Am J Transl Res 2021;13(10):11531-11539
www.ajtr.org /ISSN:1943-8141/AJTR0135705

Original Article

miR-506-3p can inhibit cell proliferation and is a diagnostic and prognostic marker of liver cancer

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Received May 26, 2021; Accepted July 29, 2021; Epub October 15, 2021; Published October 30, 2021

Abstract: Background: At the time of diagnosis, most patients with liver cancer (LC) are at advanced stage, which increases the difficulty of treatment. MiR-506-3p is considered an anti-oncogene in a wide spectrum of malignancies. This investigation aims to determine the clinical implications of miR-506-3p in diagnosis and prognosis of LC. Methods: The expression of miR-506-3p in tissues and serum samples of 92 LC patients was detected using quantitative real-time PCR (qRT-PCR), and the connection between serum miR-506-3p and pathologic features of LC patients was analyzed. The diagnostic efficacy of miR-506-3p in LC was visualized by Receiver Operating Characteristic (ROC) curves, its prognostic implications in LC were confirmed by follow-up, and its impact on LC cell proliferation was analyzed by CCK-8 assay. Results: miR-506-3p was lowly expressed in LC tissues and serum samples. Reduced serum miR-506-3p expression indicated larger tumor size, higher TNM stage, and poorer differentiation degree in LC patients. The area under the curve (AUC) of serum miR-506-3p in diagnosing LC was 0.911, and for distinguishing tumor size, TNM stage and pathologic differentiation degree, AUC was 0.751, 0.825 and 0.777, respectively. Kaplan-Meier analysis demonstrated decreased overall survival in patients presenting with reduced serum miR-506-3p. Cox proportional hazards regression model analysis revealed that TNM staging and low serum miR-506-3p expression were independent prognostic factors in patients with LC. *In vitro* experiments identified that the proliferation of LC cells decreased significantly following miR-506-3p up-regulation. Conclusion: miR-506-3p, capable of inhibiting LC cell proliferation, is a possible diagnostic and prognostic biomarker of LC.

Keywords: Liver cancer, miR-506-3p, diagnosis, prognosis

Introduction

The incidence of liver cancer (LC) increased by 4.6% from 2005 to 2015 and it ranks the fourth most deadly cancer worldwide [1]. The incidence of LC varies from region to region, but mostly occurs in developing countries. Heavy drinking, virus infection and diet are risk factors for the occurrence of LC [2]. Due to non-specific symptoms of early LC and lack of specificity, patients are usually diagnosed at advanced stages [3]. Surgery is considered the most effective treatment strategy, but unfortunately, more than 80% of patients were deprived of the opportunity for surgical treatment when diagnosed [4]. Moreover, due to the insensitivity of patients with advanced LC to clinical treatment strategies, the average overall survival (OS) is only 3-16 months [5]. Therefore, exploring the markers related to the diagnosis and prognosis of LC and its pathogenesis carries huge clinical implications.

As endogenous single-stranded non-coding RNAs with a length of 18-25 nucleotides, microRNAs (miRNAs) can specifically bind to 3'-untranslated regions (UTRs) of mRNAs and induce direct degradation or translation destruction of these genes [6]. The critical role of miRNAs in cell proliferation, differentiation, and metastasis has been well documented [7]. Their aberrant expression profiles are related to the occurrence and progression of many diseases, especially in malignant tumors [8]. Pre-

vious reports have shown that miR-506-3p is down-regulated in ovarian cancer, and is an anti-oncogene capable of inhibiting ovarian cancer cell proliferation by negatively modulating myotubulin-related protein 6 expression [9]. In osteosarcoma, miR-506-3p plays a tumor suppressor role to inhibit the proliferation and metastasis of osteosarcoma cells by suppressing the expression of RAB3D [10]. In non-small cell lung cancer (NSCLC), miR-506-3p is notably decreased, and its down-regulation can lead to gefitinib resistance; hence restoring miR-506-3p is a new direction to overcome gefitinib resistance in cancer cells [11]. Most previous studies focused on miRNAs in tissue specimens, while some clarified the diagnostic and prognostic potential of circulating miRNAs [12]. For example, the combination of miR-21, miR-155 and miR-145 was shown to be a valid strategy to differentiate NSCLC patients from controls, with a sensitivity, specificity and AUC of 76.5%, 81.3% and 0.87 respectively [13]. However, the diagnostic and prognostic role miR-506-3p plays in LC remains elusive.

