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ORIGINAL PAPER

Diagnostic and prognostic significance of serum miR-24-3p in HBV-related hepatocellular carcinoma

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Abstract The aim of this study was to explore the diagnostic and prognostic value of serum microRNAs (miRNAs) in hepatitis B viral (HBV)-related hepatocellular carcinoma (HCC). We retrospectively analyzed clinical data of 84 consecutive patients with HBV-related HCC who underwent curative resection. Additionally, we enrolled 46 healthy controls and 31 patients with chronic liver disease (CLD). Serum levels of miR-155-5p, miR-24-3p, miR-490-3p, miR-210-3p, and miR-335-5p were measured. Associations of serum miRNAs with clinicopathological factors were evaluated. Receiver operating characteristic curves were established for discriminating HCC patients from CLD patients, and the area under the curve (AUC) was calculated. Overall survival (OS) and disease-free survival (DFS) were examined by the Kaplan–Meier method. Prognostic factors were determined by multivariate Cox analysis. Consequently, serum miR-24-3p levels were significantly greater in HCC patients than healthy controls and CLD patients. Serum miR-24-3p was significantly associated with vascular invasion in HCC patients. Serum miR-24-3p discriminated HCC patients from CLD, with an AUC of 0.636 [95 % confidence interval (CI) 0.524–0.748]. Combined serum alpha-fetoprotein (AFP) and miR-24-3p had an increased AUC of 0.834 (95 % CI 0.745–0.923; $P < 0.001$). Elevated serum miR-24-3p was an independent poor prognostic factor for OS and DFS of HCC patients. In conclusion, the combination of serum miR-24-3p and AFP improves the diagnostic accuracy for HCC prediction compared to each biomarker alone. High serum miR-24-3p level is an independent predictor of poor OS and DFS in patients with HBV-related HCC.

Keywords Circulating miRNAs · miR-24-3p · Hepatocellular carcinoma · Diagnosis · Prognosis

Abbreviations

AFP	Alpha-fetoprotein
ANOVA	Analysis of variance
AUC	Area under the curve
CI	Confidence interval
CLD	Chronic liver disease
DCP	Des-γ-carboxy prothrombin
DFS	Disease-free survival
HBV	Hepatitis B viral
HCC	Hepatocellular carcinoma
miRNAs	microRNAs
OS	Overall survival
RFA	Radiofrequency ablation
ROC	Receiver operating characteristic
TNM	Tumor node metastasis

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths in the world [1]. Hepatitis B viral (HBV) infection represents an important risk factor

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for HCC. HBV-endemic countries particularly China have a high prevalence of HCC, accounting for about half of the global HCC burden [2]. Despite many treatment modalities such as radiofrequency ablation (RFA), tumor resection, and liver transplantation, the prognosis of HCC remains dismal largely because of late detection and high frequency of recurrence. The 5-year overall survival (OS) rate is less than 5 % [3]. Numerous serum markers including alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) have been explored for HCC detection in surveillance programs. However, the sensitivity and specificity of AFP for early detection of HCC are low, since serum AFP level is often elevated in patients with benign liver diseases such as hepatitis and cirrhosis either. The diagnostic sensitivity of DCP is modest because only half of small HCCs (<3 cm in diameter) show an elevation in the DCP level [4]. Therefore, there is an urgent need for identification of novel effective biomarkers for HCC detection.

microRNAs (miRNAs) are small (typically about 22 nucleotides in size), endogenous, noncoding RNAs that regulate gene expression by repressing translation or promoting mRNA degradation at the posttranscriptional level [5, 6]. miRNAs are involved in a variety of cellular processes such as proliferation, differentiation, development, stress response, and apoptosis [7, 8]. Tumor tissues often show abnormal expression of miRNAs, suggesting their critical roles in tumorigenesis [9–13]. In addition to solid tissues, miRNAs have also been identified in body liquids like blood. It has been suggested that cancer tissues are an important source of circulating miRNAs [14]. Additionally, miRNAs in the blood exhibit high stability even after multiple freezing–thawing processes. These features make circulating miRNAs a valuable, noninvasive biomarker for cancer detection and management.

A panel of five miRNAs (i.e., miR-155-5p, miR-24-3p, miR-490-3p, miR-210-3p and miR-335-5p) has been identified to be dysregulated in HCC tissues and cell lines [15–19]. In the present study, we investigated serum levels of the five miRNAs in patients with HBV-related HCC, patients with nonmalignant chronic liver disease (CLD), and healthy volunteers and evaluated the diagnostic and prognostic value of abnormal miRNAs in HBV-related HCC.

