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MAJOR ARTICLE

Clinical Value Evaluation of microRNA-324-3p and Other Available Biomarkers in Patients With HBV Infection–Related Hepatocellular Carcinoma

Li Zhao,[1] Qian Yang,[1] and Jianbo Liu[2]

[1]Department of Infectious Diseases, Yidu Central Hospital of Weifang, Qingzhou, Shandong, China, and [2]Public Health Division, Yidu Central Hospital of Weifang, Qingzhou, Shandong, China

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 polymerase chain reaction (PCR). Receiver operating characteristic (ROC) curves were constructed to evaluate the diagnostic performance of serum miR-324-3p, alpha-fetoprotein (AFP), and protein induced by vitamin K absence/antagonist II (PIVKA-II) in the differentiation of HBV-related HCC from healthy controls and chronic hepatitis B patients (CHB). The relationship between serum miR-324-3p and patients' clinical features was assessed using the 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 cases with HBV infection, and the combination of miR-324-3p, AFP, and PIVKA-II showed 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.

Conclusions. High-serum 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. diagnosis; hepatitis B virus; hepatocellular carcinoma; microRNA-324-3p; prognosis.

Global statistics show that there are about 240 million people infected with hepatitis B virus (HBV), and ~1 million HBV infection–related deaths occur annually [1]. HBV infection is widespread in the Asia-Pacific region, and China is a high–HBV prevalence country [2]. Despite advances in HBV vaccination and antiviral therapy, the morbidity and mortality of HBV-related diseases remain high [3]. Patients with HBV infection are at high risk of adverse sequelae, mainly including decompensated liver diseases, liver cirrhosis, and hepatocellular carcinoma (HCC) [4]. 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 [5]. 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 practice [6, 7]. However, many HCC patients had normal AFP levels, while high AFP levels (>200 ng/mL) could be found in those with non-HCC conditions [8]. Although diagnostic accuracy has been improved by the combined analysis of AFP and PIVKA-II, the sensitivity and specificity remain unsatisfying [9]. 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 noncoding RNAs, and they play important regulatory roles in many cellular processes, such as cell proliferation, migration, and invasion [10]. 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 [11, 12]. Thus, because of their aberrant expression, miRNAs have been indicated as pivotal biomarkers in various human cancers, including HCC [13, 14]. To improve the serological examination for cancer diagnosis, some serum miRNAs have been

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Correspondence: Li Zhao, MM, Department of Infectious Diseases, Yidu Central Hospital of Weifang, No.4138, Linglongshan South Road, Qingzhou, 262500, Shandong, China (caizhai38905@126.com).

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determined as potential biomarkers for HCC diagnosis, such as serum miR-106b [15], miR-638 [16], miR-130b, and miR-21 [17]. Tuo et al. investigated the functional role of miR-324-3p HCC progression and demonstrated that miR-324-3p could promote HCC tumorigenesis [18]. Another study by Wen et al. reported that miR-324-3p was significantly elevated in HBV-positive HCC patients compared with noncancerous controls [19], 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 their diagnostic and prognostic value. Additionally, the clinical value of serum miR-324-3p was compared with 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.

METHODS

Cell Culture and Transfection

A normal hepatic cell line, L02, an HBV-unrelated HCC cell line, Huh7, and an HBV-related cell line, Hep3B, were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, China) was used to culture the cells; it was supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO_2.