Accordingly, we conducted this investigation and verified that with decreased levels in LC patients, miR-506-3p had the differential diagnostic value for the early stage and severity of the disease. In addition, miR-506-3p can inhibit LC cell proliferation.

Materials and methods

Clinical information and specimen collection

The study enrolled 92 patients with liver cancer treated in the Second Affiliated Hospital of Nanchang University from February 2013 to January 2016, and the resected LC and paracancerous tissue samples were preserved for analysis. Additionally, 92 healthy controls with no tumor-related diseases or other major diseases from the Hepatobiliary Surgery of the Second Affiliated Hospital of Nanchang University during the same period were selected.

Inclusion criteria: Patients who were diagnosed with hepatocellular carcinoma by histology or pathology [14]; Patients with complete clinical and pathological data; Patients without history of prior surgery, radiotherapy, chemotherapy or antibiotic treatment.

Exclusion criteria: those patients with other malignant tumors, mental diseases or dysfunction of important organs were excluded.

Cubital venous blood (5 mL) was drawn from each participant and stored in gel tubes, which were then centrifuged at 1500 g within 30 min. The serum was then stored in 1.5 mL test tubes at -80°C for later use. This retrospective study was in line with the *Declaration of Helsinki*, with approval obtained from the Ethics Review Committee of the Second Affiliated Hospital of Nanchang University. Before inclusion, all participants signed an informed consent.

qRT-PCR

Isolation of total RNA from tissues, serum, and cells was performed with TRIzol kits (Invitrogen company, USA) strictly following the manufacturer's instructions. The determination of RNA concentration and integrity employed NanoDrop ND-1000 spectrophotometer (Nanodrop, USA) and 15% denaturing polyacrylamide gel. Then, the RNA samples were reverse transcribed using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, USA). Thereafter, on the ABI 7500 Real-Time PCR system, qPCR experiment was run in triplicate with the TaqMan Universal PCR Master Mix (Applied Biosystems, USA). With U6 was as the endogenous control, the calculation of cycle threshold (Ct) was conducted using SDS2.0.1 software, and miRNA expression was standardized. $2^{-\Delta\Delta Ct}$ was used to calculate gene expression profiles [15]. Primer sequence designing and synthesizing employed Thermo Fisher Scientific, Shanghai, CHN. miR-506-3p forward: 5'-ACACTCATAAGGCACCCTTC-3', reverse: 5'-TCTACTCAGAAGGGGAGTAC-3'; U6 forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell culture and transfection

Human normal hepatocytes THLE-3 and LC cells CSQT-2, SK-HEP-1 (Otwo Biotech, Shenzhen, CHN) were cultured in DMEM (Acmec Biotech, Shanghai, CHN) containing 10% FBS, 50 U/mL penicillin and 0.1 mg/mL streptomycin. All cell cultures were carried out in a moist and 5% CO_2 incubator at 37°C. Following the instructions of Lipofectamine 3000 reagent (Invitrogen, USA), cell transfection with either miR-506-3p mimics or its corresponding ne-

gative control (NC-mimics; Genechem, Shanghai, CHN) was performed, and the concentration of each miRNA transfected in each well was 50 nM. Cells were gathered for subsequent experiments 48 h after transfection. miR-506-3p mimics (5'-UAAGGCACCCUUCUGAGUAGA-3') and NC mimics (5'-UGUGCGACGCGGCUGGAUGCG-3') were purchased from Thermo Fisher Scientific Co., Ltd., Shanghai, CHN.

Cell proliferation assay

Cell proliferation was evaluated by CCK-8 (Acmec Biotech, Shanghai, CHN) assay. The cells were inoculated in a 96-well plate (3000 cells/well), and immersed in 10 μL CCK-8 solution after culture for 0, 24, 48 and 72 h. Then they were cultured for 2 h and rinsed with PBS, followed by measurement of the absorbance (OD) by a microplate reader at 450 nm (Multiskan FC, Thermo Fisher Scientific, Shanghai, CHN).

Statistical processing

Data were statistically analyzed using GraphPad Prism 6.0 (GraphPad Software, USA) and SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P<0.05$. Counted data were expressed as number of cases/percentage [n (%)] and compared by the Chi-square test. Measured data were recorded in the form of Mean ± SD and compared by the independent samples T-test. Diagnostic performance of each parameter was visualized by the receiver operating characteristic (ROC) curve. OS was the time from the confirmed diagnosis to death or the final follow-up. The visualization and comparison of patient survival were performed by Kaplan-Meier and Log-rank test, respectively. Cox regression was used to analyze the prognostic factors of patients with LC.

