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Serum lincRNA-p21 expression in primary liver diseases and liver metastatic diseases

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ABSTRACT

Background: LincRNA-p21 is involved in the initiation and progression of many human diseases. We aimed to investigate the expression of LincRNA-p21 in different types of liver diseases.
Methods: Serum from patients with primary liver diseases (chronic HBV or HCV infection, hepatitis B virus-related cirrhosis, hepatitis B virus-related HCC, non-HBV/HCV-related HCC, alcoholic liver disease) and HBV negative liver metastatic cancer and control healthy individuals was collected and serum lincRNA-p21 levels were determined by RT-qPCR. Clinicopathological characteristics of the patients were also recorded.
Results: Serum lincRNA-p21 levels in patients with chronic HBV infection, hepatitis B cirrhosis, hepatitis B virus-related HCC, chronic hepatitis B virus infection, non-HBV/HCV-related HCC, and alcoholic liver disease were higher than those in the control individuals ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.002$, respectively). The serum lincRNA-p21 level was not significantly different between patients with HBV negative liver metastatic cancer and the normal control ($P = 0.80$). LincRNA-p21 level was negatively correlated with HBV DNA ($P = 0.02$), ALT ($P = 0.01$) and AST ($P = 0.01$) in patients with liver disease, but not correlated with gender ($P = 0.24$), age ($P = 0.11$) and AFP level ($P = 0.84$). Serum lincRNA-p21 in hepatocellular carcinoma patients was higher than that in liver metastatic cancer patients ($P < 0.001$).
Conclusion: Serum lincRNA-p21 may serve as a potential biomarker for liver cell damage in patients with hepatitis virus infection, hepatitis B cirrhosis, HBV-related HCC and alcoholic liver disease.

1. Introduction

Liver diseases, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infections associated cirrhosis and hepatocellular carcinoma (HCC), and alcoholic liver disease (ALD), are major causes of illness and death in China, and affect approximately 300 million people [1]. Unfortunately, early diagnosis of these diseases remains difficult because of the absence of symptoms in the early stages. At present, the most accurate diagnostic methods for these diseases, such as endoscopic ultrasonography-guided fine needle aspiration, are invasive. Non-invasive serum biomarkers for the diagnosis, prognosis, or therapeutic response of liver disease, as well as direct targets for therapeutic intervention are major unmet clinical needs.

Long intergenic noncoding RNAs (lincRNAs) that lack significant protein-coding capacity regulate various physiological and pathological processes, and many lincRNAs are known to regulate the progression of liver disease, which facilitate the identification of candidate prognostic biomarkers and provide new therapeutic strategies [2,3]. Among these lincRNAs, long intergenic non-coding RNA-p21 (lincRNA-p21), which is about 3.0 kb length, locates approximately 15 kb upstream of the cell-cycle regulator gene p21/Cdkn1a. LincRNa-p21, as a transcriptional target of p53 and a potential diagnostic marker, is involved in proliferation, cell cycle, metabolism and reprogramming [4]. It was reported that lincRNA-p21 suppressed the development of human prostate cancer through inhibiting PKM2 [5]. LincRNA-p21 predicts favorable clinical outcome following anti-cancer therapy [6]. LincRNA-p21 was reported to be negatively correlated with the stages of liver fibrosis in HBV infected patients, and was downregulated in HCC [7,8]. In this study, we aimed to investigate the expression of lincRNA-p21 in different types of liver diseases.

Abbreviations: lincRNA, long intergenic non-coding RNA; lincRNA-p21, long intergenic non-coding RNA-p21; HBV, hepatitis B virus; HCV, hepatitis C virus; ALD, alcoholic liver disease; qRT-PCR, quantitative real-time polymerase chain reaction; HCC, hepatocellular carcinoma

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Table 1
Clinicopathological characteristics.

