that BCLC stage, TNM stage and serum miR-324-3p were independent prognostic biomarkers (all $P < 0.05$).

Discussion

Owing to the large number of HBV infected patients, the incidence and mortality of HCC have been increasing $^{20}$. The purpose of this study was to identify a novel serum biomarker to screen HBV-related HCC patients from healthy individuals and CHB patients. This study first demonstrated that the expression of miR-324-3p was significantly increased in HBV-related HCC cell line Hep3B compared with normal hepatic cell line L02 and HBV-unrelated HCC cell line Huh7. In addition, the overexpression of miR-324-3p was confirmed to promote Hep3B cell proliferation, migration and invasion. A previous study by Tuo et al. has reported the expression and functional role of miR-324-3p in HCC, and found that miR-324-3p was increased in Huh7 and Hep3B cell lines, and contributed to HCC tumorigenesis $^{18}$. These previous results were consistent with our study. However, the previous studies only focused on the role of miR-324-3p in pure HCC cases, but not HBV-related HCC. Wen et al. has reported that miR-324-3p expression was higher in HBV-positive HCC patients compared with the noncancerous controls $^{19}$. In the present study, the markedly increased miR-324-3p in Hep3B compared to Huh7 cells might indicate that miR-324-3p expression may be affected by HBV infection.

Serological examination is a quick and easy method for the diagnosis of malignant tumors, and many serum biomarkers have been applied in clinical practices $^{21}$. AFP is one of the most widely used serological biomarkers for HCC diagnosis with a sensitivity of 60% at a cutoff value of 20 ng/mL $^{22}$. However, statistics show that only one-third early stage HCC patients can be identified using AFP detection, which because that the patients with significantly elevated AFP levels account only 60%-80% of all cancer cases, and high AFP levels can also be found under non-HCC conditions $^{23}$. PIVKA-II is a newly applied biomarkers, and has improved the diagnosis of HCC with relatively high sensitivity and specificity $^{24}$. However, specific biomarkers to diagnose HCC cases with positive HBV infection are still lacking.
Numerous studies have demonstrated that aberrant miRNAs in various disease are involved in disease development and progression, so that many deregulated serum miRNAs have been determined as candidate biomarkers for disease diagnosis and prognosis. For example, serum miR-10b-5p was elevated in HCC patients, and had relatively high diagnostic accuracy for distinguishing early stage HCC cases. HCC patients had significantly decreased serum miR-129-5p, which served as a potential biomarker for diagnosis and prognosis. In HBV-related HCC, several serum miRNAs have also been identified to be related with disease development and progression, such as miR-375 and miR-223-3p. In the current study, serum expression of miR-324-3p was also investigated. Our findings revealed that the expression of serum miR-324-3p was increased in the order from healthy controls to patients with CHB and HBV-unrelated HCC, and finally, patients with HBV-related HCC. The highest serum miR-324-3p was observed in HBV-related HCC patients, which was consistent with the expression results in HCC cell lines. According to the ROC curves based on serum miR-324-3p and two HCC biomarkers (AFP and PIVKA-II) in the same serum samples, we found that the AUCs of serum miR-324-3p were higher than those of AFP and PIVKA-II for the diagnosis of HBV-related HCC from healthy controls or CHB patients. Subsequently, the diagnostic performance of the synthetic role of serum miR-324-3p, AFP and PIVKA-II was evaluated, and the AUC, sensitivity and specificity results were all improved after combining the 3 biomarkers. Thus, serum miR-324-3p might serve as a biomarker for HBV-related HCC patients, and had a potential to improve the diagnostic value of AFP and PIVKA-II. Nevertheless, these conclusion needs to be validated using larger cohorts in future studies.