Results

Expression of miR-506-3p in LC patients and LC cells

The expression of miR-506-3p in LC was detected by qRT-PCR. LC tissues present markedly decreased miR-506-3p than tumor-adjacent tissues (**Figure 1A**). Next, we detected serum miR-506-3p in LC patients and controls and found distinctly down-regulated miR-506-3p in LC patients (**Figure 1B**). Similarly, compared to human normal hepatocytes THLE-3, miR-506-3p was down-regulated in LC cell strains CSQT-2 and SK-HEP-1 (**Figure 1C**). Pearson correlation coefficient identified a positive connection between tissue and serum expression of miR-506-3p (**Figure 1D**).

Correlation between miR-506-3p and patient characteristics

We collected the clinical information of patients, see **Table 1**. The results indicated that the expression of miR-506-3p was not associated with gender, age, liver cirrhosis, HBV infection, or AFP level in LC patients. Statistical analysis identified that patients with reduced miR-506-3p expression had larger tumor size (P=0.029), higher TNM stage (P=0.002), and poorer differentiation degree (P=0.015).

Diagnostic implications of serum miR-506-3p in LC

Next, we generated ROC curves to assess the diagnostic potential of serum miR-506-3p as a non-invasive marker for LC. ROC data are presented in **Table 2**. ROC analysis determined that serum miR-506-3p was of diagnostic value in distinguishing LC patients and controls, with AUC value, sensitivity, and specificity of 0.911 (95% CI=0.870-0.953), 89.13%, and 80.43% respectively (**Figure 2A**). Serum miR-506-3p had an AUC of 0.751 (95% CI=0.645-0.858), a sensitivity of 87.50%, and a specificity of 51.67% in distinguishing tumor size (**Figure 2B**). The AUC value of serum miR-506-3p for distinguishing TNM staging was 0.825 (95% CI=0.743-0.907), the sensitivity was 97.67%, and the specificity was 53.06% (**Figure 2C**). The AUC value of serum miR-506-3p for distinguishing pathologic differentiation degree was 0.777 (95% CI=0.684-0.871), the sensitivity was 96.77%, and the specificity was 50.82% (**Figure 2D**).

Relationship between serum miR-506-3p and survival in patients with LC

In order to further evaluate whether serum miR-506-3p can be used as a prognostic indicator for patients with LC, we generated ROC curves for prognosis of patients with LC. The follow-up showed that 54 LC patients survived and 38 died. The prognostic AUC value was 0.758 (95% CI=0.661-0.855), the sensitivity was 57.89%, and the specificity was 81.48% (**Figure 3A**).

