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Serum microRNA-210 levels in different groups of chronic hepatitis B patients

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ABSTRACT

Background: It has been reported that hepatitis B virus (HBV) replication can be suppressed by microRNA-210 (miR-210). However, whether serum miR-210 levels can serve as disease parameters in patients with chronic hepatitis B (CHB) remains unclear.
Methods: Serum miR-210 levels were quantified in 115 CHB patients and 20 healthy controls by real-time PCR.
Results: We found that serum miR-210 levels can discriminate the different groups of CHB patients from healthy control ($P < 0.05$), as well as patients with HBe antigen positive from those with HBe antigen negative ($P < 0.05$). Serum miR-210 levels correlated with HBV DNA and HBs antigen ($r = 0.525$, $P < 0.001$ and $r = 0.348$, $P < 0.001$). Notably, inactive carrier patients with high (>3500 IU/mL) or low (<3500 IU/mL) levels of HBs antigen were differentiated by serum miR-210 levels ($P < 0.05$). Moreover, serum miR-210 levels correlated with liver inflammatory activity markers including alanine aminotransferase (ALT) and HAI score. However, there was no correlation of serum miR-210 levels with parameters of liver function including serum albumin, international normalized ratio and bilirubin, as well as the stages of liver fibrosis.
Conclusions: Serum miR-210 can be used as an indicator of HBV replication and translation, and a potential marker of necroinflammation in patients with CHB.

1. Introduction

Chronic infection with the hepatitis B virus (HBV), accounting for 350–400 millions of patients worldwide, is a predominant etiological factor for liver disease in China [1,2]. In particular, about 7.8% of China population are HBV carriers (93 million, two-thirds of the world's total number of carriers) [3]. Once the infection becomes chronic, chronic hepatitis B (CHB) patients are at the risk of developing liver cirrhosis and hepatocellular carcinoma (HCC). The increased risks of liver cirrhosis and HCC associated with HBV DNA level and the persistence of HBe antigen have been well documented [4].

MicroRNAs (miRNAs) are evolutionarily highly conserved, small (18–24 nucleotides long) non-coding RNAs and play a vital role in many physiological processes, including cellular development, differentiation, metabolism, apoptosis and proliferation [5]. They are additionally implicated in pathogenesis of chronic inflammation, cardiovascular disease and cancer [6–8]. Notably, it has been demonstrated that miRNAs have entered the stage of virology. For example, Zhang et al. found that miR-210 negatively regulates HBV replication by targeting the HBV pre-S1 region in HepG2 2.2.15 cells under normoxic condition [9]. Coppola et al. reported that liver hsa-miR-125a-5p correlates with HBV replication and disease progression [10]. Recently, it has been demonstrated that cell-free miRNAs could circulate in the blood and serve as stable biomarkers for diseases [11–14]. Here, we aimed to study the significance of serum miR-210 levels in treatment-naive patients with different stages of CHB and compared them with surrogate markers of viral replication and translation.

Abbreviations: HBV, hepatitis B virus,; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; miRNA, microRNA; HAI, histological activity index; ALT, alanine aminotransferase; INR, international normalized ratio; cccDNA, covalently closed circular DNA; HIV, human immunodeficiency virus; HCV, hepatitis C virus; Ct, cycle threshold.

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2. Methods

2.1. Patient selection

Serum samples were obtained from patients attending the First Affiliated Hospital of Wenzhou Medical University in Wenzhou and Ningbo Yinzhou Second Hospital in Ningbo from 2007.1 to 2012.8 (Table 1). 115 treatment-naive patients with CHB were included in this study and underwent liver biopsy. Inclusion criteria were CHB defined by detectable serum HBs antigen and serum HBV DNA for more than six months. Exclusion criteria were: (i) patients aged less than 16 years, (ii) co-infection with human immunodeficiency virus (HIV), (iii) coexistence of liver injury caused by other etiologies, including hepatitis C virus (HCV) infection, drug intake, alcohol consumption and auto-immune hepatitis, (iv) severe systematic diseases, and (v) pregnancy and lactation [15]. Demographic and clinical information was obtained, and blood samples collected from all patients on the day of diagnostic liver biopsy. 20 healthy controls (with normal liver biochemistry, no history of liver disease or alcohol abuse and no viral hepatitis) served as control patients. Written informed consent for the use of blood samples was obtained from all participants. The project was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). All procedures were performed in accordance with the current international guidelines, standards on human experimentation of the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China), and the Helsinki Declaration of 1975, revised in 1983.

Table 1
Patient characteristics.

