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ORIGINAL ARTICLE

Expression of miR-187 and miR-509-3p in serum of primary hepatocellular carcinoma patients and its evaluation of prognosis

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Summary

Purpose: *Hepatocellular carcinoma (HCC) is a histological type of primary liver cancer, with high recurrence and mortality rates worldwide. At the moment, there are no diagnostic and prognostic markers. microRNAs (miRs) are short-chain non-coding RNAs, and play a vital role in tumor diagnosis and prognosis.*

Methods: *The miR-187 and miR-509-3p expression in primary HCC was evaluated via qRT-PCR and starBase, and the diagnostic and prognostic values were analyzed via receiver operating characteristic (ROC) curve and Kaplan-Meier method.*

Results: *qRT-PCR and starBase analysis showed that the miR-187 expression was low in the tissues and serum of primary HCC patients, while that of miR-509-3p increased. ROC analysis manifested that the area under the curves (AUCs) of miR-187 and miR-509-3p in primary HCC were 0.842 and 0.866, respectively, and that of joint diagnosis was>0.9. The 5-year survival rates of miR-187 low expression group and miR-509-3p high expression group decreased markedly. Cox regression analysis identified that pathological differentiation, clinical stage and miR-187 were independent prognostic factors of primary HCC patients.*

Conclusion: *miR-187 and miR-509-3p can be potential diagnostic and prognostic indicators of primary HCC.*

Key words: *primary hepatocellular carcinoma, miR-187, miR-509-3p, prognosis, diagnosis*

Introduction

Primary liver cancer (LC) is a kind of malignancy which occurs mostly in hepatocellular carcinoma (HCC) or intrahepatic bile duct [1]. In a recent epidemiological survey of tumors [2], there are more than 840,000 new LC patients every year, and more than 800,000 will die from it. Research also found [3] that the affected population is younger and regional, and the number of those aged 40-50 is gradually increasing. The most common risk factor of HCC worldwide is chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) [4]. In addition, alcoholism, obesity and type 2 diabetes are related to HCC's increased risk [5]. At this stage, surgery is the first choice in HCC treatment [6]. However, because the incidence of LC is relatively hidden and the clinical symptoms are not obvious, it is often overlooked by patients. When the patients were admitted to hospital, most of them had been diagnosed as late stage, so that they missed the best operation period. It may be one of the reasons for the increased mortality [7,8]. Therefore, it is necessary to identify specific and sensitive biomarkers for LC early diagnosis.

MicroRNAs (miRs) are short-chain non-coding RNAs 19-25nt long [9-11]. Recent studies [12,13] have confirmed that miRs can target downstream

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genes through 3'-UTR to participate in tumorigenesis. With the early studies verified, more and more experiments have found that miRs have outstanding performance in tumor diagnosis [14,15]. For example, Shao et al [16] found that miR-155 could be used as a biomarker with high value in LC diagnosis and prognosis. In addition, Shi et al [17] discovered that serum miR-629 was a new molecular marker for diagnosis and prognosis of pancreatic cancer. miR-187 and miR-509-3p, early detection of miR, are located on human chromosome 18q12.2 and Xq27.3, respectively. Early studies [18] have found that miR-187 has low expression in HCC, and it can inhibit metastasis and epithelial-mesenchymal transition (EMT) through targeting S100A4. Another research [19] has demonstrated that miR-509-3p is highly expressed in HCC. At present, there are few researches on the diagnosis and prognosis of miR-187 and miR-509-3p in HCC.

This study mainly explores miR-187 and miR-509-3p's diagnostic and prognostic values in HCC, thereby providing potential reference for clinicians.