Table 1 Primer sequences used in this study

miRNA	Primer sequences
miR-155-5p	5′-TTAATGCTAATCGTGATAGGGGT-3′
miR-24-3p	5′-TGGCTCAGTTCAGCAGGAAC-3′
miR-490-3p	5′-CAACCTGGAGGACTCCATGC-3′
miR-210-3p	5′-CTGTGCGTGTGACAGCGG-3′
miR-335-5p	5′-TCAAGAGCAATAACGAAAAATGT-3′

Patients and methods

Subjects and study design

We reviewed the medical records of 84 consecutive patients with HBV-related HCC who had undergone curative surgical resection between January 2009 and December 2011 at Anhui Provincial Hospital (Hefei, China). The HCC patients were randomly assigned to a training set ($n = 12$) and a validation test ($n = 72$). All the cases of HCC were confirmed by pathology. Preoperative serum samples and complete follow-up data were available for each HCC patient. Clinical characteristics of the patients are summarized in Table 1. Additionally, we enrolled 46 healthy controls ($n = 12$ for the training set and $n = 34$ for the validation set) and 31 patients with CLD. All subjects included in this study gave their written

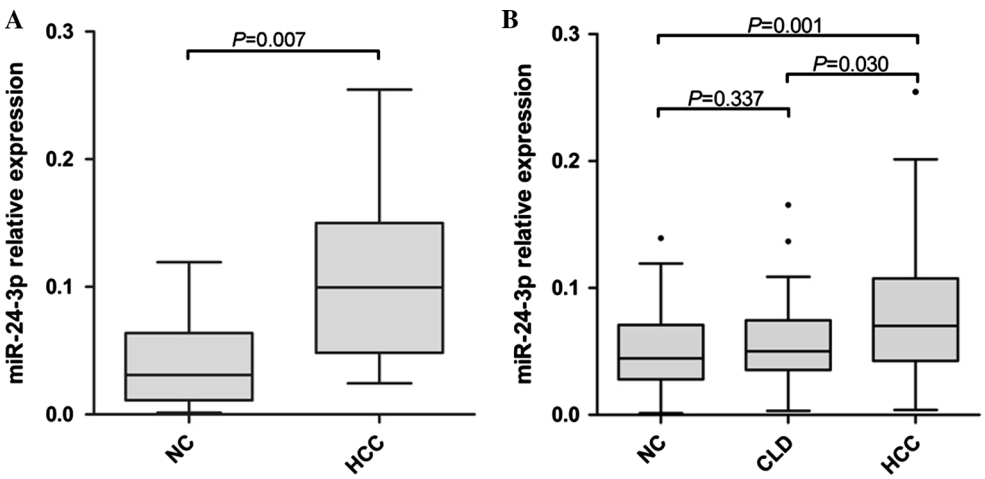

Fig. 1 Significant increase of serum miR-24-3p levels in hepatocellular carcinoma (HCC) patients. **a** Measurement of serum miR-24-3p levels in HCC patients and normal controls (NC) in the training set. **b** Measurement of serum miR-24-3p levels in HCC patients, normal controls (NC), and CLD patients in the validation set. Serum miR-24-3p levels were significantly elevated in HCC patients compared with healthy controls in the training set ($P < 0.05$)

Table 2 Associations of serum miR-24-3p levels with clinicopathologic parameters

Parameters	*n*	miR-24-3p		χ^2	*P* value
		Low	High		
Age (years)					
>60	25	15	10	1.532	0.216
≤60	47	21	26		
Gender					
Male	57	27	30	0.758	0.384
Female	15	9	6		
Tumor size (cm)					
>5	40	21	19	0.225	0.635
≤5	32	15	17		
Tumor nodule					
Multiple	14	5	9	1.419	0.234
Single	58	31	27		
Vascular invasion					
Yes	24	6	18	9.000	0.003
No	48	30	18		
Edmondson grade					
I–II	38	22	16	2.006	0.157
III–IV	34	14	20		
Tumor capsula					
Complete	44	25	19	2.104	0.147
None	28	11	17		
Liver cirrhosis					
Present	65	30	35	2.532[a]	0.112
Absent	7	6	1		
Child-Pugh grade					
A	64	33	31	0.141[a]	0.708
B	8	3	5		
AFP (ng/ml)					
>20	53	24	29	1.787	0.181
≤20	19	12	7		
TNM stage					
I–II	47	28	19	0.182	0.670
III–IV	25	8	17		

[a] χ^2 Value after continuity correction

informed consent. This study was performed in accordance with the ethical guidelines of the declaration of Helsinki and approved by the ethics committee of Anhui Provincial Hospital.

