European Review for Medical and Pharmacological Sciences 2018; 22: 8431-8437

Expressions of MiR-132 in patients with chronic hepatitis B, posthepatitic cirrhosis and hepatitis B virus-related hepatocellular carcinoma

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Abstract. – OBJECTIVE: To explore the expressions of miR-132 in patients with chronic hepatitis B, posthepatitic cirrhosis and hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC), and to investigate its possible mechanism affecting the function of the body.

PATIENTS AND METHODS: Among 125 patients with HBV, there were 44 cases of chronic hepatitis, 42 cases of liver cirrhosis and 39 cases of liver cancer. Their liver function and HBV-deoxyribonucleic acid (HBV-DNA) viral load as well as the expressions of micro ribonucleic acid-132 (miR-132), phosphoinositide 3-kinase (PI3K), phosphorylated-protein kinase B (p-Akt) and hepatitis B X protein (HBx), were detected.

RESULTS: There were significant differences in some liver function indexes and the HBV-DNA level among the three groups of patients ($p < 0.05$). The HBV-DNA level was 6.91 Lg copies/mL in the liver cancer group and 5.34 Lg copies/mL in the chronic hepatitis B group. Differences in the expression level of miR-132 among the three groups were notable ($p < 0.05$), but this expression level had a negative correlation with the HBV-DNA level. The expressions of PI3K and p-Akt proteins and messenger ribonucleic acids (mRNAs) were markedly different among the three groups ($p < 0.05$). HBx was expressed in the three groups of patients, and liver cancer patients with the highest expression degree of HBx accounted for 46%.

CONCLUSIONS: Differences in the expression of miR-132 among the three groups are evident, which may be associated with differences in liver function, the HBV-DNA level, HBx and the expressions of PI3K and p-Akt proteins to a certain degree.

Key Words:
miR-132, Chronic hepatitis B, Posthepatitic cirrhosis, Patients with hepatitis B virus-related hepatocellular carcinoma.

Introduction

According to the estimation of the World Health Organization (WHO) in 2016, over 240 million people have long been infected with hepatitis B virus (HBV), which has developed into chronic hepatitis B, and more than 680,000 people die of the disease or complications induced by the disease each year in China[1]. HBV is one of the primary infectious diseases in China[2]. HBV infection is one of the main causes of liver cirrhosis and liver cancer. Multiple indexes should be selected and their correlations with the disease should be investigated, so as to further better evaluate the disease progression and status, thus providing a reference for accurately judging the progressing stage of the disease. Micro ribonucleic acids (miRNAs) are a class of small, non-coding and endogenous RNA molecules. They interact with targets, regulate gene expression, inhibit protein production and widely regulate the biological processes of the body by facilitating the instability of mRNAs and silent translation. Thousands of related molecules have been discovered so far, and it has been revealed that diversified miRNA molecules can inter-coordinate with each other and play roles in the entire cell physiological process, which is of extreme importance for the body development, homeostasis, neurobiology, immunobiology and infection control[3,4]. It has been confirmed that a variety of miRNAs exert crucial effects in the metastasis of multiple tumors[5]. The regulatory effect of miRNAs manifests that transcripts are differentially expressed in biological processes, which can act as new-type molecular markers for a variety of different pathologies

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or abnormalities of the disease. MiRNAs have been applied as biomarkers for the diagnosis and prognosis of infectious diseases in some recent studies, such as human tuberculosis caused by infection with mycobacterium tuberculosis, sepsis triggered by various infectious agents and viral hepatitis. These non-coding transcripts have become crucial molecular regulators of host immune responses during infection[6,7]. According to researches, messenger RNAs (mRNAs) can be regulated by miRNAs in most mammals[8], and it is known that multiple miRNAs are capable of regulating the toll-like receptor 4 (TLR-4) pathway in the host innate immune responses[9-13]. Further studies, therefore, are required for fully clarifying how miRNAs affect biomolecular signaling networks.

Patients and Methods

Patients

A total of 125 patients with HBV admitted to Tangdu Hospital from January 2017 to December 2017 were selected, including 61 males and 64 females, aged 39-68 years old. After the admission and treatment, the patients were subjected to routine blood biochemistry, liver function and HBV-deoxyribonucleic acid (HBV-DNA) examinations to assess the physiological status of the patient and to exclude patients with hepatitis C virus, hepatitis E virus and other hepatitis viruses. Among them, there were 44 cases of chronic hepatitis, 42 cases of liver cirrhosis and 39 cases of liver cancer base on the inclusion criteria[14]. The patients in the group were examined with liver function, CT, color Doppler ultrasound and diagnosed with HBV DNA examination. Exclusion criteria: (1) patients with previous hepatitis C, hepatitis E virus infections or other hepatitis infection; (2) other malignant tumors; (3) other metabolic diseases. All patients received liver biopsy to obtain some liver tissues for subsequent tests and detection. Detailed data are shown in Table I. This research program was carried out after it was approved by the Ethics Committee of Tangdu Hospital. Each patient was informed of the condition and signed the informed consent.