Parameter	chronic HBV infection (46)	HBV cirrhosis (34)	HBV HCC (56)	chronic HCV infection (34)	not HBV or HCV-related HCC (27)	alcoholic liver disease (34)	liver metastatic cancer (24)	normal control (48)
Gender, m/f, n	34/12	24/10	41/15	24/10	19/8	32/2	13/11	28/20
Age, years	44(29.75–50.00)	51(43.5–59)	62(55–67.75)	58.5(49–67)	69(50–72)	56.5(47.25–65.25)	63.5(59.25–67)	42(26.5–55.75)
ALT	81(31–186.8)	34(23.5–79)	36.5(22.5–66.5)	34.5(24.75–54.25)	25(19–36)	24.5(19–34.5)	25.5(16.75–82.25)	
AST	79(53.25–163.8)	60(37.25–104.8)	46.5(29.5–95)	62.5(35.25–78.25)	87(39–112)	38(29–60)	27(20.25–77.25)	
AFP	24.03(3.26–205.9)	5.95(2.49–36.61)	208.5(12.69–4512)	7.56(3.57–53.28)	27.06(3.65–89.50)	3.72(2.54–15.26)	4.00(2.32–5.10)	
HBV/HCV DNA positive, n (%)	34(73.9)	22(64.7)	13(23.2)	27(79.4)				
LincRNA-p21	2.25(1.36 ~ 3.17)	1.69(1.19 ~ 3.61)	1.51(1.24 ~ 2.19)	1.64(1. ~ 2.23)	1.69(1.37 ~ 2.66)	1.72(0.94 ~ 2.20)	1.07(0.53 ~ 1.56)	0.96(0.79 ~ 1.26)

2. Materials and methods

2.1. Ethics statement

Informed consent was obtained from all participants before sample collection. The Human Research Ethics Committees from the Second Hospital of Shandong University approved this study. All procedures were performed in accordance with the Helsinki Declaration of 1975 (2008 revision).

2.2. Research objects

Two hundred and fifty-five hospitalized Chinese patients with liver disease (68 female; median (interquartile range) age, 49.5 (34.5–60.75) years) were recruited from the Department of liver diseases at the Second Hospital of Shandong University between 2015 and 2016, including 46 cases of chronic HBV infection, 34 cases of hepatitis B virus-related cirrhosis, 56 cases of hepatitis B virus-related HCC, 34 cases of chronic HCV infection, 27 cases of non-HBV/HCV-related HCC, 34 cases of alcoholic liver disease and 24 cases of other cancers that spread to liver with HBV and HCV negative. The patients did not undergo surgery, radiotherapy and chemotherapy before sample collection. Diagnosis of liver cirrhosis or HCC was based on typical morphological findings using CT or ultrasound and pathomorphological observation, according to the Strategy for Prevention and Therapy of Viral Hepatitis reported in 2000. Patients with more than one type of liver diseases were excluded. Clinicopathological characteristics of patients, such as HBV DNA, ALT, AST, and AFP levels, were recorded.

The control group consisted of 48 age- and gender-matched healthy individuals (20 female; median (interquartile range) age, 42 (26.5–55.75) years). Patients and controls were residents of the same area and came from the same human subpopulation.

2.3. Sample collection and preparation

For serum lincRNA-p21 detection, venous blood was drawn from patients and healthy donors and centrifuged at 3500 rpm for 10 min within 2 h of blood draw. The supernatant serum was collected and further centrifuged at 12,000 rpm for 15 min to remove cell debris. The whole process was strictly controlled to avoid hemolysis, and the serum was stored at −80℃ until RNA extraction.

2.4. RNA isolation and RT-qPCR analysis

Total RNA from serum samples was extracted using TRIzol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The quantity and quality of RNA were measured by NanoDrop spectrophotometer. The reverse transcription (RT) reactions were performed using a Prime Script™ RT Reagent Kit (Takara, Dalian, Liaoning). After mixing 1 μg of template RNA, 4 μL of 5× Prime Script Buffer Mix, 1 μL of Prime Script RT Enzyme Mix I, 1 μL of Oligo dT Primer and RNase-free dH_2O in a final volume of 20 μL, the reaction solution was incubated at 37℃ for 30 min, followed by 85℃ for 5 s and 4℃ for 60 min. RT-qPCR was run on a CFX96™ real-time system (Bio-Rad, CA, American). 2 μL of diluted generated cDNAs was mixed with 12.5 μL of SYBR Premix Ex TaqTM, 0.5 μL of Dye II, 1 μL forward and reverse primers (10 μM) and 9 μL of nuclease-free water in a final volume of 25 μL according to the manufacturer's instructions (Takara, Dalian, China). The reaction solution was incubated at 95℃ for 30 s, followed by 45 cycles of 95℃ for 5 s and 60℃ for 34 s. Each experiment was repeated at least three times. Melting curve analysis was performed to evaluate the specificity of the RT-qPCR products. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative gene expression level normalized by endogenous control, GAPDH. The primer sequences were synthesized by RiboBio (Guangzhou, China). LincRNA-p21 primer sequences: forward, 5′-GGGTGGCTCACTCTTCTGGC-3′; reverse, 5′-TGGCCTTGCCCG