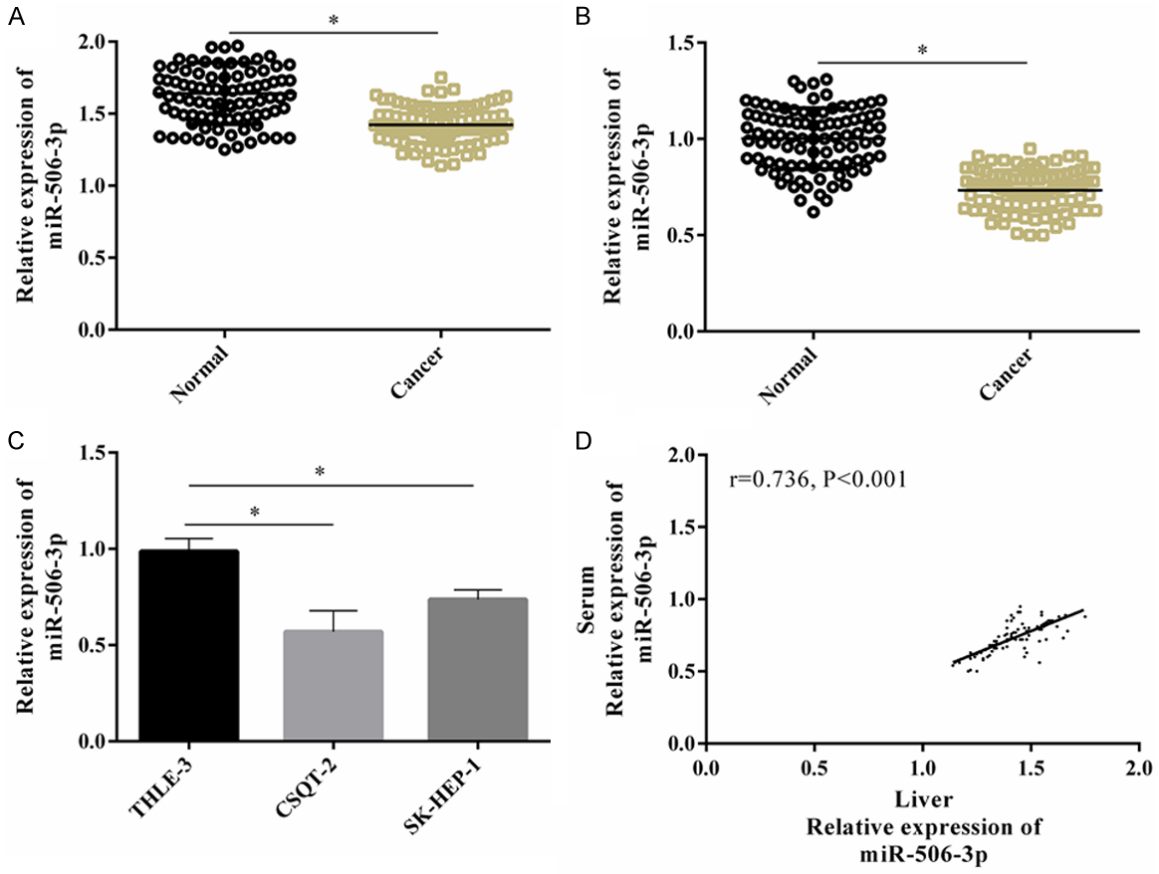

Figure 1. Expression of miR-506-3p in liver cancer tissues and liver cancer cells. A: Expression of miR-506-3p in liver cancer tissues by qRT-PCR. B: Expression of miR-506-3p in serum of patients with liver cancer by qRT-PCR. C: Expression of miR-506-3p in human normal hepatocytes THLE-3 and hepatoma cells CSQT-2 and SK-HEP-1 by qRT-PCR. D: Correlation between miR-506-3p expression in liver cancer tissues and serum samples by Pearson. Note: *P<0.05.

Subsequent Kaplan-Meier survival analysis identified LC patients with decreased serum miR-506-3p had significantly shortened OS (**Figure 3B**). In addition, Cox proportional hazard regression analysis revealed that by univariate analysis, poor prognosis of LC patients was related to TNM stage, differentiation degree, AFP level, and serum miR-506-3p expression. Of note, multivariate analysis showed that TNM stage and low serum miR-506-3p expression were independent prognostic factors for LC patients. This suggests that miR-506-3p can be used as an independent prognostic indicator for the OS of patients with LC (**Tables 3**, **4**).

MiR-506-3p reduced LC cell proliferation

MiR-506-3p was transiently transfected into CSQT-2 and SK-HEP-1 cells to verify its role in LC. MiR-506-3p-mimic transfection led to significantly elevated miR-506-3p in LC cells, compared with NC-mimic transfection (**Figure 4A**). CCK-8 assay identified that the proliferation of LC cells was reduced significantly after miR-506-3p up-regulation (**Figure 4B**).

Discussion

The prognosis of LC patients is still poor even after surgical resection and chemoradiotherapy [16]. Hence, early diagnosis and prognosis evaluation of LC can assist doctors in developing appropriate timely treatment, which is of great importance for survival.

It is increasingly confirmed that the dysregulation of miRNAs is vital in the onset and progression of cancer [17]. Evidence has shown that miR-506 is strongly associated with drug resistance, proliferation, invasion, and metastasis

Table 1. Relationship between serum miR-506-3p expression and clinicopathologic features of patients with LC