Blood Samples Collection

Patients receiving fasting hemospasia and liver tissues were obtained by liver biopsy. 3 ml venous blood were extracted from each patient at room temperature. After standing at room temperature for 30 min, the samples were centrifuged at 20°C 3000 g for 10 minutes. The serum was separated and stored in the refrigerator at -20°C.

Detection of Liver Function

The blood biochemical indexes of patients in each group were determined by blood biochemistry instrument (Jinan Yuxin Biotechnology Co., Ltd., Jinan, China) after fasting venous blood was drawn from patients, and the procedure was carried out strictly according to the protocol of blood biochemistry test in laboratory.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qPCR)

TRIzol method was adopted to extract the total RNA in the liver tissue or blood via the one-step lysis. The quality of RNA was confirmed by OD detection and agarose gel electrophoresis. After that, the mRNA in the total RNA was reversely transcribed using the reverse transcription kit Moloney Murine Leukemia Virus reverse transcription system (Vazyme Biotechnology Co., Ltd., Nanjing, China), with U6 RNA as miRNA internal reference control. The reverse transcription PCR primer for miR-132 was purchased from Ribobio (Guangzhou, China). Real-time PCR was conducted using the kit on the StepOne Plus system with β-actin as an internal reference. The primer sequences used are as follows: phosphoinositide 3-kinase (PI3K):

Table I. Data of patients.

Group	N	Male/female (n)	Minimum age (years old)	Maximum age (years old)	Average age (years old)
Chronic hepatitis	44	21/23	42	68	52
Liver cirrhosis	42	22/20	43	61	51
Liver cancer	39	18/21	39	67	53
Total	125	61/64	/	/	/
p		> 0.05	> 0.05	> 0.05	> 0.05

F: 5'-ATGGGGATGATTTACGGC-3' and R: 5'-TCTCCTTTGTTCTTGTCTTTGA-3', β-action: F: 5'-TCCTGTGGCATCCACGAAACT-3' and R: 5'-GAAGCATTTGCGGTGGACGAT-3', and protein kinase B (Akt): F: 5'-TCTATGGCGCTGAGATTGTG-3' and R: 5'-CTTAATGTGCCCGTGCTTCA-3'. Real-time PCR was carried out in triplicate and included no template control. The relative expression and quantification were expressed as $2^{-\Delta\Delta Ct}$.

Western Blotting (WB) Analysis

After weighing the liver tissue weight of patients in each group, RIPA lytic solution was added according to the weight/volume ratio of 100 mg/1 mL. Tissue was homogenized using ultrasonic homogenizer homogenate. Next, the tissue was centrifuged at 8000 g for 10 min at 4°C. The supernatant was the total protein. Protein concentration was determined with protein quantitative kit (Beyotime Biotechnology Co., Ltd, Wuhan, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed to separate the proteins, which were then transferred onto polyvinylidene difluoride (PVDF) membranes and block with 5% milk at 20°C for 1 h. Next, the band was incubated with primary rabbit anti-human PI3K, p-AKT, β-actin monoclonal antibodies (1:600; Cell Signaling Technology, Danvers, MA, USA) for 2 h at 4°C. The membrane was washed with Tris-buffered saline and Tween-20 (TBST) for 3 times. Subsequently, blots reacted with goat anti-rabbit or secondary polyclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) for 1 h at 20°C, whose color was developed using the enhanced chemiluminescence. The monoclonal antibodies applied in this study included anti-PI3K, phosphorylated-Akt (p-Akt) and β-actin antibodies, in which β-actin was regarded as an internal reference control.