GGCTTGTC-3′. GAPDH primer sequences: forward, 5′-CGCTCTCTGCT CCTCCTGTT-3′; reverse, 5′-CCATGGTGTCTGAGCGATGT-3′.

2.5. Statistical analysis

Data were presented as the median (interquartile range). Statistical significance was assessed by comparing the median (interquartile range) values between groups using the nonparametric Mann-Whitney U test. Statistical comparisons among multiple groups were made using one-way ANOVA analysis of variance. Nonparametric Spearman rank correlations were used to determine the relationships between lincRNA-p21 expression and clinicopathological characteristics. The probability value $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS 19.0 software and Graphpad.cameyo.

3. Results

3.1. Serum lincRNA-p21 levels in patients

Clinicopathological characteristics of the patients are showed in Table 1. Serum median lincRNA-p21 levels in patients with chronic HBV infection, hepatitis B cirrhosis, hepatitis B virus-related HCC, chronic HCV infection, non-HBV/HCV-related HCC, and alcoholic liver disease were 2.25,1.69,1.51,1.64,1.69 and 1.72, respectively, which were higher than that of control healthy individuals (0.96) ($P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.002$, respectively) (Fig. 1). The serum lincRNA-p21 level was not significantly different between patients with HBV negative liver metastatic cancer and normal control (1.07 VS 0.96, $P = 0.73$). LincRNA-p21 level in hepatitis B virus-related HCC patients was lower than that in patients with chronic HBV infection ($P = 0.003$). The lincRNA-p21 level was not significantly different between patients with hepatitis B cirrhosis and those with hepatitis B virus-related HCC ($P = 0.09$).

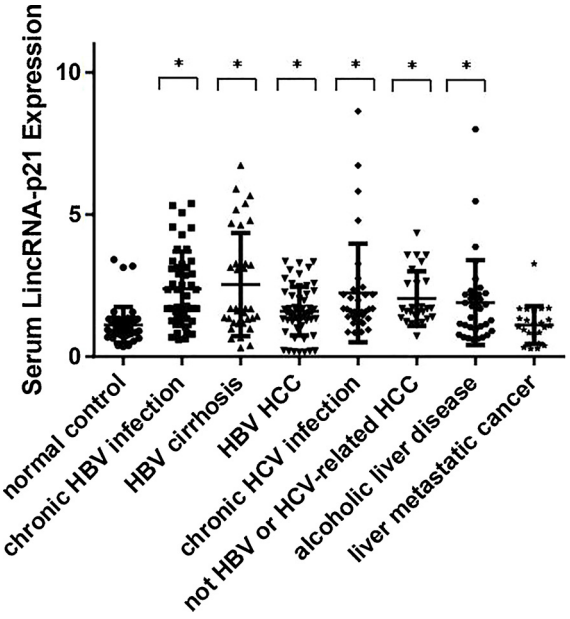

Fig. 1. Quantitative RT-PCR analysis of serum lincRNA-p21 levels in normal control (n = 48) and in patients with chronic HBV infection (n = 46), hepatitis B cirrhosis (n = 34), hepatitis B virus-related HCC (n = 56), chronic HCV infection (n = 34), non-HBV/HCV-related HCC (n = 27), alcoholic liver disease (n = 34), and HBV negative liver metastatic cancer (n = 24). Statistical comparison of the serum lincRNA-p21 levels between normal control and patients was made using the nonparametric Mann-Whitney U test. *$P < 0.01$ vs. the normal control.

Table 2
The correlation between serum lincRNA-p21 and clinicopathological characteristics in liver diseases.