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'-ACUGCCCCAGGUGCUGCUGG-3'; mimic NC: 5'-UUCUCCGAACGUGUCACGU-3'. miR-324-3p mimic and mimic NC were transfected into Huh7 and Hep3B cells, respectively, using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), and the transfection efficiency was evaluated using quantitative real-time PCR (qRT-PCR) at 48 hours 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 were 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 who underwent routine physical examination without malignant tumor history or hepatitis virus infection. The demographic and clinical data of the participants are summarized in Table 1. There were no statistical differences in age or 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

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, Wilmington, DE, USA). The RNA was then reverse-transcribed into cDNA using an miRNA cDNA synthesis kit (CWBiotech, Beijing, China) following the manufacturer's protocols. Expression of miR-324-3p was determined using an miRNA quantitative PCR (qPCR) assay kit (CWBiotech, Beijing, China) and measured on an Applied Biosystems 7900 Real-Time PCR system (Foster City, CA, USA) with setting as follows: 95°C for 10 minutes, 40 cycles of 95°C for 20 seconds, 58°C for 15 seconds, 72°C for 20 seconds. The expression quantitation of miR-324-3p was done using the $2^{-\Delta\Delta Ct}$ 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% CO_2. CCK-8 reagent (10 μL) was added into the wells at preset time points: 0, 24, 48, and 72 hours. After 1 hour 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

Table 1. Clinicopathological Characteristics of the Study Population

Features	Healthy Controls	CHB Patients	HBV-Unrelated HCC Patients	HBV-Related HCC Patients	*P* Value
No.	76	72	52	96	-
Age, y	53 (42–59)	50 (38–61)	52 (43–62)	55 (41–67)	.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)	<.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)	<.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)	<.001[a]
PIVKA-II, mAV-mL	22.7 (12.5–33.6)	26.8 (13.6–36.2)	312.7 (38.9–668.8)	277.6 (37.7–594.9)	<.001[a]
Gender					.928[a]
Females	32	32	20	41	
Males	44	40	32	55	
Liver cirrhosis					<.001[a]
Negative	76	34	31	37	
Positive	0	38	21	59	
Tumor size, cm					.693[b]
≤5	/	/	31	54	
>5	/	/	21	42	
Differentiation					.627[b]
Well and moderate	/	/	33	57	
Poor	/	/	19	39	
BCLC stage					.480[b]
A	/	/	35	59	
B	/	/	17	37	
TNM stage					.616[b]
I–II	/	/	32	55	
III	/	/	20	41	

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PIVKA-II, protein induced by vitamin K absence/antagonist II; TNM, Tumor Node Metastasis.

[a]Comparison between 4 groups.

[b]Comparison between HCC patients with negative and positive HBV infection.

(24-well plates, 8 μm pore size; Corning, NY, USA) precoated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used for the invasion assay, and those with no need of Matrigel were used for the 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-hour incubation in a humidified incubator at 37°C, the cells that had moved to the lower membrane surface were stained with 0.1 crystal violet for 15 minutes. 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, AFP and PIVKA-II levels were measured using a fully automated chemiluminesent microparticle immunoassay (CMIA) system (Abbott, San Diego, CA, USA) according to the manufacturer's protocols.

Statistical Analysis

Each experiment was repeated at least 3 times, and the 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 as numbers. Differences in continuous variables were compared using the Kruskal-Wallis H test or 1-way analysis of variance followed by Tukey's test, and categorical variables were analyzed using the chi-square test. 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 the chi-square test. Receiver operating characteristics (ROC) curves were plotted based on serum miR-324-3p, AFP, and PIVKA-II levels, and the area under the curve (AUC) was calculated. The follow-up survival information was analyzed using the Kaplan-Meier method, and a log-rank test was used to compare the differences between survival curves. miR-324-3p levels and other available markers of HCC were included in a Cox regression model to confirm the independence of miR-324-3p as a prognostic indicator in patients with HBV-related HCC. A *P* value of <.05 indicated statistical significance.