Immunohistochemical Staining

Immunohistochemical staining was conducted to detect the expression levels of hepatitis B X protein (HBx) in patients with chronic hepatitis B, liver cirrhosis and liver cancer. After that, dewaxing was carried out, and the tissue sections were placed in an incubator for 10-15 min, followed by hydration with different concentrations of alcohol and antigen repair. The tissue in the sections was sealed with blocking solution through dropwise addition, covered with a sealing film and placed in a humidor for about 30 min. After that, the tissue was added with primary antibodies, covered with a sealing film, and placed in a humidor at 4°C overnight. After washing with 0.1% phosphate-buffered saline and Tween-20 (PBST) for 3 times, the tissue was added dropwise with an appropriate amount of secondary antibodies including horseradish peroxidase (HRP), covered with a sealing film and placed into a humidor for 1 h of reaction. After washing for 3 times, color developing solution was added dropwise to completely cover the tissue. Subsequently, staining was performed at room temperature for 15-20 min, the reaction was terminated, and the tissue was counterstained for 2 min. The results were examined and determined via a microscope.

Statistical Analysis

Statistical Product and Service Solutions 16.0 software (SPSS Inc., Chicago, IL, USA) was adopted for data statistical analysis, and the difference was analyzed using analysis of variance (ANOVA) and the least significant difference (LSD) post-hoc test. $p < 0.05$ was considered as statistically significant.

Results

Comparisons of Liver Function Indexes Among Each Group of Patients

The serum of 44 patients with hepatitis, 42 patients with liver cirrhosis and 39 patients with liver cancer was detected so as to explore whether there were differences in liver function among different groups of patients. Some liver function indexes are shown in Table II. There were significant differences in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate (ALP), gamma-glutamyltransferase (GGT), total protein (TP), albumin (ALB) and total bilirubin (T-BIL) between hepatitis patients and liver cancer patients ($p < 0.05$). AST, GCT and ALB were significantly different between patients with hepatitis and cirrhosis ($p < 0.05$). There were huge differences in AST, TP and ALB between cirrhosis and liver cancer patients ($p < 0.05$). The other indexes were not significantly different among the three groups.

Levels of HBV and miR-132 in Each Group of Patients

The HBV-DNA level was 5.34 Lg copies/mL in the liver cancer group, 5.89 Lg copies/mL in the liver cirrhosis group and 6.91 Lg copies/mL in the chronic hepatitis B group, showing obvious

Table II. Comparisons of liver function indexes among each group of patients.

Item	Group		
	Chronic hepatitis B group	Liver cirrhosis group	Liver cancer group
ALT (U/L)	56.5 ± 8.4	81.3 ± 15.7*	92.6 ± 15.2*
AST (U/L)	33.2 ± 11.1	51.3 ± 8.6*	79.2 ± 10.1*#
ALP (U/L)	91.5 ± 13.1	114.3 ± 25.7	136.7 ± 21.4*
GGT (U/L)	34.6 ± 5.5	39.9 ± 7.2	51.2 ± 8.7*
TP (g/L)	66.2 ± 13.7	59.3 ± 14.4	47.1 ± 10.6*#
ALB (g/L)	44.6 ± 8.2	33.7 ± 7.1*	28.3 ± 7.6*#
T-BIL (μmol/L)	22.1 ± 7.2	29.3 ± 8.3	30.2 ± 5.4*
Conjugated bilirubin (μmol/L)	3.8 ± 1.9	5.9 ± 1.7	6.4 ± 2.5
C-reactive protein (mg/L)	4.2 ± 1.8	4.5 ± 1.4	4.3 ± 1.5
Erythrocyte sedimentation rate (mm/h)	13.2 ± 6.3	13.8 ± 7.2	14.3 ± 5.9

Note: *Compared with chronic hepatitis B group, the difference is significant ($p < 0.05$). #Compared with liver cirrhosis group, the difference is significant ($p < 0.05$).

Table III. Levels of HBV-DNA and miR-132 in patients of each group.

Item	Group		
	Chronic hepatitis B group	Liver cirrhosis group	Liver cancer group
HBV-DNA (Lg copies/mL)	5.34 ± 1 .25	5.89 ± 1.63*	6.91 ± 1.75*#
miR-132	0.81 ± 0.42	0.74 ± 0.33*	0.52 ± 0.31*#

Note: *Compared with chronic hepatitis B group, the difference is significant ($p < 0.05$). #Compared with liver cirrhosis group, the difference is significant ($p < 0.05$).

differences among the three groups ($p < 0.05$). There were notable differences in the level of miR-132 among the three groups, but its level had a negative correlation with the HBV-DNA level (Table III).

Expression Levels of PI3K and Akt in the Patient's Blood of Each Group

The expression levels of PI3K protein in liver tissues of patients with hepatitis, liver cirrhosis and liver cancer were (0.18 ± 0.085), (0.24 ± 0.16) and (0.49 ± 0.15), respectively, and differences among the three groups were evident ($p < 0.05$). Besides, the expression levels of p-Akt protein in the three groups were also significantly different ($p < 0.05$) (Figure 1 and Table IV).