Serum samples were collected from each participant. The levels of miR-155-5p, miR-24-3p, miR-490-3p, miR-210-3p and miR-335-5p in serum samples were measured using quantitative real-time polymerase chain reaction (qRT-PCR) assay as described below. The diagnostic and prognostic significance of serum miRNAs was explored.

qRT-PCR analysis

Total RNA was isolated from serum samples using the miRNeasy Serum/Plasma Kit (#217184; QIAGEN, Germany) following the manufacturer's protocol. Cel-miR-39 mimic (#219610; QIAGEN) was added into the serum samples as an external control for qRT-PCR analysis. cDNA was synthesized from total RNA with the miScript II RT Kit (#218161; QIAGEN). Serum miRNAs levels were measured using the miScript SYBR® Green PCR Kit (#218073; QIAGEN) on the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). miRNA-specific forward primers (Table 1) were designed on the basis of the miRNA sequences obtained from the miRBase database (http://www.mirbase.org/). PCR cycling conditions were as follows: initial denaturation at 95 °C for 15 min and 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s. Relative serum miRNA levels were calculated after normalizing against cel-miR-39 [14, 20] using the $2\text{-}\Delta\Delta C_t$ method, where $\Delta C_t = C_t(\text{target}) - C_t(\text{Cel-miR-39})$.

Statistical analysis

Statistical analysis was done with SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The difference in serum miRNA levels was determined by the Mann–Whitney test or analysis of variance (ANOVA) followed by Tukey's test. Associations of serum miR-24-3p levels with clinicopathologic parameters were analyzed using the χ^2 test. Receiver operating characteristic (ROC) curves were established for discriminating patients with HCC from those with CLD, and the area under the curve (AUC) was calculated. Survival curves were plotted using the Kaplan–Meier method, and differences in survival rates were analyzed using the log-rank test. Prognostic relevance of each variable to overall survival (OS) and disease-free survival (DFS) was evaluated using the Cox regression model. A two-side *P* value <0.05 was considered as statistically significant.

Results

Increased serum levels of miR-24-3p in HCC patients

Serum miR-24-3p levels were significantly elevated in HCC patients compared with healthy controls in the training set ($P < 0.05$; Fig. 1a). However, the other 4 miRNAs showed comparable serum levels in HCC patients versus healthy controls ($P > 0.05$; data not shown). We confirmed the significant increase of serum miR-24-3p levels in HCC patients in the validation set comprising 60

HCC patients, 34 healthy controls, and 31 CLD patients (Fig. 1b).

Associations of serum miR-24-3p levels with clinicopathologic parameters of HCC

Based on the median serum miR-24-3p level, our HCC patients were divided into two subgroups: low miR-24-3p group (≤0.0702) and high miR-24-3p group (>0.0702). Our results revealed that serum miR-24-3p was significantly associated with vascular invasion, but not with other clinicopathologic parameters including age, gender, TNM stage, Child-Pugh grade classification, serum AFP level, tumor multiplicity, tumor size, capsula status, TNM stage, and Edmondson grade (Table 2).

Diagnostic potential of serum miR-24-3p levels in HCC

To evaluate whether serum level of miR-24-3p can be used as a diagnostic marker for HCC, ROC curve analyses were performed. We found that serum levels of miR-24-3p discriminated HCC patients from CLD, with an AUC of 0.636 [95 % confidence interval (CI) 0.524–0.748; $P = 0.029$] (Fig. 2). The AUC of serum AFP alone and in combination with serum miR-24-3p for predicting HCC patients in the validation set was 0.789 (95 % CI 0.692–0.885; $P < 0.001$) and 0.834 (95 % CI 0.745–0.923; $P < 0.001$), respectively.