Clinicopathologic feature	n=92	miR-506-3p		χ^2	P
		High (n=46)	Low (n=46)		
Gender				0.708	0.400
Female	40	22 (47.83)	18 (39.13)		
Male	52	24 (52.17)	28 (60.87)		
Age (years)				0.438	0.508
<65	31	17 (36.96)	14 (30.43)		
≥65	61	29 (63.04)	32 (69.57)		
Tumor size (cm)				4.792	0.029
≤4	60	35 (76.09)	25 (54.35)		
>4	32	11 (23.91)	21 (45.65)		
TNM staging				9.824	0.002
I-II	49	32 (69.57)	17 (36.96)		
III-IV	43	14 (30.43)	29 (63.04)		
Differentiation degree				5.887	0.015
High/moderate differentiation	61	36 (78.26)	25 (54.35)		
Low differentiation	31	10 (21.74)	21 (45.65)		
Cirrhosis				1.577	0.209
No	50	28 (60.87)	22 (47.83)		
Yes	42	18 (39.13)	24 (52.17)		
HBV infection				2.029	0.154
No	24	15 (32.61)	9 (19.57)		
Yes	68	31 (67.39)	37 (80.43)		
AFP (μg/L)				1.181	0.277
<400	33	19 (41.30)	14 (30.43)		
≥400	59	27 (58.70)	32 (69.57)		

Table 2. ROC measurements

Measures	AUC	95% CI	Cut-off	Specificity (%)	Sensitivity (%)
miR-506-3p					
Liver cancer	0.911	0.870-0.953	0.86	80.43	89.13
Tumor size	0.751	0.645-0.858	0.78	51.67	87.50
TNM staging	0.825	0.743-0.907	0.80	53.06	97.67
Differentiation degree	0.777	0.684-0.871	0.79	50.82	96.77

of tumor cells [18]. Research by Sun et al. indicated miR-506-3p, an anti-oncogene in ovarian cancer, can increase the reaction to poly-ADP-ribosome polymerase inhibitor and cisplatin in serous cystadenocarcinoma of the ovary by targeting EZH2/β-catenin [19]. Wen et al. indicated that miR-506, with down-regulated expression in around 80% of cervical cancer samples, can be a tumor suppressor by directly targeting hedgehog pathway transcription factor Gli3 [20]. In our study, we found that the expression of miR-506-3p was reduced in LC tissues, serum samples, and cells, suggesting that miR-506-3p is an anti-oncogene in LC. Further, we studied the correlation between serum miR-506-3p and patient clinicopathologic features. Larger tumor size, poorer differentiation degree and higher TNM stage were evident in patients presenting with under-expressed miR-506-3p. Similarly, Guo proposed that miR-506-3p, with down-regulated expression in NSCLC, was capable of predicting adverse prognosis and was related to advanced TNM stage and larger tumor size [21]. Therefore, miR-506-3p can be