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 and HBV-related and -unrelated HCC cells. The results, shown in Figure 1A, indicate that the 2 HCC cell lines Huh7 and Hep3B had significantly higher

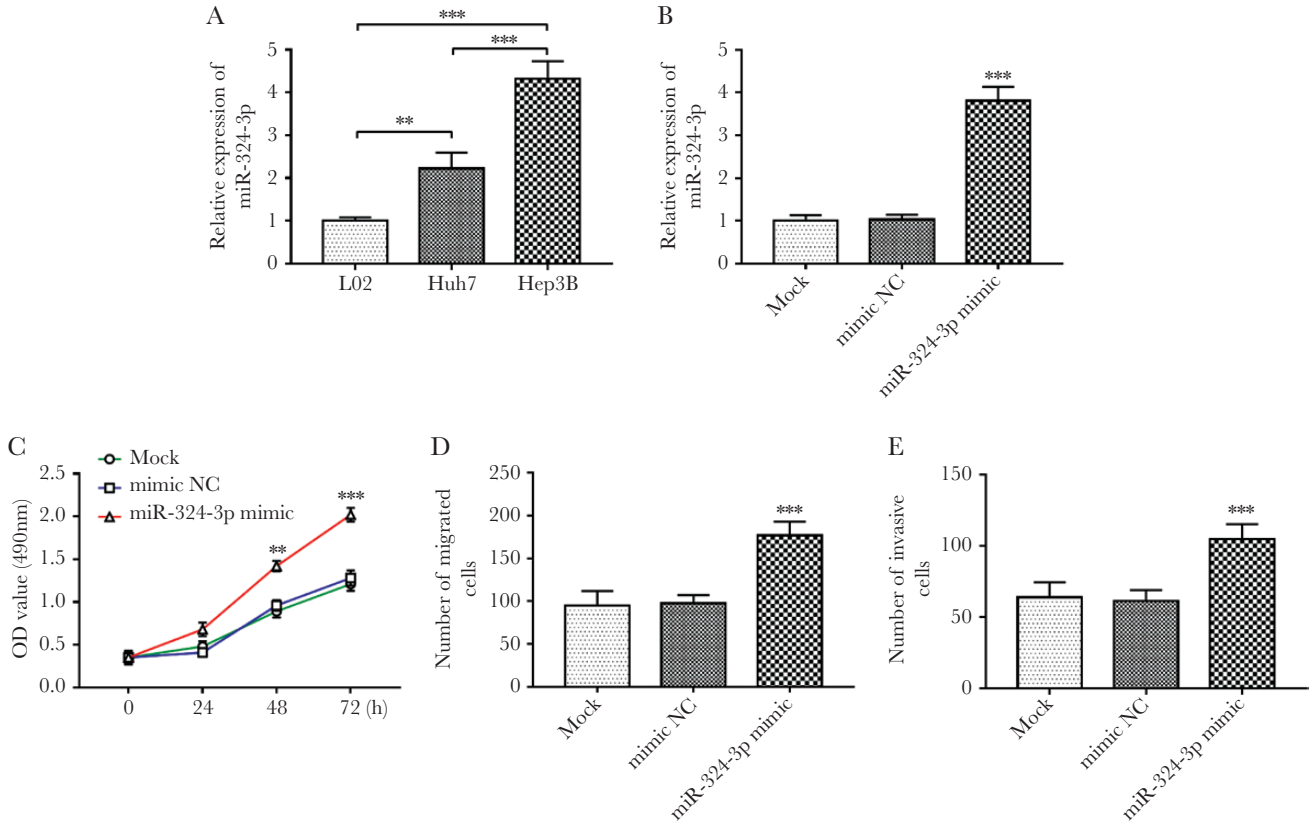

Figure 1. Expression and biological function of miR-324-3p in HBV-related HCC cell line Hep3B. A, Hep3B had higher miR-324-3p expression than the L02 and Huh7 cell lines. B, miR-324-3p mimic significantly increased miR-324-3p expression in Hep3B cells. C–E, The overexpression of miR-324-3p promoted Hep3B cell proliferation (C), migration (D), and invasion (E). $^{**}P < .01$; $^{***}P < .001$. Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p.

miR-324-3p expression than the normal cell line L02 ($P < .01$ for Huh7, $P < .001$ for Hep3B). In addition, the expression of miR-324-3p was clearly elevated in HBV-related HCC cell line Hep3B when compared with the HBV-unrelated HCC cell line Huh7 ($P < .001$).