Methods

Clinical data

Eighty-four primary HCC patients admitted in our hospital from May 2013 to May 2014 were included into the study. During the operation, their tumor and paracancerous tissues were collected, and the peripheral blood was collected after diagnosis and before treatment. Besides, 40 cases of chronic HBV infection (CHB), 59 of liver cirrhosis (LC) and 50 healthy subjects (HD) were collected in our hospital at the same time, and the peripheral blood samples were selected as control observation. They were left at room temperature for 45 min, then centrifuged for 30 min at 1000 rpm, and the supernatant was collected and stored at -70℃. This research was approved by the Medical Ethics Committee of our hospital, and conformed to the Declaration of Helsinki [20]. Primary HCC patients did not receive antitumor treatment before, and they were pathologically diagnosed. In CHB patients, the copy number/ml and alanine aminotransferase (ALT) level increased based on the continuous DNA quantification higher than 10. The diagnosis of LC patients includes liver biology examination, clinical manifestation of ascites, portal hypertension, splenomegaly and laboratory examination. There were no abnormalities in blood tests, abdominal ultrasound and other malignancies of HD. Patient age and gender in each group had no remarkable difference ($p>0.05$) (Table 1).

qRT-PCR detection

The total RNA was extracted from serum and tumor tissues by EasyPure miRNA Kit (TransGen Biotech, Beijing, China, ER601-01). The purity, concentration and integrity were tested via ultraviolet spectrophotometer and agarose gel electrophoresis. It was reversely transcribed using 5×TransScript® All-in-One SuperMix for PCR and TransScript® miRNA RT Enzyme Mix to obtain cDNA. The test was strictly in line with the manufacturer's kit. The TransScript Two-Step RT-PCR SuperMix (TransGen Biotech, Beijing, China, AQ201-01) Kit was usd for PCR amplification. The ABI 7500PCR amplifier was put into use, and the amplification system and conditions were configured based on the instructions. Three repeat holes were set, and the experiment was conducted three times in total. U6 was employed as amplification internal reference, and data were assessed by $2^{-\Delta\Delta ct}$ [21]. The miR primer was designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd (Table 2).

Table 1. Baseline data of patients

Factor	*Primary hepatocellular carcinoma (n=84)*	*CH (n=40)*	*LC (n=59)*	HD (n=50)	p value
Age (years)	55.30±4.20	54.75±4.21	54.24±5.83	53.42±4.18	0.147
Gender (male/female)	52/32	28/12	34/25	33/27	0.468
AFP (ng / ml)	403.5±34.8	280.0±31.2	90.3±23.4	14.8±2.9	<0.001
Tumor diameter (cm)	4.2±1.8	-	-	-	
Pathological differentiation (low/medium/high)	28/18/38	-	-	-	
Clinical stage (I/II/III/IV)	18/35/17/14	-	-	-	

Table 2. Primer sequence

Gene	*Upstream primer 5'-3'*	*Downstream primer 5'-3'*
miR-187	GCCGAGTCGTGTCTTGTGTT	CTCAACTGGTGTCGTGGA
miR-509-3p	UGAUUGGUACGUCUGUGGGUAGTT	CUACCCACAGACGUACCAAUCATT
U6	CTCGCTTCGGCAGCACA	ACGCTTCACGAATTTGCGT

Follow-up of patients

In this study, primary HCC patients were followed-up for 5 years, usually in January, March, June, September, December of each year. In view of miR-187 and miR-509-3p's median expression, they were divided into high and low expression groups. The relationship between the two and patient survival was further observed.

Online database analysis

The miR-187 and miR-509-3p expression of HCC in TCGA database was analyzed online by starBase [22] (http://starbase.sysu.edu.cn/).

Statistics

The collected data were statistically analyzed by SPSS20.00, and pictures were drawn by Graph Pad 8. The data distribution was analyzed by Kolmgorov-Smirnov test, in which normal distribution data were tested by t-test. The inter-group comparison was assessed by independent-samples t-test. The adoption rate of counting data was expressed by %, and was analyzed by chi-square test. When the theoretical number T<5 but T≥1 and n≥40, it was tested by continuous correction chi-square. The ranked data were evaluated by rank sum test and expressed by Z. In view of gene expression's median value, the data were divided into high and low expression groups. Patients' survival situation was assessed by Kaplan-Meier and log-rank test. The diagnostic value of miR in primary HCC patients was drawn by receiver operating characteristic (ROC) curve. When the area under the curve (AUC) was greater than 0.5, it was meaningful. There were differences when p<0.05.