This study collected the clinicopathological characteristics of patients with HBV-related HCC, and found the significant association of serum miR-324-3p with liver cirrhosis, tumor size, BCLC stage and TNM stage of the patients. These findings indicate that miR-324-3p might be involved in the development of HCC. The previous study also provide evidence for the relationship between miR-324-3p with HCC tumor size and TNM stage, but miR-324 did not show a relationship with cirrhosis in the previous study. Without appropriate treatment, HBV infection can progress to liver cirrhosis, which can lead to HCC. In our study, the HCC patients were positive HBV infection, and the relationship found between miR-324-3p and cirrhosis might suggested that miR-324-3p was involved in the progression of HBV-related hepatitis to HCC. To confirm this reasoning, the expression of miR-324-3p was compared between liver cirrhosis positive and negative patients. The analysis results revealed that liver cirrhosis positive patients had significantly higher serum miR-324-3p, and the elevated serum miR-324-3p might have potential to distinguish liver cirrhosis positive HCC patients from the negative patients. These findings further confirm the important role of miR-324-3p in the development and progression of HBV-related liver diseases. Furthermore, the association of miR-324-3p with HCC patients’ overall survival was evaluated, and high miR-324-3p was related with low survival rate, and served as an independent prognostic factor in patients with HBV-related HCC. Therefore, serum miR-324-3p might be used as a biomarker to predict the overall survival prognosis of HBV-related HCC.
In conclusion, our findings found that miR-324-3p expression was significantly increased in HBV-related HCC cell lines and patients, and that serum miR-324-3p had better diagnostic performance than AFP and PIVKA-II for distinguishing HBV-related HCC from healthy individuals and CHB patients. Serum high miR-324-3p could predict poor overall survival prognosis in patients with HBV-related HCC. Therefore, serum miR-324-3p may be involved in the progression of HBV-related hepatitis to HCC, and may serve as a biomarker for the diagnosis and prognosis of HBV-related HCC. Several limitations were included in this study, and the limited sample size is the major one. Thus, further studies are necessary to confirm the clinical value of miR-324-3p in HBV-related HCC.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Patient Consent Statement

A signed written informed consent was obtained from each patient. The experimental procedures were all in accordance with the guideline of the Ethics Committee of Yidu Central Hospital of Weifang and followed were in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association.
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Figure legends

**Figure 1.** Expression and biological function of miR-324-3p in HBV-related HCC cell line Hep3B. A. Hep3B had the highest miR-324-3p expression than L02 and Huh7 cell lines. B. miR-324-3p mimic significantly increased miR-324-3p expression in Hep3B cells. C – E. The overexpression of miR-324-3p promote Hep3B cell proliferation (C), migration (D) and invasion (E) abilities. **P < 0.01, ***P < 0.001.

**Figure 2.** Serum expression of miR-324-3p increased in order from healthy controls to patients with CHB and HBV-unrelated HCC, and finally, patients with HBV-related HCC. HBV-related HCC patients had the highest serum miR-324-3p levels. **P < 0.01, ***P < 0.001.

**Figure 3.** ROC curves based on serum miR-324-3p, AFP and PIVKA-II for patients with HBV-related HCC. A. ROC curves of miR-324-3p, AFP and PIVKA-II in discriminating HBV-related HCC from healthy controls. B. ROC curves of miR-324-3p, AFP and PIVKA-II in discriminating HBV-related HCC from CHB patients. AUC, area under the curve.

**Figure 4.** Differentially expressed miR-324-3p had potential to distinguish liver cirrhosis positive patients from negative patients. A. Serum miR-324-3p was significantly higher in liver cirrhosis positive patients compared with the negative cases. ***P < 0.001. B. A ROC curve based serum miR-324-3p levels in HBV-related HCC patients with or without liver cirrhosis (AUC = 0.886).

**Figure 5.** Kaplan-Meier survival curves for HBV-related HCC patients with different serum levels of miR-324-3p. Patients with high level of miR-324-3p had a poor overall survival than those with low miR-324-3p levels (log-rank P = 0.023).
| Features                  | Healthy controls | CHB patients | HBV-unrelated HCC patients | HBV-related HCC patients | \( P \) value |
|--------------------------|------------------|--------------|-----------------------------|--------------------------|--------------|
| Number                   | 76               | 72           | 52                          | 96                       | -            |
| Age (years)              | 53 (42-59)       | 50 (38-61)   | 52 (43-62)                  | 55 (41-67)               | 0.979\(^a\) |
| ALT (IU/L)               | 15.5 (8.9-26.8)  | 108.4 (36.2-441.5) | 87.7 (43.3-135.0)   | 119.1 (42.2-489.4)      | <0.001\(^a\) |
| AST (IU/L)               | 12.8 (9.4-29.8)  | 91.5 (32.6-289.8) | 72.3 (45.8-108.6)   | 112.3 (48.4-323.9)      | <0.001\(^a\) |
| AFP (ng/mL)              | 3.2 (1.2-7.9)    | 5.5 (1.6-16.9) | 85.8 (3.9-258.7)   | 92.8 (6.0-275.6)        | <0.001\(^a\) |
| PIVKA-II (mAV-mlL)       | 22.7 (12.5-33.6) | 26.8 (13.6-36.2) | 312.7 (38.9-668.8) | 277.6 (37.7-594.9)     | <0.001\(^a\) |
| Gender                   |                 |              |                             |                          | 0.928\(^a\) |
| Females                  | 32               | 32           | 20                          | 41                       |             |
| Males                    | 44               | 40           | 32                          | 55                       |             |
| Liver cirrhosis          |                 |              |                             |                          | <0.001\(^a\) |
| Negative                 | 76               | 34           | 31                          | 37                       |             |
| Positive                 | 0                | 38           | 21                          | 59                       |             |
| Tumor size (cm)          |                 |              |                             |                          | 0.693\(^b\) |
| \( \leq 5 \)             | /                | /            | 31                          | 54                       |             |
| > 5                      | /                | /            | 21                          | 42                       |             |
| Differentiation          |                 |              |                             |                          | 0.627\(^b\) |
| Well and moderate        | /                | /            | 33                          | 57                       |             |
| Poor                     | /                | /            | 19                          | 39                       |             |
| BCLC stage               |                 |              |                             |                          | 0.480\(^b\) |
| A                        | /                | /            | 35                          | 59                       |             |
| B                        | /                | /            | 17                          | 37                       |             |
| TNM stage                |                 |              |                             |                          | 0.616\(^b\) |
| I-II                     | /                | /            | 32                          | 55                       |             |
| III                      | /                | /            | 20                          | 41                       |             |

ALT: alanine aminotransferase; AST: aspartate aminotransferase; AFP: alpha-fetoprotein; PIVKA-II: protein induced by vitamin k absence/antagonist II; BCLC: Barcelona Clinic Liver Cancer; TNM: Tumor Node Metastasis; \( a \), comparison between four groups; \( b \), comparison between HCC patients with negative and positive HBV infection.
Table 2 Diagnostic performance of serum miR-324-3p, AFP and PIVKA-II in patients with HBV-related HCC

|                     | HBV-related HCC patients vs. healthy controls |                      | HBV-related HCC patients vs. CHB patients |                      |
|---------------------|-----------------------------------------------|----------------------|-------------------------------------------|----------------------|
|                     | Indicators | AUC | Cutoff value | Sensitivity (%) | Specificity (%) | Indicators | AUC | Cutoff value | Sensitivity (%) | Specificity (%) |
| miR-324-3p          | 0.926      | 1.608 | 77.08       | 93.42          |              | miR-324-3p          | 0.907      | 1.690 | 81.25       | 87.5          |
| AFP                 | 0.761      | 8.35  | 85.42       | 71.05          |              | AFP                 | 0.653      | 20.15 | 62.50       | 75.00         |
| PIVKA-II            | 0.884      | 37.25 | 84.38       | 86.84          |              | PIVKA-II            | 0.829      | 40.3  | 83.33       | 73.61         |
| miR-324-3p+AFP+PIVA-II | 0.966     | \    | 88.54       | 96.05          |              | miR-324-3p+AFP+PIVA-II | 0.941     | \    | 81.25       | 97.22         |