The expression levels of PI3K mRNAs in the three groups were (0.12 ± 0.091), (0.25 ± 0.083) and (0.44 ± 0.11), respectively, displaying significant differences ($p < 0.05$) and an uptrend. Differences in the expression levels of AKTK mRNAs in the three groups were also remarkable ($p < 0.05$) (Table V).

Detection of the HBx Expression in the Liver of Each Group via Immunohistochemistry

HBx was expressed in the liver tissues of the three groups of patients. Liver cancer patients with the highest expression degree of HBx ac-

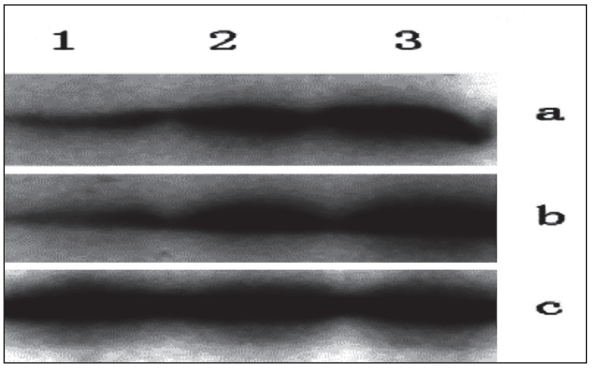

Figure 1. Detection of the expressions of PI3K and Akt in blood via WB. 1: Hepatitis. 2: Liver cirrhosis. 3: Liver cancer. a. PI3K. b: p-Akt. c: β-actin.

Table IV. Levels of PI3K and Akt proteins in blood.

	Group		
	Chronic hepatitis B group	**Liver cirrhosis group**	**Liver cancer group**
PI3K	0.18 ± 0.085	0.24 ± 0.16*	0.49 ± 0.15*#
p-Akt	0.54 ± 0.26	0.72 ± 0.35*	0.81 ± 0.44*#

Note: *Compared with chronic hepatitis B group, the difference is significant ($p < 0.05$). #Compared with liver cirrhosis group, the difference is significant ($p < 0.05$).

Table V. Expressions of PI3K and AKT mRNAs in blood.

	Group		
	Chronic hepatitis B group	**Liver cirrhosis group**	**Liver cancer group**
PI3K	0.12 ± 0.091	0.25 ± 0.083*	0.44 ± 0.12*#
Akt	0.27 ± 0.071	0.36 ± 0.15*	0.68 ± 0.34*#

Note: *Compared with chronic hepatitis B group, the difference is significant ($p < 0.05$). #Compared with liver cirrhosis group, the difference is significant ($p < 0.05$).

counted for about 46%, and chronic hepatitis B patients with the highest expression degree of HBx was only 22%, with a significant difference ($p < 0.05$) (Figure 2 and Table VI).

Discussion

HBV is capable of invading liver cells, constantly proliferating in liver cells, manifested as chronic infection, and destroying liver cells for a long term. Liver cells continue to constantly proliferate for compensating for liver function, thus causing fibrosis, and they further develop into liver cirrhosis. The constant proliferation of the cells is very likely to result in the activation of oncogenes, thus forming liver cancer. Immune responses and body functions in the host are involved in the infection, clearance and pathogenesis of HBV[15,16]. It has been shown[17] that the HBV viral load in the body of patients appears to be positively associated with the occurrence risks of liver cirrhosis and liver cancer, indicating that the patient's disease progression needs to be closely tracked. According to researches, the incidence rate of HBV-infected miRNAs in serum of individuals goes up with the severity of the disease. There were 77 chronic asymptomatic carriers, 101 patients with liver cirrhosis and

