ORIGINAL ARTICLE

Hepatitis B Virus Affected Serum MicroRNA-203a Level in Hepatocellular Carcinoma

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SUMMARY

Background: MicroRNAs have been shown to play a critical role in early diagnosis of hepatocellular carcinoma. Nevertheless, microRNAs’ functions in serum of patients with hepatocellular carcinoma (HCC) are not fully understood.

Methods: qRT-PCR was used to detect the expression level of microRNA-203a (miR-203a) in clinical serum samples of HCC and HepG2 cells. Kaplan-Meier method was used to estimate overall survival, and the cell scratch test was used to observe the migration ability of cells in vitro.

Results: Here, we first observed that serum miR-203a was significantly up-regulated in HCC patients with HBV compared to without HBV. In HCC patients, miR-203a low expression was positively related with poor overall survival. In addition, we found that HBV improved the poor prognosis of HCC patients with lower miR-203a levels. After successfully constructing HepG2 cell line carrying HBV, further studies demonstrated miR-203a expression level was increased in HepG2 with HBV compared to without HBV.

Conclusions: Lower serum miR-203a level in HCC patients led to worse overall survival, which depended on HBV. In vitro, miR-203a level was positively correlated with HBV. Therefore, our studies provided the novel insight into the role of serum miR-203a in HCC patients with HBV and potential new molecular target for early diagnosis of hepatitis B virus-related hepatocellular carcinoma.


KEY WORDS
hepatocellular carcinoma, miR-203a, HBV, serum

INTRODUCTION

Hepatocellular carcinoma (HCC) is a type of primary liver cancer with a high mortality rate and one of the most common malignant cancers in the world [1]. HCC is the third most common cause of cancer-related mortality worldwide. Genetics, hepatitis B virus infection, hepatitis C virus infection, and aflatoxin exposure are the main risk factors for HCC [2]. At present, the prognosis of HCC patients is poor, mainly due to the higher recurrence and metastasis rate. Antiviral therapy is the most basic etiological treatment of hepatitis B-related HCC, which has received much attention in clinical practice [3]. However, the most effective treatment for
HCC is the early operation of cancers, and the 5-year survival rate of patients with early diagnosis and treatment exceeds 70%, which is much higher than that of patients with advanced HCC [4]. Therefore, improving the efficiency of early screening and diagnosis and early treatment are effective ways to improve patient survival rate and prolong survival time [5]. The blood test in patients has many advantages, such as convenience, economy, small harm, and strong operability. It has great clinical value, but it has not been fully implemented due to the lack of effective early detection and diagnosis markers for HCC patients with HBV. Therefore, it is still necessary to find a sensitive and specific index for early diagnosis of HCC patients with HBV or without HBV.

MicroRNAs are a type of small RNA ribonucleotide sequence, which inhibit gene expression by binding to the 3’ untranslated region (UTR) of the target genes’ mRNA [6]. miRNAs have been found in animals, plants, and viruses. About 40% of miRNA genes are latent in introns or exons of non-protein-expressed genes or protein-coding genes, which regulate gene expression [7]. Recently, many studies have emphasized that miRNA may play a key role in HBV with hepatitis B virus [8]. Although HBV-encoded miRNA has not been verified, it has been confirmed that HBV products could affect cellular miRNA expression. Much research has focused on the measurement of miRNA in HBV-related HCC. Serum miRNAs could be the diagnostic and prognostic markers for HCC. For example, serum miR-145 and miR-199b may indicate the conversion of precancerous lesions [9]. However, circulating microRNA may not only become a molecular marker related to HBV infection and HCC, but also may become their corresponding therapeutic target.

As a member of the miRNA family, miR-203a was first discovered to be highly expressed specifically in epithelial basal cells. Abnormally expressed miR-203a, as a tumor suppressor gene or oncogene, participates in the occurrence and development of many cancers such as skin cancers, leukemia, cervical cancer, breast cancer, prostate cancer, lung cancer, and digestive system cancers [10-12]. The expression level of miR-203a was detected in the tissues of patients with HCC, and it was found that miR-203a level was lower than that of the adjacent cancer, suggesting that miR-203a was a tumor suppressor gene in HCC [13]. At present, the detection of miR-203a expression in serum and tissue has been applied to the diagnosis and prognosis judgment of some diseases [14]. There are relatively few research reports on the diagnosis of HBV-related liver diseases. Therefore, our study tested the expression level of miR-203a in peripheral blood serum of HCC patients with HBV, explored the diagnostic value of miR-203a in HBV-related HCC, and identified serum miR-203a as a molecular marker for HBV-related HCC. It provides a basis for further research on the bio-molecular mechanism of serum miR-203a in the development of new therapeutic strategies.