To investigate the biological function of miR-324-3p in HBV-related HCC cells, Hep3B cells with significant overexpression of miR-324-3p were obtained using cell transfection with miR-324-3p mimic ($P < .001$) (Figure 1B). The cell biological processes analysis results showed that overexpression of miR-324-3p could enhance Hep3B cell proliferation, migration, and invasion (all $P < .01$) (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 $P < .01$) (Figure 2). Additionally, significantly elevated miR-324-3p expression was observed in HBV-related HCC patients compared with CHB and HBV-unrelated HCC patients (both $P < .001$). 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

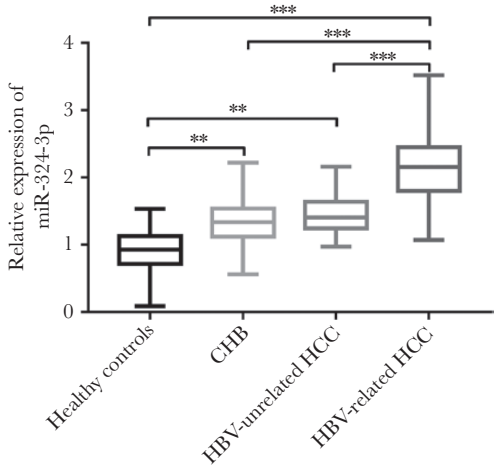

Figure 2. Serum expression of miR-324-3p increased most in HBV-related HCC, followed by patients with HBV-unrelated HCC and CHB, finally, healthy controls. HBV-related HCC patients had the highest serum miR-324-3p levels. $^{**}P < .01$; $^{***}P < .001$. Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p.

value analysis. First, the differentially expressed miR-324-3p and the available biomarkers between HBV-related HCC patients and healthy controls were analyzed, and the ROC curves are exhibited in Figure 3A. In addition, an 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 was also evaluated (Figure 3B, 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 a sensitivity and specificity of 62.5% and 75.00%, respectively. When integrating the 3 biomarkers, the diagnostic performance was improved, with an AUC of 0.941 and a 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 ($P = .003$), tumor size ($P = .010$), Barcelona Clinic Liver Cancer (BCLC) stage ($P = .003$), and Tumor Node Metastasis (TNM) stage ($P = .005$). In other words, patients who 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, or tumor differentiation (all $P > .05$).

Differentially Expressed miR-324-3p Between HCC Patients With Different Statuses of Liver Cirrhosis

An association of miR-324-3p with liver cirrhosis was found in HBV-related HCC patients by the chi-square test. In addition, serum expression of miR-324-3p was found to be significantly elevated in positive liver cirrhosis patients compared with negative cases ($P < .001$) (Figure 4A). ROC curve–based serum miR-324-3p levels were plotted for HBV-related HCC patients, and the results, shown in Figure 4B, indicated that serum miR-324-3p had the 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, Kaplan-Meier survival curves for patients with HBV-related HCC were plotted (Figure 5). It was found that patients with high-serum miR-324-3p had a significantly poorer overall survival than those with low miR-324-3p levels (log-rank $P = .023$). 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 to patients' survival (all $P < .05$). The subsequent multivariate analysis, which included the variables