Parameter	CHB patients	Healthy controls
Epidemiology		
Gender, m/f (%)	69/46 (60.0/40.0)	11/9 (55.0/45.0)
Age, years, median (range)	53 (41.7, 61.2)	50 (40.3, 59.6)
Virology		
HBs antigen (log_{10} IU/mL), median (range)	3.60 (1.50, 5.00)	
HBV DNA (log_{10} IU/mL), median (range)	3.88 (1.69, 8.91)	
HBe antigen positive, n (%)	16 (13.9)	
HBe antigen negative		
Inactive carriers, n (%)	20 (17.4)	
Low replicative, elevated ALT n (%)	9 (7.8)	
High replicative, normal ALT n (%)	31 (26.9)	
High replicative, elevated ALT n (%)	39 (33.9)	
HAI		
2, n (%)	13 (11.3%)	
3, n (%)	25 (21.7%)	
4, n (%)	8 (7.0%)	
5, n (%)	10 (8.7%)	
6, n (%)	12 (10.4%)	
7, n (%)	16 (13.9%)	
8, n (%)	12 (10.4%)	
9, n (%)	9 (7.8%)	
≥11, n (%)	10 (8.7%)	
Fibrosis		
F0, n (%)	15 (13.0%)	
F1, n (%)	19 (16.5%)	
F2, n (%)	24 (20.9%)	
F3, n (%)	20 (17.4%)	
F4, n (%)	21 (18.3%)	
F5, n (%)	6 (5.2%)	
F6, n (%)	10 (8.7%)	

Inactive carriers: patients with low replicative (HBV DNA < 2000 IU/mL) and normal ALT (<40 U/L); low replicative HBe antigen negative elevated ALT: patients with low replicative (HBV DNA <2000 IU/mL) and elevated ALT (>40 U/L); high replicative HBe antigen negative normal ALT: patients with high replicative (HBV DNA >2000 IU/mL) and normal ALT (<40 U/L); high replicative HBe antigen negative elevated ALT: patients with high replicative (HBV DNA >2000 IU/mL) and elevated ALT (>40 U/L); HBe antigen positive: patients with HBe antigen-positive hepatitis.

2.2. Liver histology

Liver biopsy was performed using a 16-gauge Menghini needle. Each liver biopsy case was advised by physicians in care, and liver specimens at least 2.0 cm in length were obtained. Samples were fixed in formalin, embedded in paraffin, and stained with hematoxylin–eosin. Results were reviewed by experienced hepatopathologists [16]. In addition, at least 8–10 portal tracts in samples are required to admit patients. Histological activity index (HAI) and fibrosis stages (F0 = no fibrosis–F6 = cirrhosis) were assessed according to the Ishak scoring system [17].

2.3. Blood sample processing

Blood samples were centrifuged at 3400 *g* for 7 min at room temperature within 4 h of acquisition. Sera were transferred into Eppendorf tubes and additionally centrifuged at 12,000 *g* for 10 min at 4 °C to remove the remaining cells. Serum samples were stored at −80 °C pending RNA extraction.

2.4. Detection of miR-210 with real-time quantitative RT-PCR

According to the manufacturer's instructions for liquid samples, total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Carlsbad, California, USA). Then DNase treatment (Qiagen, Carlsbad, California, USA) was performed to remove DNA contaminants in total RNA and final elution volume was 20 μL. In addition, serum RNA preparations were quantified using NanoDrop 1000 (Nanodrop, Wilmington, Delaware, USA) before the reverse transcription reaction. Reverse transcription was performed in a 20 μL reaction volume using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). For cDNA synthesis, reaction mixtures were sequentially incubated at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. miR-210 was quantified in triplicate via qRT-PCR using the TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA). According to the standard TaqMan MicroRNA assay protocol, real-time PCR was performed on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) under the following conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for 60 s. Each PCR mixture (20 μL) included the reverse transcription products, TaqMan 2X Universal PCR Master Mix without UNG Amperase, miRNA-specific TaqMan probes, and primers supplied by Applied Biosystems. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. Ct values were calculated with SDS 2.0.1 software (Applied Biosystems, Foster City, CA). The formula $2^{-\Delta Ct}$ was used to calculate the miRNA levels in serum, where ΔCt = mean (Ct of internal references) − Ct of target miRNA. The relative expression levels of miR-210 were calculated and normalized to miR-16 (Applied Biosystems, Foster City, CA) using the comparativeΔCt method and the equation $2^{-\Delta Ct}$, as described previously [18].

2.5. Serum HBV DNA and serum hepatitis marker analysis

For all patients, serum HBV DNA was quantified using an Artus HBV QS-RGQ Kit (Qiagen, Hilden, Germany), with a lower detection limit of 10.2 IU/mL. HBsAg, HBeAg, and antibodies against HBsAg (anti-HBs), HBeAg (anti-HBe) and hepatitis B core antigen (anti-HBc) were determined using the Roche Modular E170 Immunoassay Analyzer (Roche, Basel, Switzerland). HBsAg titres were additionally determined with the HBsAg quantitative assay (Abbott Laboratories, Abbott Park, IL, USA) based on an automated chemiluminescent microparticle immunoassay (Abbott Architect i2000SR analyzer; Abbott Laboratories), according to the manufacturer's instructions. Architect HBsAg QT measures a range of HBsAg from 0.05 to 250 IU/mL. Therefore, the dilution for samples with higher HBsAg titre is required.