Results

miR-187 expression is low in primary HCC, while that of miR-509-3p is high

We analyzed the miR-187 and miR-509-3p expression in HCC by starBase. The results manifested that the former was low in HCC, while the latter was high in HCC (Figure 1A). The qRT-PCR detection of the expression in the tumor tissues of primary HCC was consistent with the results of the online database analysis of starBase (Figure 1B). In addition, the expression of miR-187 in the serum

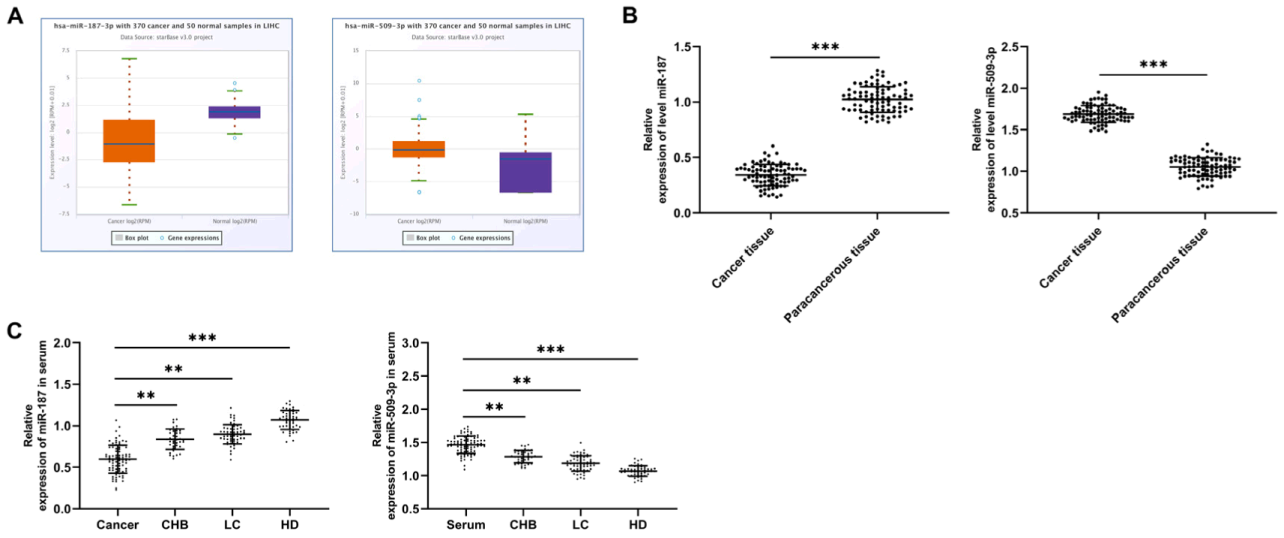

Figure 1. miR-187 and miR-509-3p's relative expression in primary HCC. **A:** miR-187 and miR-509-3p's relative expression in HCC in TCGA database is analyzed by Starbase online website. **B:** miR-187 and miR-509-3p's relative expression in tumor tissues of primary HCC patients is tested by qRT-PCR. **C:** miR-187 and miR-509-3p's relative expression in HCC, CHB, LC and HD is tested via qRT-PCR (**p<0.01, ***p<0.001).