### MATERIALS AND METHODS

#### Clinical samples

Serum samples of 60 HCC patients with hepatitis B virus and 60 HCC patients without HBV who underwent surgical treatment in our hospital from February 2014 to February 2015 were collected. All patients were diagnosed postoperatively, and they were in the early stage of liver cancer. All patients with hepatitis A, C, D, E virus or mixed virus infection should be excluded. Before taking the medicine and performing surgical treatment, when patients were on an empty stomach in the morning, 4 mL of peripheral venous blood was collected in a 5 mL test tube (sterile, RNase-free, and anti-coagulant-free) at room temperature. Let it coagulate naturally to separate the serum. After these samples were centrifuged at 3,000 r/min for 10 minutes, the upper serum was drawn and filled into sterile and RNase-free 1.5 mL centrifuge tubes. After standing at room temperature for 5 minutes, serum was kept at -80°C. The study was approved by the Ethics Committee of Yichun City People’s Hospital, and the patients or family members signed an informed consent.

#### Cell culture and plasmid transfection

HepG2 cells were cultured in 10% fetal bovine serum and 100 U/mL penicillin in DMEM culture medium. The culture conditions were 37°C and 5% CO2, and the cells were passaged when they grew to 80% in the plate. The hepatitis B virus full-length DNA (HBV-DNA) plasmid was cloned into the adenovirus shuttle plasmid vector (pAdTrack-CMV). Green fluorescent protein (GFP) was added to the plasmid, finally the recombinant adenovirus plasmid (pAd-HBV-GFP) was obtained. The plasmid was produced by the Sangon Biotech company in Shanghai. pAd-HBV-GFP expression vector and pAdTrack-CMV were transfected into HepG2 cells by liposome (Lipofectamine™ LTX and Plus Reagent 3000). After 6 hours of starvation, the cells continue to be cultured for 24 hours.

#### Extraction of total RNA from serum and cells

The dissolved serum samples were centrifuged at 1,500 r/min at 4°C for 5 minutes, and the supernatant was transferred to a new tube. Trizol kit was used to extract the total RNA of cells and serum. RNAs were concentrated by isopropanol, and RNA concentration was determined by NanoDrop (Thermo Scientific, Shanghai, China). The blood test in patients has many advantages, such as convenience, economy, small harm, and strong operability. It has great clinical value, but it has not been fully implemented due to the lack of effective early detection and diagnosis markers for HCC patients with HBV or without HBV.

#### Primer design

Primers were designed according to the gene sequence of Genbank. The specific primers are as follows: miR-203a upstream primer 5'-GTCAACAGTTCTGACGCA-3', downstream primer 5'-CCGGTGTTAGTGCTCCTAAA-3'; RNU6B upstream primer

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5'-TCGCTTCGCGCAGCAC-3',
5'-AACGCTTCAGAATTGGC-3'.

qRT-PCR
To avoid RNA degradation, all operations should be quickly performed. The total RNA was reverse-transcribed into cDNA using miScript II RT Kit (Qiagen, Germany). The reaction conditions: 37°C 15 minutes, 85°C 5 seconds. cDNA was used as template for PCR amplification. PCR reaction conditions on ABI 7300 real-time fluorescence quantitative machine: 95°C 30 seconds, 95°C 5 seconds, 40 cycles at 60°C for 30 seconds. The mRNA level was represented by 2-ΔΔCT. For qRT-PCR, RNU6B as the internal reference.

Cell migration assay
Cells, 5 x 10^4, were resuspended in serum-free medium and placed in the upper chambers. Use the tip of the pipette to compare with a ruler, as far as possible to scratch the horizontal line behind, the tip should be vertical, and cannot be inclined. Wash the cells 3 times with PBS, remove the scratched cells, and add serum-free medium. Put in a 37°C 5% CO2 incubator and cultivate. Press 0 and 24 hours to take samples and take pictures.