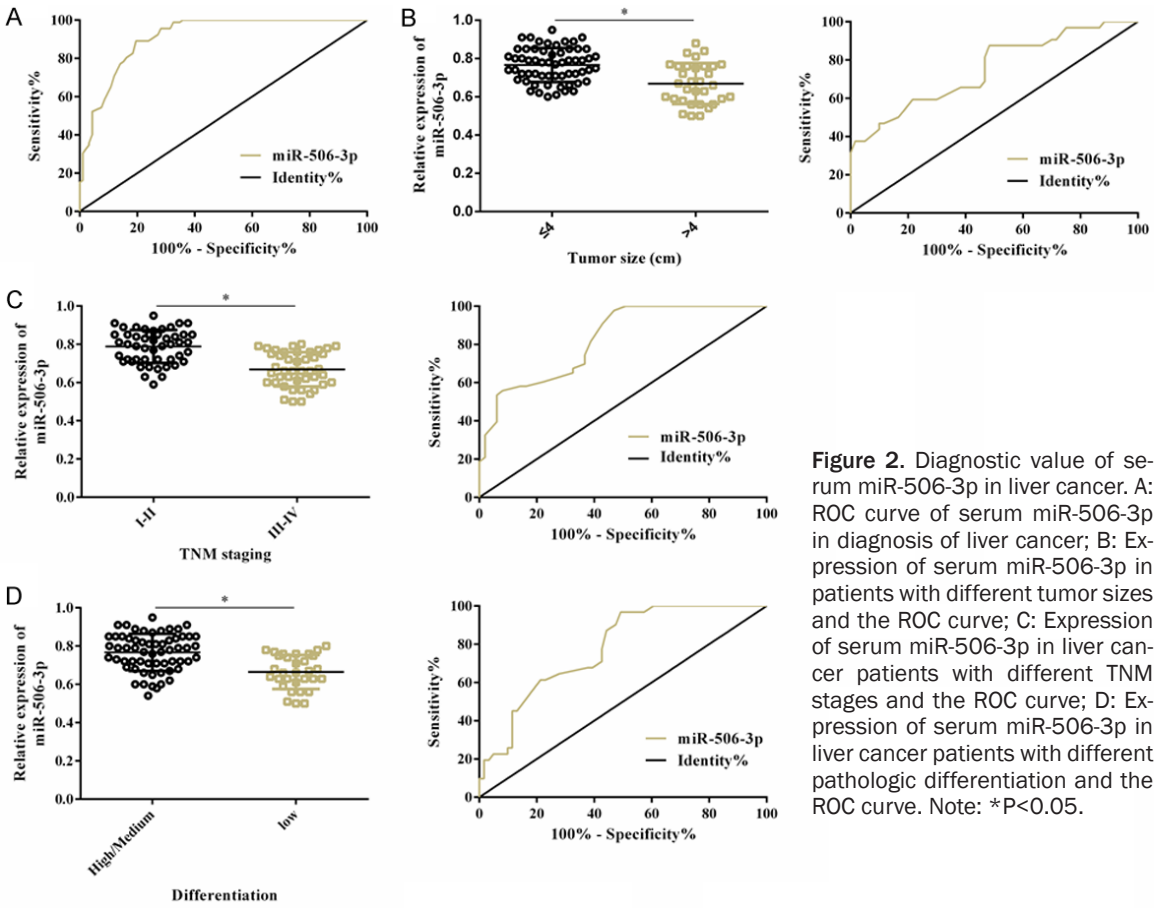

Figure 2. Diagnostic value of serum miR-506-3p in liver cancer. A: ROC curve of serum miR-506-3p in diagnosis of liver cancer; B: Expression of serum miR-506-3p in patients with different tumor sizes and the ROC curve; C: Expression of serum miR-506-3p in liver cancer patients with different TNM stages and the ROC curve; D: Expression of serum miR-506-3p in liver cancer patients with different pathologic differentiation and the ROC curve. Note: *P<0.05.

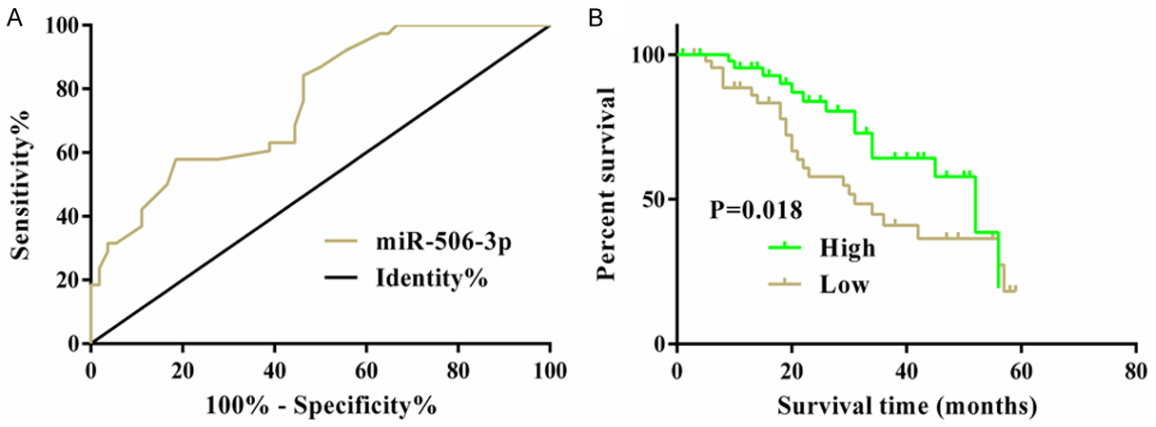

Figure 3. Relationship between serum miR-506-3p and survival of patients with liver cancer. A: ROC curve of prognosis of patients with liver cancer. B: Kaplan-Meier survival curve.

a biomarker with clinical implications for LC patients, and targeted delivery of miR-506-3p may be an effective strategy for the treatment of LC.