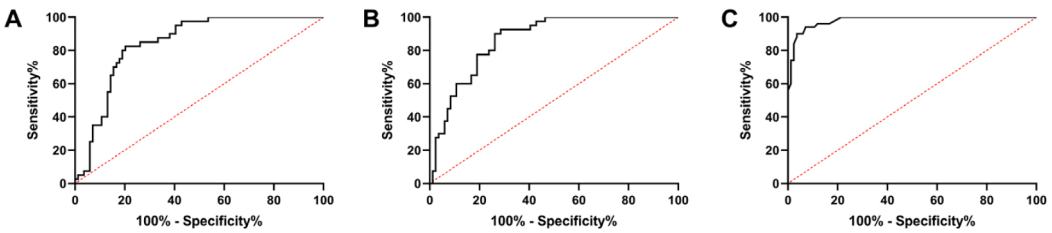

Figure 2. ROC curve analysis of diagnostic value of miR-187 and miR-509-3p in primary HCC. **A:** miR-187's ROC curve in diagnosing primary HCC. **B:** miR-509-3p's ROC curve in diagnosing primary HCC. **C:** ROC curve of miR-187 combined with miR-509-3p in diagnosing primary HCC.

of primary HCC, CHB, LC and HD was detected by qRT-PCR which revealed that the miR-509-3p expression in the serum of primary HCC was obviously lower than that of CHB, LC and HD (Figure 1C). This suggested that miR-187 and miR-509-3p might be potential diagnostic and prognostic indicators of primary HCC.

Diagnostic value of miR-187 and miR-509-3p

The above study confirmed the miR-187 and miR-509-3p expression in primary HCC. To further determine the diagnostic value of the two, we performed ROC analysis. Through analysis, we found that the AUCs of miR-187 and miR-509-3p were 0.842, 0.866, respectively (Figure 2A-B), and that of joint diagnosis was>0.9 (Figure 2C), suggesting that both had high diagnostic value in primary HCC (Table 3).

miR-187 and miR-509-3p have high prognostic value in primary HCC

In order to analyze miR-187 and miR-509-3p's prognostic values in primary HCC, we divided patients into high and low expression groups in the light of their median expression, and further analyzed the relationship between the two and patients' 5-year survival. This showed that the 5-year survival rate of miR-187 low expression group and miR-509-3p high expression group decreased dramatically suggesting that the two might be potential prognostic indicators of primary HCC (Figure 3).

Cox regression analysis

At the end of the study, we also conducted Cox regression analysis to observe the independent prognostic factors of primary HCC patients. Firstly, through univariate analysis, we found that patho-

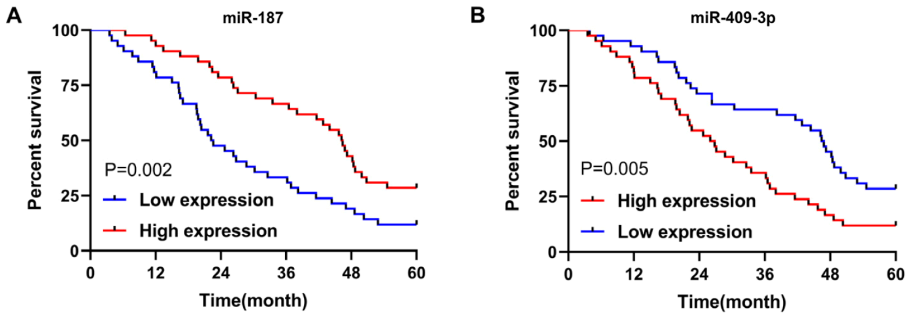

Figure 3. The 5-year survival rate of miR-187 low expression group and miR-509-3p high expression group decreased dramatically in both groups.

Table 3. ROC curve data

Factor	*AUC*	*95CI%*	*Specificity, %*	*Sensitivity, %*	*Youden index, %*	*Cut-off*
miR-187	0.840	0.774-0.911	82.50	79.76	62.26	>0.729
miR-509-3p	0.866	0.804-0.927	92.50	71.43	63.93	<1.407
Joint curve	0.981	0.964-0.998	94.00	92.86	86.86	<1.270