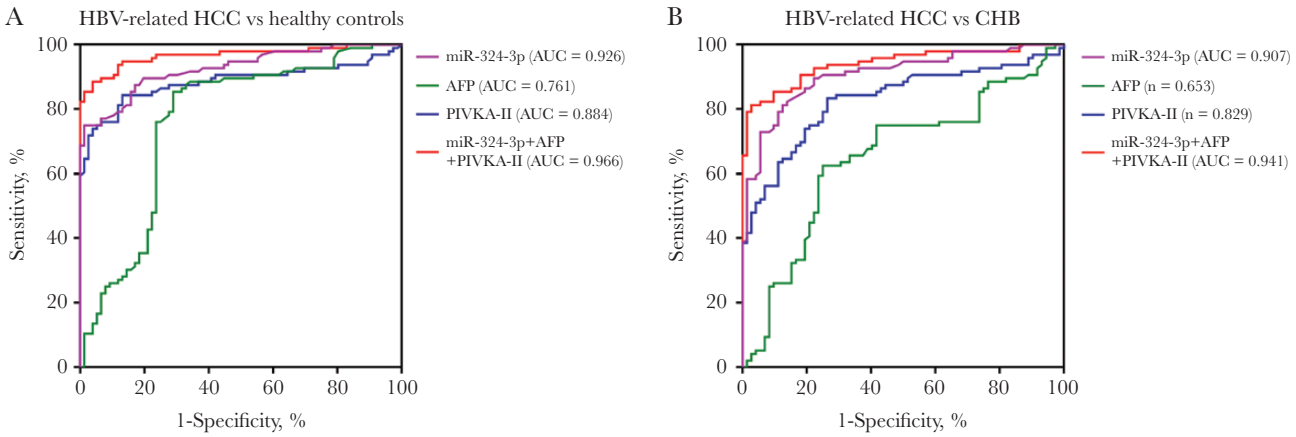

Figure 3. ROC curves based on serum miR-324-3p, AFP, and PIVKA-II for patients with HBV-related HCC. A, ROC curves of miR-324-3p, AFP, and PIVKA-II in discriminating HBV-related HCC patients from healthy controls. B, ROC curves of miR-324-3p, AFP, and PIVKA-II in discriminating HBV-related HCC patients from CHB patients. Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; CHB, chronic hepatitis B; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p; PIVKA-II, protein induced by vitamin K absence/antagonist II; ROC, receiver operating characteristics.

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				
Indicators	AUC	Cutoff Value	Sensitivity, %	Specificity, %
miR-324-3p	0.926	1.608	77.08	93.42
AFP	0.761	8.35	85.42	71.05
PIVKA-II	0.884	37.25	84.38	86.84
miR-324-3p+AFP+PIVKA-II	0.966	\	88.54	96.05
HBV-Related HCC Patients vs CHB Patients				
Indicators	AUC	Cutoff Value	Sensitivity, %	Specificity, %
miR-324-3p	0.907	1.690	81.25	87.5
AFP	0.653	20.15	62.50	75.00
PIVKA-II	0.829	40.3	83.33	73.61
miR-324-3p+AFP+PIVKA-II	0.941	\	81.25	97.22

Abbreviations: AFP, alpha-fetoprotein; AUC, area under the curve; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p; PIVKA-II, protein induced by vitamin K absence/antagonist II.

with significant results from the univariate analysis, indicated that BCLC stage, TNM stage, and serum miR-324-3p were independent prognostic biomarkers (all $P < .05$).

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. reported on the expression and functional role of miR-324-3p in HCC; it found that miR-324-3p was increased in the 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. reported that miR-324-3p

Table 3. Relationship Between Serum miR-324-3p and Clinicopathological Characteristics in Patients With HBV-Related HCC

Features	Total No.	Low miR-324-3p	High miR-324-3p	*P* Value
No.	96	44	52	
Age, y				.148
≤60	32	18	14	
>60	64	26	38	
Gender				.617
Females	41	20	21	
Males	55	24	31	
Liver cirrhosis				.003*
Negative	37	24	13	
Positive	59	20	39	
Tumor size, cm				.010*
≤5	54	31	23	
>5	42	13	29	
Differentiation				.434
Well and moderate	57	28	29	
Poor	39	16	23	
BCLC stage				.003*
A	59	34	25	
B	37	10	27	
TNM stage				.005*
I–II	55	32	23	
III	41	12	29	

Abbreviations: BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; TNM, Tumor Node Metastasis.
*$P < .05$.