Statistical analysis
Chi-square test was used to compare the different clinicopathological factors between HCC patients with HBV and without HBV. Paired t-test for the two groups and one-way ANOVA for more than two groups were used to analyze miR-203a expression data. When data did not follow normal distribution, Mann-Whitney U test between two groups and Kruskal-Wallis H test for three or more groups should be used. Kaplan-Meier analysis and the log rank test were used for survival analysis. Furthermore, univariate and multivariate analyses were performed using the logistic regression model. All of the data were analyzed using GraphPad Prism 6.0 and SPSS 26.0. A p-value of < 0.05 indicated statistically significant changes. Each experimental design included more than three biological repeats.

RESULTS

The relationship between HBV and clinical features in patients with hepatocellular carcinoma
One hundred twenty patients with early-stage liver cancer have been diagnosed, aged from 44 to 72 years, with an average of 57.8 years. There were 60 patients in the hepatitis B virus group, including 49 males and 11 females, with an average age of 55.8. There were 60 patients in the non-HBV group, including 32 males and 28 females, with an average age of 59.8. Comparing the different clinical characteristics of the two groups of patients, there are more male patients in the hepatitis B virus group, and the AFP level is higher than that in the non-hepatitis B virus group. There are no differences between the two groups with other characteristics, such as cirrhosis, liver function, tumor site, and TNM classification, as shown in Table 1.

In HCC patients with HBV, the overall survival rate was 29.9%. In HCC patients without HBV, the overall survival rate was 41.7%. Comparing the 5-year overall survival rate of the two groups of patients, there was a significant difference after the log-rank test, the overall survival rate with HBV was significantly lower than that without HBV, shown in Figure 1. In addition, for cox proportional hazards regression model, univariate and multivariate analysis indicated that ALP, TNM staging, and HBeAg were independent risk factors that affected the overall survival of HCC. Their Hazard Ratio (HR) was 1.88, 2.33, and 1.89 during univariate analysis, and HR values during multivariate analysis were 1.59, 1.96, and 1.67, shown in Table 2.

MiR-203a was increased in HCC patients with HBV compared to those without HBV
In serum of HCC patients, we detected the miR-302a level by qRT-PCR and found that the overall level of miR-203a in 60 HCC patients with HBV was higher than that in 60 HCC patients without HBV. In 60 HCC patients with HBV, the miR-203a mean level was 1.69, while it was 0.70 in 60 HCC patients without HBV, shown in Figure 2A. Serum miR-203a mean level with HBV was more than twice that in patients without HBV, suggested that serum miR-203a level was increased by HBV.

The effect of serum miR-203a level on overall survival rate of hepatocellular carcinoma patients depended on HBV
Both groups were divided into two subgroups according to the mean level of miR-203a, as “more than mean level” and “less than mean level”. In the 60 HCC patients without HBV, 26 patients’ serum miR-203a levels were more than the mean level, and 34 patients’ levels were less than the mean level. Comparing the 5-year survival rates of the two subgroups, it was found that the “more than mean level” group was survived longer than “less than mean level”, 50% vs. 30.4%, suggested that lower serum miR-203a level has the worse prognosis in HCC patients without HBV (Figure 2B). In the 60 HCC patients with HBV, 33 patients’ serum miR-203a levels were more than the mean level, and 27 patients’ levels were less than the mean level. Comparing the 5-year survival rates of the two subgroups, it was found that there was no significant difference between the “more than mean level” and “less than mean level” group, 32.1% vs. 28.1%, showed that HBV did not affect the poor prognosis of HCC patients with lower miR-203a levels (Figure 2C).
Table 1. Relationship between HBV and clinicopathological features in early stages of HCC patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>With HBV/HBeAg(+)</th>
<th>Without HBV/HBeAg(-)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>p = 0.271</td>
</tr>
<tr>
<td>&lt; 60</td>
<td>24</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>≥ 60</td>
<td>36</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>p = 0.001 *</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>49</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>AFP (ng/mL)</td>
<td></td>
<td></td>
<td>p &lt; 0.001 *</td>
</tr>
<tr>
<td>&lt; 400</td>
<td>16</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>≥ 400</td>
<td>44</td>
<td>22</td>
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</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Absence</td>
<td>46</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Liver function</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Child-Pugh A</td>
<td>39</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Child-Pugh B and C</td>
<td>21</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Lobe</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Right</td>
<td>43</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>17</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>TNM</td>
<td></td>
<td></td>
<td>p = 0.568</td>
</tr>
<tr>
<td>I/II</td>
<td>37</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>III/IV</td>
<td>23</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05.