AUC: area under the curve, Cut-off: optimal cut-off point

Table 4. Cox regression analysis

Factor	*Univariate Cox*			*Multivariate Cox*		
	p value	*HR*	*95%CI*	p value	HR	95%CI
Gender	0.714	1.096	0.671-1.790			
Age	0.830	1.056	0.645-1.728			
Tumor diameter	0.257	1.321	0.816-2.140			
Pathological differentiation	0.004	0.653	0.489-0.872	0.001	0.605	0.450-0.814
Clinical stage	0.016	1.341	1.056-1.703	0.028	1.294	1.028-1.628
miR-187	0.003	0.483	0.297-0.785	0.001	0.425	0.257-0.701
miR-409-3p	0.006	0.501	0.307-0.817			

HR: risk ratio, 95% CI: 95% confidence interval.

logical differentiation, clinical stage, miR-187 and miR-509-3p were the prognostic factors of patients. Further analysis manifested that pathological differentiation, clinical stage and miR-187 were the independent prognostic factors (Table 4).

Discussion

More and more studies show that miRs take part in a series of biological processes, especially the development and progression of tumors [23-25]. Furthermore, other research has also found that miR's abnormal expression in tumor tissues, blood and cells is relevant to tumor development and prognosis [26-28]. In this research, we found that miR-187 and miR-509-3p's differential expression in primary HCC could be a potential diagnostic and prognostic indicator.

miR-187, also known as miR-187-3p, belongs to miR-187 family and is differentially expressed in various tumors. Wu et al, for example, discovered [29] that FGF9 expression targeted by miR-187-3p enhanced the sensitivity of breast cancer cells to gemcitabine. Other researchers [30] found that miR-187-3p could improve non-small cell LC development by regulating BCL6. However, there are relatively few studies on miR-187 in primary HCC. We found that the miR-187 expression in HCC was obviously lower than that in CHB, LC and HD, which indicated that it might be a potential diagnostic indicator.

As a member of miR-506 family, miR-509-3p can regulate many kinds of tumors like other members of miR-506 family, especially in tumor drug resistance. For instance, Chen et al [31] reported that miR-509-3p advanced cisplatin-induced apoptosis of ovarian cancer cells by regulating anti-apoptosis genes. What's more, research found that miR-509-3p could enhance the sensitivity of ovarian cancer to platinum drugs [32]. At present, however, only Wang et al [19] have mentioned that cancer protein HBXIP participates in HCC development by regulating E2F1 and SCG3 up-regulated by miR-509-3p. miR-509-3p's clinical value in primary HCC is still vague. Besides, we first found that the miR-509-3p expression in HCC was markedly higher than that in CHB, LC and HD, suggesting that it might be involved in HCC development.

miR-187 and miR-509-3p expression has been confirmed in the above study. To further analyze their clinical value in primary HCC, we performed ROC curve analysis and Kaplan-Meier survival analysis respectively. Firstly, ROC analysis showed that miR-187 and miR-509-3p's AUCs in diagnosing primary HCC is>0.7, but through joint prediction analysis, we found that the AUC ROC drawn by miR-187 and miR-509-3p is>0.9, with great value. The improvement of the survival of cancer patients has always been a problem that puzzles clinicians. At the moment, the clinical prognosis of primary HCC is not ideal. We found that the 5-year survival rate of patients with low expression of miR-187 and high expression of miR-509-3p decreased obviously. In addition, Cox regression analysis found that pathological differentiation, clinical stage and miR-187 were independent prognostic factors of primary HCC. These experiments show that miR-187 can be a potential indicator for diagnosis and prognosis.

However, this study still has some limitations. In the first place, this study did not analyze the miR-187 and miR-509-3p expression in drug-resistant primary HCC patients. Early studies found that both of them played an important role in drug resistance. In the second place, the mechanism of miR-187 and miR-509-3p in primary HCC is unclear, and whether they participate in tumor development and progression needs further exploration. Therefore, we'll carry out more experiments in future research to perfect our research conclusions.

All in all, miR-187 and miR-509-3p can be potential diagnostic and prognostic indicators of primary HCC.

Conflict of interests

The authors declare no conflict of interests.

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