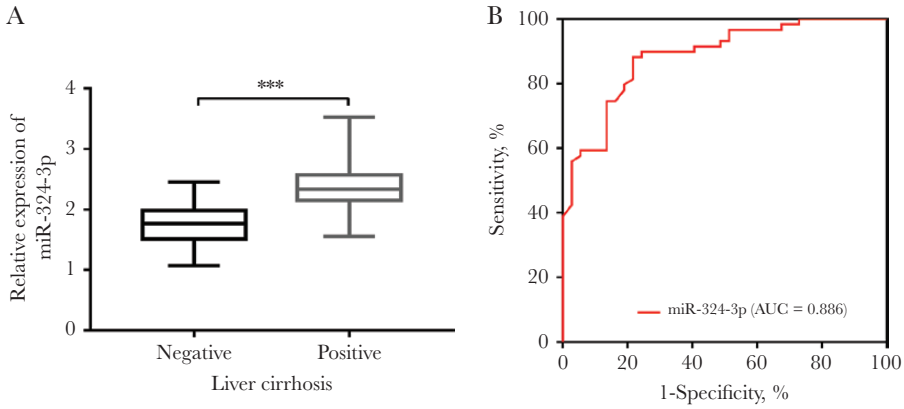

Figure 4. Differentially expressed miR-324-3p had the potential to distinguish liver cirrhosis–positive patients from negative patients. A, Serum miR-324-3p was significantly higher in liver cirrhosis–positive patients compared with negative cases. $^{***}P < .001$. B, ROC curve–based serum miR-324-3p levels in HBV-related HCC patients with or without liver cirrhosis (AUC, 0.886). Abbreviations: AUC, area under the curve; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p; ROC, receiver operating characteristics.

expression was higher in HBV-positive HCC patients compared with noncancerous controls [19]. In the present study, the markedly increased miR-324-3p in Hep3B compared with Huh7 cells might indicate that miR-324-3p expression is 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 practice [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 of early-stage HCC patients can be identified using AFP detection, because the patients with significantly elevated AFP levels account for only 60%–80% of all cancer cases, and high AFP levels can also be found in non-HCC conditions [23]. PIVKA-II is a newly applied biomarker and has improved the diagnosis of HCC, with relatively high sensitivity and specificity [24]. However, specific biomarkers to diagnose HCC cases with HBV infection are still lacking.

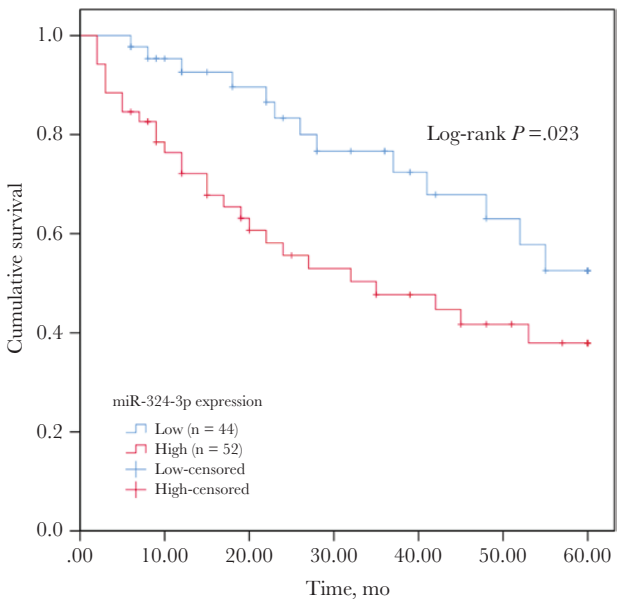

Figure 5. Kaplan-Meier survival curves for HBV-related HCC patients with different serum levels of miR-324-3p. Patients with high levels of miR-324-3p had worse overall survival than those with low miR-324-3p levels (log-rank $P = .023$). Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; miR-324-3p, microRNA-324-3p.