Serum miRNAs have been shown to be biomarkers of multiple diseases and are a promising field of clinical diagnostic research [22, 23]. They have been thoroughly studied and can diagnose and predict the prognosis of cancer patients [24]. According to Bhattacharya, serum miR-30e and miR-223 were significantly down-regulated in LC patients and are new non-invasive biomarkers for LC [25]. Our rese-

Table 3. Univariate Cox regression analysis of overall survival

Measures	β	SE	Wald	P	Exp (B)	95.0% CI	
						Upper	Lower
Gender	0.399	0.351	1.292	0.256	1.49	0.749	2.965
Age (years)	0.176	0.374	0.222	0.638	1.193	0.573	2.482
Tumor size (cm)	0.555	0.33	2.832	0.092	1.742	0.913	3.327
TNM staging	1.214	0.399	9.25	0.002	3.367	1.54	7.361
Differentiation degree	1.035	0.334	9.586	0.002	2.814	1.462	5.418
Cirrhosis	0.358	0.334	1.145	0.285	1.43	0.743	2.755
HBV infection	0.864	0.451	3.67	0.055	2.372	0.98	5.741
AFP (μg/L)	1.091	0.445	6.012	0.014	2.978	1.245	7.126
miR-506-3p	1.551	0.421	13.608	0.001	4.717	2.069	10.756

Table 4. Multivariate Cox regression analysis of overall survival

Measures	β	SE	Wald	P	Exp (B)	95.0% CI	
						Upper	Lower
TNM staging	0.858	0.41	4.386	0.036	2.359	1.057	5.268
Differentiation degree	0.713	0.338	4.451	0.035	2.041	1.052	3.959
AFP (μg/L)	0.423	0.475	0.791	0.374	1.526	0.601	3.876
miR-506-3p	1.153	0.45	6.566	0.01	3.168	1.311	7.655

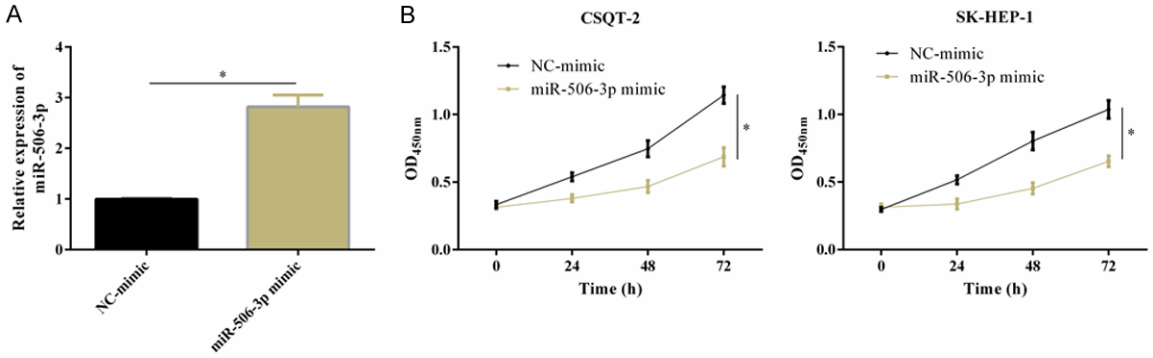

Figure 4. miR-506-3p inhibited the proliferation of liver cancer cells. A: Expression of miR-506-3p in CSQT-2 and SK-HEP-1 cells transfected with miR-506-3p mimic and NC-mimic by qRT-PCR. B: Cell proliferation ability by CCK-8 assay. Note: *P<0.05.

arch revealed that serum miR-506-3p had good diagnostic value for LC; moreover, it had differential value for tumor size, TNM stage, and differentiation degree. Li pointed out that decreased miR-506 in ovarian, gastric, and pancreatic cancers was related to the low a OS rate [26]. However, the relationship between serum miR-506-3p and outcome of LC patients has not been studied. The follow-up revealed a close association between the down-regulation of miR-506-3p and the poor survival of LC patients, indicating its role as a diagnostic and prognostic marker for LC. However, there are still some discrepancies in the role of miR-506-3p in tumor progression [27, 28]. As reported by Xiang, miR-124-3p and miR-506-3p, both under-expressed in LC tissues and cell lines, can decrease SIRT1 protein expression and suppress LC cell growth, migration and invasion by up-regulating their expression profiles [29]. Our study preliminarily found that miR-506-3p, capable of inhibiting the proliferation of LC cells, can be used as a new biomarker of LC. However, there is still room for improvement in this study. First, the downstream targeting gene of miR-506-3p has not been studied in depth.

Second, the construction of animal models is warranted to verify the mechanism of miR-506-3p in LC *in vivo*. These deficiencies are the focus of our future research.

In sum, this paper argues that miR-506-3p, capable of inhibiting the proliferation of LC cells, can be used as a novel diagnostic and prognostic biomarker for LC.

Disclosure of conflict of interest

None.

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