AFP: alpha-fetoprotein; PIVKA-II: protein induced by vitamin K absence/antagonist II; AUC: area under the curve.
Table 3 Relationship between serum miR-324-3p and clinicopathological characteristics in patients with HBV-related HCC

| Features             | Total number | Low miR-324-3p | High miR-324-3p | $P$ value |
|----------------------|--------------|----------------|-----------------|-----------|
| Number               | 96           | 44             | 52              |           |
| Age (years)          |              |                |                 | 0.148     |
| ≤ 60                 | 32           | 18             | 14              |           |
| > 60                 | 64           | 26             | 38              |           |
| Gender               |              |                |                 | 0.617     |
| Females              | 41           | 20             | 21              |           |
| Males                | 55           | 24             | 31              |           |
| Liver cirrhosis      |              |                |                 | 0.003*    |
| Negative             | 37           | 24             | 13              |           |
| Positive             | 59           | 20             | 39              |           |
| Tumor size (cm)      |              |                |                 | 0.010*    |
| ≤ 5                  | 54           | 31             | 23              |           |
| > 5                  | 42           | 13             | 29              |           |
| Differentiation      |              |                |                 | 0.434     |
| Well and moderate    | 57           | 28             | 29              |           |
| Poor                 | 39           | 16             | 23              |           |
| BCLC stage           |              |                |                 | 0.003*    |
| A                    | 59           | 34             | 25              |           |
| B                    | 37           | 10             | 27              |           |
| TNM stage            |              |                |                 | 0.005*    |
| I-II                 | 55           | 32             | 23              |           |
| III                  | 41           | 12             | 29              |           |

BCLC: Barcelona Clinic Liver Cancer; TNM: Tumor Node Metastasis; *$P < 0.05$. 
## Table 4 Analysis of prognostic indicators for HBV-related HCC patients using Cox regression analysis

| Indicators            | Univariate analysis |          |          | Multivariate analysis |          |          |
|-----------------------|---------------------|----------|----------|-----------------------|----------|----------|
|                       | HR                  | 95% CI   | P value  | HR                    | 95% CI   | P value  |
| Age                   | 1.756               | 0.685-3.085 | 0.745    | -                     | -        | -        |
| Gender                | 1.869               | 0.699-3.307 | 0.658    | -                     | -        | -        |
| Liver cirrhosis       | 2.217               | 0.856-4.581 | 0.148    | -                     | -        | -        |
| Tumor size            | 1.669               | 0.819-2.890 | 0.274    | -                     | -        | -        |
| Differentiation       | 2.098               | 0.985-3.332 | 0.056    | -                     | -        | -        |
| BCLC stage            | 2.569               | 1.628-3.874 | **0.016*** | 2.369                 | 1.382-3.587 | **0.032*** |
| TNM stage             | 2.271               | 1.448-3.197 | **0.022*** | 2.007                 | 1.318-2.949 | **0.036*** |
| AFP                   | 1.987               | 0.874-2.408 | 0.185    | -                     | -        | -        |
| PIVKA-II              | 1.953               | 1.267-2.949 | **0.041*** | 2.017                 | 0.973-3.228 | 0.068    |
| miR-324-3p            | 2.885               | 1.841-4.008 | **0.008*** | 2.594                 | 1.789-3.884 | **0.012*** |

AFP: alpha-fetoprotein; PIVKA-II: protein induced by vitamin K absence/antagonist II; BCLC: Barcelona Clinic Liver Cancer; TNM: Tumor Node Metastasis; *P < 0.05.
Figure 2

Relative expression of miR-324-3p

Healthy controls  CHB  HBV-unrelated HCC  HBV-related HCC

***  ***  ***  **  **
Figure 5

Cum survival

Log-rank P=0.023

miR-324-3p expression
- Low (n=44)
- High (n=52)
- Low-censored
- High-censored

Months