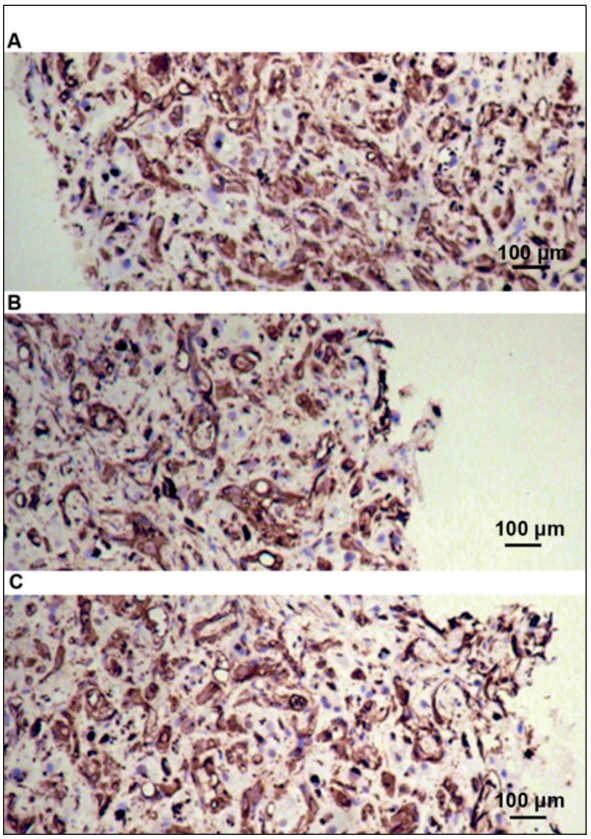

Figure 2. Detection of the HBx expression in liver tissues. ***A:*** Hepatitis. ***B:*** Liver cirrhosis. ***C:*** Liver cancer.

Table VI. HBx expressions in liver tissues of different patients.

Group	Expression degree/ratio		
	Positive (+)/(%)	Positive (++)/(%)	Positive (+++)/(%)
Chronic hepatitis B group	21/(47%)	14/(31%)	10/(22%)
Liver cirrhosis group	15/(36%)	12/(29%)	16/(39%)
Liver cancer group	9/(23%)*	12/(31%)	18/(46%)*

Note: *Compared with chronic hepatitis B group, the difference is significant ($p < 0.05$). #Compared with liver cirrhosis group, the difference is significant ($p < 0.05$).

135 patients with HBV-related acute-chronic liver failure[18]. MiR-210[19] and miR-124[20] have correlations with the disease severity. Additionally, it has been reported in some studies that miR-345-3p, miR-371a-5p and miR-2861 can be applied as positive indicators for fibrosis, but miR-486-3p and miR-497-5p appear to be lowly expressed in all stages of fibrosis compared with patients with non-fibrotic chronic hepatitis B patients[21]. Other studies have revealed that miR-132 has certain associations with many bacterial and viral infections as well as tumor pathogenesis. Based on previous studies, miR-132 has a certain relationship with viral infection. An increase in the expression level of miR-132 can inhibit the expression of some proteins in the virus, so as to decrease the expression of the antiviral protein. However, in liver cancer related to HBV infection, the expression level of miR-132 is downregulated, indicating that miR-132 may participate in the development of liver cancer[22]. Comparative analysis was performed for some liver function indexes of the three groups of patients in order to explore their miR-132 distribution. The results demonstrated that there were significant differences in some liver function indexes of patients in the chronic hepatitis B group, the posthepatitic cirrhosis group and the HBV-related hepatocellular carcinoma group, which may be related to the different progression states of the three groups of patients. It has shown in previous studies that miR-132 exerts different effects on different viruses. In this study, the level of HBV-DNA was compared among the three groups of patients, and the level of miR-132 was detected at the same time. The results manifested that significant differences were found in the HBV level among the three groups, in which the HBV-DNA level was the highest in the liver cancer group and the lowest in the chronic hepatitis B group. The level of miR-132 was remarkably different among the three groups, but this level had a negative correlation with the HBV-DNA level. The above results indicate that miR-132 may resist viruses through activating the innate immune responses of the body, and the level of miR-132 may indicate the disease state of patients to some extent. In this study, related proteins and genes that regulate the cell apoptosis were also examined to further investigate the possible effects of miR-132 on other regulatory molecules in the three groups of patients. Significant differences were detected in the expression levels of PI3K and p-Akt proteins in the liver tissues of patients with hepatitis, liver cirrhosis and liver cancer, and obvious differences in the expression levels of PI3K and p-Akt mRNAs in them. It has been revealed in a variety of studies that HBx can actively attack or resist the body's immune system. In this study, HBx was expressed in the liver tissues of the three groups of patients, in which the expression degree of HBx in liver cancer patients was the highest, suggesting that HBx may interact with miR-132.

Conclusions

We found miR-132 may be associated with liver function in patients to a certain degree. In the three groups of patients, the expression level of miR-132 may interact with the HBV level and the HBx protein level. Further data and more in-depth studies are needed to investigate the correlations of miR-132 with liver function and different progressions of the disease, thus further scientifically evaluating the interaction between miR-132 and HBV.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Foundation
Shaanxi Natural Science Basic Research Program (2017JM8011).

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