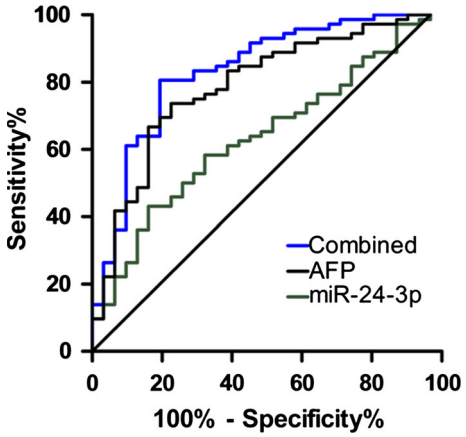

Fig. 2 ROC curves of serum AFP and miR-24-3p alone or in combination in discriminating HCC patients from CLD patients

Prognostic significance of serum miR-24-3p levels in HCC

As shown in Fig. 3, patients with high serum miR-24-3p levels had a significantly shorter median OS (15 vs. 33 months; $P = 0.002$) and DFS (15 vs. 29 months; $P = 0.007$) than those with low serum miR-24-3p levels. To determine whether serum miR-24-3p level was an independent risk factor for prognosis, the Cox proportional hazard regression model was employed. Univariate and multivariate survival analysis indicated that elevated serum miR-24-3p had a negative prognostic impact on OS (HR = 2.141, 95 % CI 1.158–3.960; $P = 0.015$; Table 3) and DFS (HR = 2.055, 95 % CI 1.114–3.792; $P = 0.021$; and Table 4).

Discussion

Since circulating miRNAs were discovered in 2008 [21], they hold promise as useful biomarkers for the diagnosis and prognosis of human cancers. miR-24 is frequently deregulated in a variety of malignancies such as breast cancer [22], pancreatic cancer [23], and non-small cell lung cancer [24]. In this study, we demonstrated that serum miR-24-3p levels were significantly higher in HBV-related HCC patients than healthy controls and CLD patients. In agreement with our results, Liu et al. [25] reported that

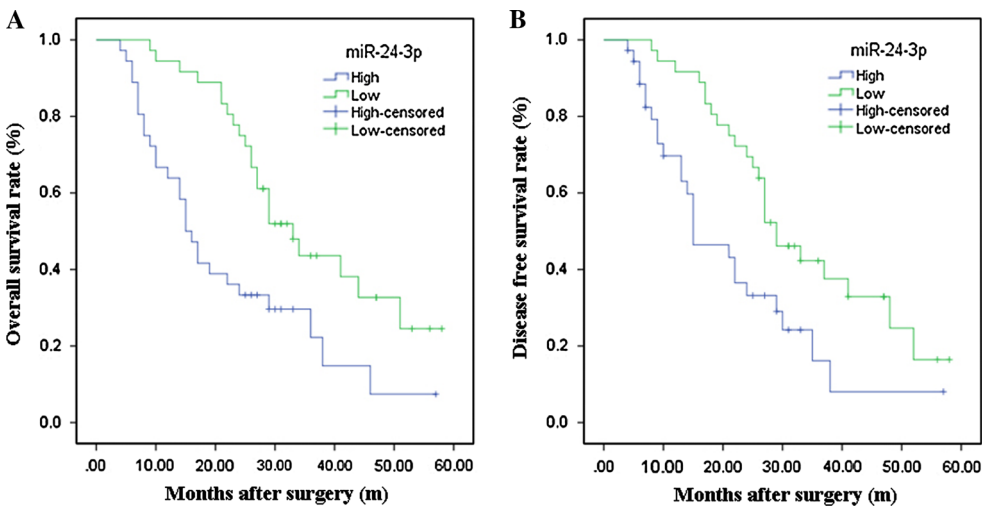

Fig. 3 OS and DFS rates of HCC patients with high versus low serum miR-24-3p levels. **a** OS rate of HCC patients with high versus low serum miR-24-3p levels. **b** DFS rate of HCC patients with high versus low serum miR-24-3p levels. Patients with high serum miR-24-3p levels had a significantly shorter median OS (15 vs. 33 months; $P = 0.002$) and DFS (15 vs. 29 months; $P = 0.007$) than those with low serum miR-24-3p levels

Table 3 Univariate and multivariate analysis of prognostic factors for OS

Variables	Univariate			Multivariate		
	HR	95 % CI	*P*	HR	95 % CI	*P*
miR-24-3p (high vs. low)	2.364	1.341–4.167	0.003	2.141	1.158–3.960	0.015
Tumor nodule (multiple vs. single)	2.980	1.540–5.766	0.001	1.391	0.575–3.366	0.464
Vascular invasion (yes vs. no)	2.655	1.489–4.735	0.001	2.358	1.163–4.783	0.017
TNM stage (III–IV vs. I–II)	4.417	2.397–8.139	<0.001	2.788	1.251–6.210	0.012
Edmondson grade (III–IV vs. I–II)	2.711	1.514–4.852	0.001	2.011	1.029–3.930	0.041
Tumor capsula (none vs. complete)	1.934	1.099–3.404	0.022	1.723	0.920–3.226	0.089
Child-Pugh grade (B vs. A)	4.541	2.069–9.967	<0.001	4.209	1.699–10.424	0.002
Serum AFP (ng/ml) (>20 vs. ≤20)	2.493	1.223–5.080	0.012	3.238	1.520–6.896	0.002