Table 2. Univariate and multivariate analyses of overall survival in HCC patients.

<table>
<thead>
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<th>Parameters</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age (≥ 60/&lt; 60)</td>
<td>1.22</td>
<td>0.82 - 1.87</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>0.78</td>
<td>0.43 - 1.32</td>
</tr>
<tr>
<td>Cirrhosis (absence/presence)</td>
<td>1.23</td>
<td>0.67 - 2.59</td>
</tr>
<tr>
<td>Liver function (Child-Pugh A/Child-Pugh B and C)</td>
<td>1.12</td>
<td>0.62 - 2.02</td>
</tr>
<tr>
<td>Lobe (right/left)</td>
<td>0.87</td>
<td>0.62 - 1.36</td>
</tr>
<tr>
<td>AFP (ng/mL) (≥ 400/&lt; 400)</td>
<td>1.88</td>
<td>1.01 - 3.21</td>
</tr>
<tr>
<td>TNM (I, II/III, IV)</td>
<td>2.33</td>
<td>1.47 - 3.34</td>
</tr>
<tr>
<td>HBeAg (positive/negative)</td>
<td>1.89</td>
<td>1.25 - 3.12</td>
</tr>
</tbody>
</table>

** p < 0.01, * p < 0.05.

The miR-203a expression levels in HepG2 cells was affected by HBV

In order to explore the relationship between HBV and miR-203a levels in vitro, we constructed a viral plasmid containing the HBV genome. The gelose gel electrophoresis indicated that this large plasmid with HBV genome was about 7 kb, shown in Figure 3A. After being stably transfected into the HepG2 cells, we used PCR to verify the success of the cell model with HBV, shown in Figure 3A. Meanwhile, through the GFP reporter...
Figure 1. The five-year overall survival rate of HCC patients.
Black line expressed patients without HBV, grey line showed patients with HBV.

Figure 2. The relationship between HBV and serum miR-203a level in HCC patients.
A. The comparison of serum miR-203a level between HCC patients with HBV and without HBV, black points expressed HCC patients without HBV, grey points expressed HCC patients with HBV.
B. When serum miR-203a level was used as the cutoff, HCC patients without HBV were divided into two groups, “miR-203a more than mean level” and “miR-203a less than mean level”. The five-year overall survival rate of the two groups were shown, black line expressed “miR-203a more than mean level”, grey line expressed “miR-203a less than mean level”.
C. HCC patients with HBV were also divided into two groups. The five-year overall survival rate of the two groups were shown, black line expressed “miR-203a more than mean level”, grey line expressed “miR-203a less than mean level”. * p < 0.01.
Figure 3. HepG2 cells line carrying HBV and GFP promoted the progression of HCC.

A. Specific endonuclease digestion and PCR verified the effectiveness of recombinant DNA plasmid.
B. Directly observe the effect after transfection under a fluorescence microscope (magnification × 200).
C. Observe the effects of HBV on the invasion and migration of HepG2 cells through cell scratch experiments (magnification x 100).
Figure 4. The relationship between HBV and miR-192-2 level in HepG2 cells.

Detected the miR-203a expression level between HepG2 cells with HBV and without HBV by qRT-PCR.

* p < 0.05.

gene, the HepG2 cells model carrying HBV could be more clearly established successfully, shown in Figure 3B. Moreover, we found that the proliferation and migration ability of HepG2 with HBV was stronger than that of HepG2 without HBV by cell migration experiments, shown in Figure 3C, suggested that HBV could increase the cell proliferation of hepatocellular carcinoma. Finally, we detected miR-203a expression level in HepG2 with HBV, found that miR-203a in HepG2 with HBV had higher expression than that in HepG2 without HBV, shown in Figure 4, indicated that the expression of miR-203a in vitro was up-regulated by HBV.