Parameter	serum lincRNA-p21	
	correlation coefficients (r)	*P* value
Gender	−0.07338	0.24
Age	−0.1013	0.11
ALT	0.1537	0.01
AST	0.1716	0.01
AFP	0.0128	0.84
HB/HCV DNA	0.1834	0.02

Nonparametric Spearman correlation was used.

3.2. Associations between lincRNA-p21 expression and clinicopathological characteristics

LincRNA-p21 levels were negatively correlated with HBV DNA ($P = 0.02$), ALT ($P = 0.01$) and AST ($P = 0.01$) in patients with liver disease. However, we did not find any correlation between lincRNA-p21 levels and other clinicopathological features, including gender ($P = 0.24$), age ($P = 0.11$) and AFP level ($P = 0.84$) (Table 2). The difference in lincRNA-p21 level between HBeAg-positive hepatitis B patients and HBeAg-negative hepatitis B patients was also not significant ($P = 0.49$). There were 56 cases of hepatitis B virus-related HCC, including 21 cases of early (I or II) stage HCC and 35 cases of late (III or IV) stage HCC. The serum lincRNA-p21 content was not significantly different between early stage HCC patients and late stage HCC patients ($P = 0.05$).

3.3. LincRNA-p21 expression in primary hepatocellular carcinoma patients and liver metastatic cancer patients

Serum lincRNA-p21 in 83 primary hepatocellular carcinoma patients was higher than that in 24 liver metastatic cancer patients ($P < 0.001$) (Fig. 2). ALT and AST in primary hepatocellular carcinoma patients were higher than those in liver metastatic cancer patients (median (interquartile range) 33 (21–56) U/L VS 19 (14.25–68.5) U/L, $P = 0.04$; 49 (30–92) U/L VS 27 (20–66) U/L, $P < 0.001$). There was no significant difference in serum lincRNA-p21 between hepatitis B virus-related HCC and non-HBV/HCV-related HCC ($P = 0.08$).

3.4. LincRNA-p21 expression in alcoholic liver disease patients

Serum lincRNA-p21 levels in patients with alcoholic liver disease

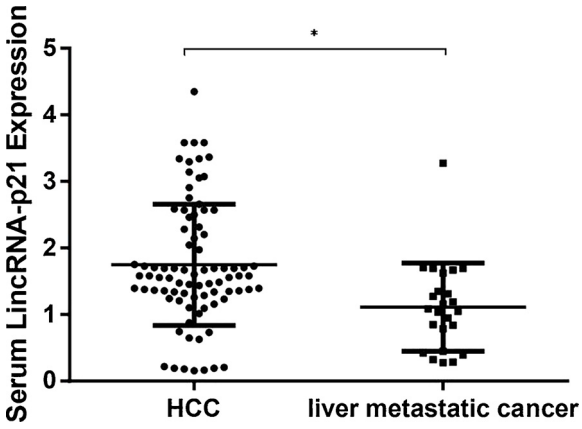

Fig. 2. Quantitative RT-PCR analysis of serum lincRNA-p21 levels in 83 hepatocellular carcinoma patients and 24 liver metastatic cancer patients. Statistical comparison of the serum lincRNA-p21 levels between two group was made using the nonparametric Mann-Whitney U test. *$P < 0.01$.

were lower than those in patients with chronic HBV infection ($P = 0.04$) (Fig. 1). There was no significant difference in serum lincRNA-p21 between HCC patients and alcoholic liver disease patients ($P = 0.79$).

4. Discussion

Most patients with HCC have an underlying chronic liver inflammation, which causes a continuous liver damage, leading to liver cirrhosis and eventually HCC. In China, the number of chronic hepatitis B patients who receive anti-virotic treatment is very low, and many Hepatitis B patients do not receive antiviral therapy until cirrhosis or HCC occurred.

Many LincRNAs were reported in the clinic as biomarkers for diagnosing liver disease. LINC00152, RP11-160H22.5 and XLOC014172 were different between HCC patients and chronic hepatitis patients or healthy controls. The risk score analysis revealed that combination of the three lncRNAs with AFP could distinguish HCC patients from chronic hepatitis patients and healthy control individuals with AUC of 0.986 and 0.985, respectively [9]. It was also found that long non-coding RNA HOTTIP/HOXA13 expression was associated with the disease progression and predicted the outcome in hepatocellular carcinoma patients [10]. LincRNa-p21 is related to the development and progression of human diseases, particularly in cancer. The expression of lincRNa-p21 was often inconsistent in different reports. LincRNA-p21 was reported significantly upregulated in NSCLC tissues and cells by Yang T [11], but lincRNA-p21 was reported down-regulated in tumor tissue in NSCLC by Castellano JJ [12]. In liver disease the expression of lincRNa-p21 was even more complex because of the viral infection and antiviral drug uses. Fujun Yu found that lincRNA-p21 expression was significantly down regulated in liver tissues during liver fibrosis, and could be used as a potential biomarker of liver fibrosis in chronic hepatitis B patients [13,14]. It was reported that lincRNA-p21 was downregulated in HCC tumor tissue and lincRNA-p21 overexpression inhibited tumor invasion through Notch signaling-induced EMT [15]. In our study, lincRNA-p21 expression in samples from patients with more types of liver diseases was investigated to improve the potential diagnostic application of this biomarker. We found that serum lincRNA-p21 level was significantly higher in patients with hepatitis virus infection, hepatitis B cirrhosis, and hepatitis B virus-related HCC, compared with the healthy control. This is inconsistent with previous reports, probably because most of the patients in our hospital have poor economic conditions and pay insufficient attention to hepatitis B virus infection. At the time of consultation, they have liver fibrosis or even HCC, and have never received antiviral treatment. LincRNA-p21 was also repoted upregulated in the hepatocyte during liver fibrosis by Xiaolong Tu [16].

LincRNA-p21 levels were correlated with HBV DNA in patients with hepatitis B virus-related disease, indicating that the serum lincRNA-p21 may associate with viral replication. LincRNA-p21 levels were also negatively correlated with ALT and AST in patients with hepatitis B virus-related disease. The livers of the 24 patients who had cancers that spread to liver were not damaged by alcohol or viruses, and the lincRNA-p21 levels in these patients were not as low as those in 83 primary hepatocellular carcinoma patients. Maybe the level of lincRNA-p21 is associated with hepatocyte injury. Our findings suggest that serum lincRNA-p21 level may serve as a potential biomarker for liver cell damage.

LincRNAs regulate a wide range of biological processes through diverse molecular mechanisms including chromatin modification, transcriptional regulation, and posttranscriptional regulation. Some lincRNAs in HCC tissues demonstrated a good correlation with those in plasma of HCC patients, such as small nucleolar RNA host gene 1 [17]. There was no report regarding the correlation of lincRNA-p21 level in tissues and in plasma of patients with liver diseases. However, down-regulation of lincRNA-p21 in human hepatocellular carcinoma tissues and liver fibrosis tissues were reported [18,13]. Yoon JH reported that lincRNA-p21 suppresses target mRNA translation [19]. Kobayashi S found that p21 mRNA expression was up-regulated to control cell cycle under regeneration stress, and once HCC develops in the liver, p21 mRNA expression decreases [20]. In our study, serum lincRNA-p21 level was higher in patients with chronic HBV infection and in hepatitis B virus-related HCC patients. LincRNA-p21 up-regulation and p21 down-regulation may act as a guard to prevent hepatocytes from tumorgenicity. Further study is needed to find out the exact role of lincRNA-p21 in tumorigenesis.

Alcohol has become the second leading cause of hepatic lesion after hepatitis virus in these years. Dong-Hyung Noh reported that alcohol intake induced piecemeal necrosis involving activation of the TGF-β1/p-Smad2/3/p21 signaling pathway in hepatocytes and alcohol administration and HCV core protein had a synergistic effect in the progression of hepatic degeneration [21]. This is the first report showing that serum lincRNA-p21 level was higher in patients with ALD. Maybe alcohol intake can cause liver cell damage and down-regulation of lincRNA-p21.

Collectively, our study demonstrated that serum lincRNA-p21 was higher in patients with primary liver diseases. Serum lincRNA-p21 may serve as a potential biomarker for liver cell damage in hepatitis virus infection, hepatitis B cirrhosis, HBV-related HCC and alcoholic liver disease.

Conflict of interest

All other authors declare no conflict.

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