Table 4 Univariate and multivariate analysis of prognostic factors for DFS

Variables	Univariate			Multivariate		
	HR	95 % CI	*P*	HR	95 % CI	*P*
miR-24-3p (high vs. low)	2.117	1.197–3.744	0.010	2.055	1.114–3.792	0.021
Tumor nodule (multiple vs. single)	2.758	1.372–5.544	0.004	1.773	0.725–4.334	0.210
Vascular invasion (yes vs. no)	2.707	1.484–4.938	0.001	2.374	1.170–4.820	0.017
TNM stage (III–IV vs. I–II)	4.764	2.447–9.274	<0.001	2.580	1.157–5.756	0.021
Edmondson grade (III–IV vs. I–II)	2.560	1.423–4.605	0.002	2.283	1.210–4.306	0.011
Tumor capsula (none vs. complete)	2.138	1.210–3.777	0.009	1.845	0.984–3.460	0.056
Serum AFP (ng/ml) (>20 vs. ≤20)	2.659	1.266–5.583	0.010	3.134	1.432–6.862	0.004

miR-24 is upregulated in HCC tumor tissues relative to adjacent noncancerous liver tissues. The upregulation of miR-24 in HCC suggests its contribution to the development of this disease. Indeed, miR-24 overexpression has been shown to accelerate the proliferation and induce apoptotic death of HCC cells [25]. It has been documented that the miR-23a ~ 27a ~ 24 cluster is upregulated in HCC tissues and exerts antiapoptotic and pro-proliferative effects on HCC cells [19]. Hatziapostolou et al. [26] reported that increased miR-24 expression promotes hepatocellular oncogenesis through a miRNA-inflammatory feedback loop circuit consisting of miR-124, IL6R, STAT3, miR-24, and miR-629.

It has been reported that upregulated miR-24-3p promotes tumor cell invasion by modulating EGF signaling and the expression of Sprouty 2 [22, 27]. Interestingly, both EGF signaling and Sprouty 2 are dysregulated in HCC and implicated in invasion and metastasis. Therefore, miR-24 may also affect HCC cell invasion. This hypothesis is supported by our finding that increased serum miR-24-3p levels were significantly correlated with vascular invasion in HCC patients.

Circulating miRNAs are emerging as novel noninvasive biomarkers for detection and prognosis of HCC [28]. Zhang et al. [29] showed that serum miR-143 and miR-215 concentrations have diagnostic value in the differentiation of HCC patients from healthy controls. Consistently, our data revealed the diagnostic significance of serum miR-24-3p in HCC, with an AUC of 0.636 (95 % CI 0.524–0.748). Of particular interest, the combination of serum miR-24-3p and AFP levels was more accurate in detecting HCC than each biomarker alone, with a statistically significant increase in the AUC. Serum miR-24 also presents diagnostic potential in patients with lung cancer [30]. Our data further demonstrated that higher serum miR-24-3p concentrations were significantly associated with shorter OS and DFS. Multivariate analysis indicated an independent poor prognostic impact of elevated serum miR-24-3p concentrations in HCC patients. Taken together, we identified serum miR-24-3p as a novel diagnostic and prognostic factor for HCC.

miR-155-5p, miR-490-3p, miR-210-3p, and miR-335-5p have been recently reported to be deregulated in HCC tissues [15–18]. However, our results showed no significant differences in their serum levels between HCC patients and healthy controls. This inconsistency may reflect that the four miRNAs in the serum are not specifically derived from HCC tissues. Indeed, inflammatory tissues have been shown to express high level of miR-155 [31].

A major limitation of this single-institute study is relatively small sample size. Additionally, there is a potential selection bias inherent to any retrospective study. A prospective study with a larger cohort of patients is thus needed to confirm the present findings.

In summary, our present data indicate that HCC patients have significantly higher levels of serum miR-24-3p than normal controls and CLD patients. The combination of serum miR-24-3p and AFP improves the diagnostic accuracy for HCC prediction compared to each biomarker alone. High serum miR-24-3p level is an independent predictor of poor OS and DFS in HCC patients receiving curative surgery. These findings warrant large-scale, prospective studies to confirm the diagnostic and prognostic utility of serum miR-24-3p in HCC patients.

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Conflict of interest The authors declare that they have no competing financial interests.

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