Numerous studies have demonstrated that aberrant miRNAs in various diseases are involved in disease development and progression, so that many deregulated serum miRNAs have been determined as candidate biomarkers for disease diagnosis and prognosis [25]. For example, serum miR-10b-5p was elevated in HCC patients and had relatively high diagnostic accuracy for distinguishing early-stage HCC cases [26]. HCC patients had significantly decreased serum miR-129-5p, which served as a potential biomarker for diagnosis and prognosis [27]. In HBV-related HCC, several serum miRNAs have also been identified as being related to disease development and progression, such as miR-375 [28] and miR-223-3p [29]. In the current study, serum expression of miR-324-3p was also investigated. Our findings revealed that expression of serum miR-324-3p was lowest in healthy controls, higher in patients with CHB and HBV-unrelated HCC, and highest in patients with HBV-related HCC. The highest serum miR-324-3p was observed in HBV-related HCC patients, which was consistent with the expression in HCC cell lines. According to the ROC curves based on serum miR-324-3p and 2 HCC biomarkers (AFP and PIVKA-II) in the same serum samples, the AUCs of serum miR-324-3p were higher than those of AFP and PIVKA-II for the diagnosis of HBV-related HCC in healthy controls and 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

Table 4. Analysis of Prognostic Indicators for HBV-Related HCC Patients Using Cox Regression Analysis

	Univariate Analysis			Multivariate Analysis		
Indicators	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	1.756	0.685–3.085	.745	-	-	-
Gender	1.869	0.699–3.307	.658	-	-	-
Liver cirrhosis	2.217	0.856–4.581	.148	-	-	-
Tumor size	1.669	0.819–2.890	.274	-	-	-
Differentiation	2.098	0.985–3.332	.056	-	-	-
BCLC stage	2.569	1.628–3.874	.016*	2.369	1.382–3.587	.032*
TNM stage	2.271	1.448–3.197	.022*	2.007	1.318–2.949	.036*
AFP	1.987	0.874–2.408	.185	-	-	-
PIVKA-II	1.953	1.267–2.949	.041*	2.017	0.973–3.228	.068
miR-324-3p	2.885	1.841–4.008	.008*	2.594	1.789–3.884	.012*

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; PIVKA-II, protein induced by vitamin K absence/antagonist II; TNM, Tumor Node Metastasis.

* $P < .05$.

HCC patients and had the potential to improve the diagnostic value of AFP and PIVKA-II. Nevertheless, these conclusions need to be validated using larger cohorts in future studies.

This study collected the clinicopathological characteristics of patients with HBV-related HCC and found a significant association of serum miR-324-3p with liver cirrhosis, tumor size, BCLC stage, and TNM stage. These findings indicate that miR-324-3p might be involved in the development of HCC. A previous study also provided evidence for the relationship between miR-324-3p and HCC tumor size and TNM stage [18], 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 could lead to HCC [30]. In our study, HCC patients were positive for HBV infection, and the relationship found between miR-324-3p and cirrhosis might suggest that miR-324-3p was involved in the progression of HBV-related hepatitis to HCC. To confirm this, the expression of miR-324-3p was compared between liver cirrhosis–positive and –negative patients. The results revealed that liver cirrhosis–positive patients had significantly higher serum miR-324-3p and that elevated serum miR-324-3p might have the potential to distinguish liver cirrhosis–positive HCC patients from –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 to 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 of HBV-related HCC patients.

In conclusion, we 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 patients from healthy individuals and CHB patients. High-serum miR-324-3p could predict poor overall survival 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. This study had several limitations, 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.

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Potential conflicts of interest. The authors declare that they have no conflicts of interest. All authors: no reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Patient consent. 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 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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