DISCUSSION

Hepatitis B virus replication is an important factor causing hepatitis, cirrhosis, liver cancer and other diseases [15]. A large number of clinical studies have shown that the high expression of hepatitis B virus DNA levels is positively correlated with the occurrence of HCC [16]. For patients with HBV infection, active antiviral therapy can reduce the risk of HCC, especially for HBV patients with positive hepatitis B e antigen [17]. Of course, early diagnosis can significantly improve the survival rate and prognosis of HBV-related HCC patients. The detection of serum bio-markers is of great clinical value in the early screening and diagnosis of HCC. Alpha-fetoprotein (AFP) is currently the only clinical serum marker, but its detection sensitivity and accuracy have not reached the ideal level [18]. The serum markers discovered in recent years, such as alpha-fetoprotein heteroplasma, de-γ-carboxy-prothrombin, Golgi glycoprotein 73, and osteopontin which make up for the deficiency of AFP in diagnostic sensitivity and specificity [19]. For these bio-markers, Direct evidence from clinical research was lacking so they have not been effectively used in clinical diagnosis. Therefore, the discovery and application of new serum markers to improve the efficiency of early diagnosis of HBV-related HCC has become an urgent need in clinical.

Since miRNA was first discovered in Caenorhabditis elegans in 1993 [20], great progress has been made in the study of miRNA in human carcinogenesis. Studies have shown that miRNA played the role of oncogene or tumor suppressor gene, and simultaneously regulated multiple genes and participated in the regulation of tumor development by combining target mRNA. In addition, miRNA showed a high degree of stability in serum or plasma due to the protective effect of RNase [21]. Therefore, it was believed that miRNA presented significant differential expression levels in HCC and normal serum samples, which had a potential indicator role in the screening and diagnosis of HBV-related HCC [22]. In HCC, miR-122 level was decreased, which could inhibit the target sequence of HBV replication [23] so that the interaction of miRNA and HBV mRNA maybe cause chronic diseases. Many recent studies have re-
ported many HBV-related miRNAs in HCC [24], such as miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801. In addition, the expression levels of miR-25, miR-92a, miR-206, miR-375, and let-7f in serum samples of HCC patients were significantly increased compared with controls [25]. MiR-125b-5p and miR223-3p showed certain expression specificity in patients with HBV-related liver disease [26], which suggested that the specificity of using miRNA to diagnose patients with HBV-positive HCC was feasible. These studies confirmed that serum miRNA as a potential biomarker for the diagnosis of HBV-related HCC had a very important clinical value in the study of early diagnosis of HBV-related HCC.

Moreover, over-expression of miR-203a significantly inhibits the proliferation and migration of liver cancer cells [27]. The low expression of miR-203a also plays an important role in the occurrence and development of HCC through the release of cancer-promoting targets [28]. The dynamic detection of serum miR-203a in HCC patients will provide some guiding significance for clinical individualized treatment and prognosis for patients. Abnormal expression was found in serum miR-203a in hepatitis C virus-associated chronic liver disease patients [29]. However, the miR-203a level in the serum of HCC patients with HBV has not been reported. Therefore, our study is the first to detect the expression of miR-203a in the serum of HBV-related HCC patients.

In our study, we first compared the level of serum miR-203a between HCC patients with HBV and without HBV. We found that serum miR-203a level was higher in HCC patients with HBV. After analyzing the overall survival rate, we first showed that the effect of serum miR-203a level on survival rate of hepatocellular carcinoma patients depended on HBV. Moreover, we constructed a stable HepG2 cell line with HBV, and after confirming the transfection effect by fluorescence microscopy and PCR. The HepG2 cell line was used in the later stage. Then, HepG2 with HBV had stronger proliferation and migration ability. Finally, we further verified that HBV could increase the level of miR-203a in HepG2 cells.

CONCLUSION

In summary, serum small molecules have gradually become a hot spot for early diagnosis of cancers. Our studies first confirmed that serum miR-203a was related to HBV in HCC. We further studied the expression of miR-203a affected the progression of HCC patients. In particular, the study confirmed that the effect of serum miR-203a level on overall survival of HCC patients depended on HBV and miR-203a expression level was up-regulated by HBV in HepG2. However, serum miR-203a level could act as a new biomarker for the early diagnosis and prognosis assessment. Therefore, this study elaborated the new function of serum miR-203a in HCC and provided new theoretical and experimental basis for early diagnosis of HCC patients with hepatitis B virus.

Ethics Approval and Informed Consent to Participate:
The inclusion of human participants, as well as the use of human data and human samples in this study, was approved by the Ethics Committee of the Jiujiang University Affiliated Hospital. The patients or family members all signed an informed consent.

Declaration of Interest:
The authors declared that they had no conflicts of interest.

References:


