The Interaction of lncRNA-HEIH and lncRNA-HULC with HBXIP in Hepatitis B Patients

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Received 13 July 2018; Revised 6 October 2018; Accepted 23 October 2018; Published 4 December 2018

Academic Editor: Haruhiko Sugimura

Hepatitis B virus (HBV) infection is a major public health concern, affecting more than 350 million people worldwide, despite the advent of effective vaccines and other control measures [1–3]. Chronic HBV infection is characterized by the detection of serum hepatitis B surface antigen (HBsAg) after 6 months of infection with continuous liver inflammation and activation of fibrogenic processes, which can lead to hepatic cirrhosis (HC), decompensated (symptomatic) liver disease, and the development of hepatocellular carcinoma (HCC) in 25%–40% of HBV carriers [3]. Thus, it is urgent to identify new and promising diagnostic markers and therapeutic targets for HBV-related diseases. HBV is a double-stranded DNA virus containing four partially overlapping open reading frames, encoding the C, S, and X proteins, and a viral DNA polymerase [4]. Among these four proteins, only the X protein (HBX) has been clearly associated with tumorigenesis [5]. The hepatitis B X-interacting protein (HBXIP) was originally identified by its interaction with the C-terminus of the HBX, which has been found to enhance the growth of hepatoma cells and promote tumorigenesis [6].

Long noncoding RNAs (lncRNAs), a class of RNA segments >200 nucleotides in length, have been considered to be the “noise” of genome transcription owing to their limited protein-coding capacity [7]. With the continued development of viral research, it has been revealed that lncRNAs play important roles in the regulation of protein coding genes, stem cell differentiation, allelic expression, cell

1. Introduction

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Long noncoding RNAs (lncRNAs), a class of RNA segments >200 nucleotides in length, have been considered to be the “noise” of genome transcription owing to their limited protein-coding capacity [7]. With the continued development of viral research, it has been revealed that lncRNAs play important roles in the regulation of protein coding genes, stem cell differentiation, allelic expression, cell
cycle control, and cell death [8–11]. Moreover, the aberrant expression of lncRNAs has been widely associated with the physiological and pathological processes of many diseases. Therefore, the potential of lncRNAs as therapeutic targets has been raised and tested in several recent studies. A 16 kb lncRNA found at chromosomal location 6p24.3, which is highly upregulated in liver cancer (lncRNA-HULC) and HCC, is composed of one intron and two exons [12, 13]. In addition, a lncRNA highly expressed in HCC (lncRNA-HEIH) has been associated with disease recurrence and, thus, has been investigated as an independent prognostic factor for overall survival of patients with hepatitis B-related HCC [11]. Although the expression levels of lncRNA-HULC and lncRNA-HEIH were found to be increased in HCC, the underlying molecular mechanisms remain unclear.

The aims of this study were to quantify the expression levels of lncRNA-HULC, lncRNA-HEIH, and HBXIP in patients with HBV infection and hepatitis B-related diseases.

2. Materials and Methods

2.1. Ethics Statement. The study protocol was approved by the Ethics Committee of People’s Hospital of Anji and conducted in accordance with the tenets of the Declaration of Helsinki and the ethical guidelines for medical and health research involving human subjects as established by the National Institutes of Health and the Committee on Human Research of People’s Hospital of Anji. Written informed consent was obtained from all patients or their lineal relatives for the use of peripheral blood and liver tissues.

2.2. Peripheral Blood Samples. Peripheral blood samples were collected from 75 HBV-positive patients and 25 HBV-negative normal controls who received treatment or medical examinations at People’s Hospital of Anji. Patients with infections of HBV or any other virus (e.g., hepatitis virus A, C, D, E, and I) or liver diseases (e.g., hepatic cyst and hepatic metastasis) were excluded from analysis. The 100 study participants were allotted to one of the four following groups (n = 25 each): a HBV-positive group (14 females and 11 males with no HBV-related disease); a HBV + HC group (12 females and 13 males with HBV-related disease and HC); a HBV + HCC group (10 females and 15 males with HBV-related disease and HCC); or a control group (13 female and 12 male age-matched controls who were HBV negative with no history of an infectious, psychiatric, neurological, or metabolic disease). The clinical features of all study participants are summarized in Table 1.

2.3. Liver Tissues. HBV-positive HCC tissues and corresponding adjacent noncancerous liver tissues (NT) were obtained from patients in the HBV + HCC, HBV-HCC, HBV + NT, and HBV-NT groups who underwent resection in our hospital. On account of the ethical limitation, liver tissues were not collected from the normal controls, HBV carriers, or HBV-positive patients with HC. Thus, normal and cirrhotic liver tissues were not assessed in this study. The clinicopathological characteristics of the study participants are summarized in Table 2.

2.4. RT-qPCR. RT-qPCR was used to detect the expression of lncRNA-HULC and lncRNA-HEIH. Total RNA was isolated from the peripheral blood samples, liver tissues, and HepG2.2.15 cells using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) and then reverse transcribed into cDNA with the Veriti 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA) using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). Each RT-qPCR reaction contained 2 μL of cDNA, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 10 μL of SYBR Green qPCR Master Mix (TaKaRa Biotechnology (Dalian) Co. Ltd., Dalian, China). The threshold cycle (CT) values were used to calculate the relative expression levels of target genes with the comparative CT method using GAPDH as the reference gene.

Table 1: Clinical features of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HBV</th>
<th>HBV + HC</th>
<th>HBV + HCC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>14</td>
<td>12</td>
<td>10</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>11</td>
<td>13</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>45.07 ± 15.06</td>
<td>40.18 ± 16.25</td>
<td>42.31 ± 8.56</td>
<td>45.62 ± 11.28</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>55.58 ± 12.01</td>
<td>91.46 ± 12.96</td>
<td>121.77 ± 16.89</td>
<td>157.29 ± 21.78</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ALB (g/L)</td>
<td>48.44 ± 8.88</td>
<td>43.32 ± 7.58</td>
<td>28.21 ± 6.69</td>
<td>30.37 ± 6.84</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TBIL (μmol/L)</td>
<td>15.23 ± 3.67</td>
<td>22.24 ± 4.52</td>
<td>33.52 ± 7.17</td>
<td>40.44 ± 8.03</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>DBIL (μmol/L)</td>
<td>4.28 ± 1.13</td>
<td>8.44 ± 1.61</td>
<td>17.22 ± 3.35</td>
<td>27.24 ± 5.98</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AFP (μg/L)</td>
<td>4.95 ± 1.13</td>
<td>26.83 ± 4.67</td>
<td>52.79 ± 7.76</td>
<td>122.74 ± 18.76</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>25.48 ± 6.68</td>
<td>181.67 ± 21.90</td>
<td>69.28 ± 11.11</td>
<td>78.24 ± 10.94</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>22.14 ± 5.11</td>
<td>196.75 ± 20.29</td>
<td>89.89 ± 13.22</td>
<td>102.25 ± 14.11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HBV-DNA (copies/mL)</td>
<td>0</td>
<td>(7.12 ± 0.28) × 105</td>
<td>(2.23 ± 0.47) × 104</td>
<td>(7.35 ± 0.55) × 104</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

HBsAg: hepatitis B surface antigen; ALP: alkaline phosphatase; ALB: albumin; TBIL: total bilirubin; DBIL: direct bilirubin; AFP: alpha-fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase.
2.5. RNA Immunoprecipitation (RIP). RIP analyses were performed to detect the interaction between LncRNA-HEIH/LncRNA-HULC and HBXIP using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (EMD Millipore Corporation, Billerica, MA, USA) in accordance with the manufacturer’s instructions. Briefly, liver tissues or harvested cells were lysed in RIP lysis buffer and then incubated with RIP buffer containing magnetic beads conjugated to antibodies (Abs) against HBXIP (Abcam, Cambridge, UK) or normal rabbit immunoglobulin (IgG (Abcam). The samples were incubated with proteinase K to digest the proteins, then the coprecipitated RNA was detected by RT-qPCR.

2.6. Western Blot Analysis. Total protein was extracted from the liver tissues and HepG2.2.15 cells, then quantified, diluted with 5× loading buffer to the same concentration, denatured at 95°C, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene fluoride membranes, which were blocked with 5% skimmed milk at room temperature for 2 h and then incubated with Abs against HBXIP (dilution, 1:1000; Abcam) and GAPDH (dilution, 1:3000; Abcam) overnight at 4°C. Afterward, the membranes were washed three times with Tris-buffered saline with Tween 20, incubated with horseradish peroxidase-conjugated goat anti-rabbit Ab (dilution, 1:4000; Abcam), and washed three times with Tris-buffered saline with Tween 20. Then, the protein bands were visualized using an enhanced chemiluminescence reagent (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and quantified by densitometric scanning with the Fusion-FX7 system (Vilber Lourmat, Collégien, France). The mean optical density of the samples was normalized to that of GAPDH.

2.7. Statistical Analysis. All data are expressed as the mean ± standard deviation and were analyzed using the t-test, chi-squared test, or one-way analysis of variance with SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA) to determine the significance. A probability (p) value of <0.05 was considered statistically significant.

3. Results

3.1. Expression Levels of LncRNA-HEIH and LncRNA-HULC in Peripheral Blood and Liver Tissues. According to the RT-qPCR results, the expression levels of LncRNA-HEIH and LncRNA-HULC were significantly upregulated in the peripheral blood of HBV-positive patients (HBV, HBV + HC, and HBV + HCC groups), as compared to the control group (p < 0.05, Figure 1(a)). Moreover, this increase was more prominent in the HBV + HCC group than the HBV and HBV + HC groups (p < 0.05, Figure 1(a)). Similarly, the expression levels of LncRNA-HEIH and LncRNA-HULC were notably increased in the liver tissues of patients with HCC (HBV + HCC and HBV-HCC groups) as compared to corresponding adjacent noncancerous liver tissues (HBV + NT and HBV-NT groups) (p < 0.05, Figure 1(b)). In addition, the expression levels of LncRNA-HEIH and LncRNA-HULC were greater in the liver tissues of the HBV + HCC and HBV + NT groups than that in the HBV-HCC and HBV-NT groups, indicating that expression was greater in HBV-positive than HBV-negative liver tissues (p < 0.05, Figure 1(b)).

3.2. LncRNA-HEIH and LncRNA-HULC Coimmunoprecipitates with HBXIP. RIP assays were performed to determine whether HBXIP interacts with LncRNA-HEIH and LncRNA-HULC. RNA obtained from the RIP assay using Abs against HBXIP and IgG was subjected to RT-qPCR analysis. The results indicated that the expression levels of LncRNA-HEIH and LncRNA-HULC were greater in the samples pretreated with HBXIP.

Table 2: Clinical features of patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HBV + HCC</th>
<th>HBV-HCC</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
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<td>Female</td>
<td>10</td>
<td>12</td>
<td>&gt;0.05</td>
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<tr>
<td>Male</td>
<td>15</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>HBsAg Positive</td>
<td>0</td>
<td>25</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>45.62 ± 11.28</td>
<td>44.71 ± 9.98</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>166.29 ± 36.12</td>
<td>150.49 ± 29.98</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>34.56 ± 10.18</td>
<td>33.33 ± 17.24</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Tbil (μmol/L)</td>
<td>42.49 ± 10.36</td>
<td>45.05 ± 6.21</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>DBil (μmol/L)</td>
<td>31.75 ± 4.98</td>
<td>30.23 ± 5.69</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AFP (μg/L)</td>
<td>125.78 ± 19.27</td>
<td>126.09 ± 22.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>90.57 ± 12.14</td>
<td>91.07 ± 25.07</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>HBV-DNA (copies/mL)</td>
<td>(8.21 ± 0.69) × 10^4</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

HBsAg: hepatitis B surface antigen; ALP: alkaline phosphatase; ALT: alanine aminotransferase.
Ab as compared to those pretreated with IgG ($p < 0.05$, Figure 2(a)), which demonstrated that LncRNA-HEIH and LncRNA-HULC coimmunoprecipitated with HBXIP. Subsequently, the expression levels of HBXIP were quantified in liver tissues by western blot analysis. As shown in Figure 2(b), the expression levels of HBXIP were notably increased in the liver tissues of patients with HCC (HBV+HCC and HBV-HCC groups) as compared to corresponding adjacent noncancerous liver tissues (HBV+NT and HBV-NT groups) ($p < 0.05$) and higher in the HBV+HCC and HBV+NT groups than that in the HBV-HCC and HBV-NT groups ($p < 0.05$).

4. Discussion
With the development of high-resolution microarrays and massively parallel sequencing technology, it is widely believed that more than 90% of the human genome is actively transcribed into noncoding RNAs (ncRNAs) and less than 2% actually encodes proteins [15]. The ncRNAs are classified as small ncRNAs ($< 200$ nucleotides) and lncRNAs ($> 200$ nucleotides) according to size. Although these RNAs have been considered to be the “noise” of genome transcription and do not actually participate in gene encoding and protein synthesis directly, they may play significant regulatory roles...
in a great variety of illnesses, such as hepatitis B infection [16–19]. For example, the small ncRNA miR-137 promotes the expression of HBV genes and viral replication by targeting the expression of the protein inhibitor STAT 2 [20]. Additionally, the miR-99 family promotes HBV replication posttranscriptionally through IGFlR/P38K/Akt/mTOR/ULK1 signaling-induced autophagy [21]. In recent years, it has become increasingly obvious that lncRNAs also play critical roles in hepatitis B-related diseases [22, 23]. However, few studies have investigated the mechanisms underlying the involvement of lncRNAs in hepatitis B-related diseases, thus the underlying mechanisms remain unclear. Nonetheless, some previous studies have revealed that lncRNAs play important roles in the regulation of protein coding genes, stem cell differentiation, allelic expression, cell cycle control, and cell death [8–11]. Moreover, lncRNAs are widely involved in physiological and pathological processes, thus lncRNAs have become a main focus of research in the field of molecular biology [24].

The expression levels of lncRNA-HULC and lncRNA-HEIH are relatively high in HCC. lncRNA-HULC is located on chromosome 6p24.3 and regarded as the first lncRNA with highly specific upregulation in HCC, suggesting that it can be used as a promising noninvasive novel biomarker for the diagnosis and/or prognosis of HCC [11, 12]. Du et al. suggested that HBX could promote the expression of lncRNA-HULC in human immortalized normal liver L-O2 cells and hepatoma HepG2 cells, resulting in increased proliferation of hepatoma cells via the suppression of p18 [1, 25]. In addition, Liu et al. demonstrated that a mutation to lncRNA-HULC might contribute to decreased HCC susceptibility in persistent HBV carriers [26]. Moreover, expression of lncRNA-HULC has been positively correlated with the progression of hepatitis B infection and the occurrence of hepatitis B-related diseases, such as HC and HCC. Likewise, in the present study, gradually increased expression of lncRNA-HULC was detected in the peripheral blood samples of the control, HBV, HBV + HC, and HBV + HCC groups. Moreover, lncRNA-HULC expression was greater in the liver tissues of the HBV-positive and HBV-negative HCC groups, as compared to corresponding adjacent noncancerous liver tissues (HBV + NT and HBV-NT groups), and expression of lncRNA-HEIH and lncRNA-HULC was greater in the liver tissues of the HBV + HCC and HBV + NT groups than in the HBV–HCC and HBV-NT groups, which is consistent with the results of a previous study [27]. Similarly, lncRNA-HEIH, another highly expressed lncRNA in HCC, showed identical expression trends with lncRNA-HULC in peripheral blood and liver tissues of patient with hepatitis B or hepatitis B-related diseases. Yang et al. found that lncRNA-HEIH inhibited cell differentiation in the G0/G1 phase and accelerated the carcinogenesis of hepatitis B-related HCC [11, 28]. These results suggest that lncRNA-HULC and lncRNA-HEIH are positively correlated with hepatitis B infection and hepatitis B-related diseases. As we know, lncRNAs are involved in all aspects of gene regulation, including chromosome dosage compensation, imprinting, epigenetic regulation, nuclear and cytoplasmic trafficking, and transcription, as well as mRNA splicing and translation [29]. In the present study, the positive interactions of HBXIP with lncRNA-HULC and lncRNA-HEIH were further investigated by RIP analysis, which showed that HBXIP expression was greater in HBV-positive than HBV-negative HCC. Wang et al. indicated that HBXIP might play an important role in tumorigenesis by enhancing angiogenesis in HCC [6]. Therefore, it is possible that high expression of lncRNA-HULC and lncRNA-HEIH promotes the expression of HBXIP, which results in increased HBV replication and the occurrence of hepatitis B-related diseases.

In summary, the present findings demonstrate that the interactions of lncRNA-HULC and lncRNA-HEIH with HBXIP might be involved in the occurrence of hepatitis B-related diseases. Understanding the relationship of lncRNA-HULC and lncRNA-HEIH with HBXIP in hepatitis B and hepatitis B-related diseases may lead to the development of novel therapeutic interventions to ameliorate hepatitis network dysfunction and associated morbidities.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflict of interests to declare.

Authors’ Contributions

Lingjuan Ruan and Lifei Huang contributed equally to this work.

Acknowledgments

We thank the patients and families for the donation of peripheral blood and liver tissues. This work was supported by Public Welfare Application Research Project of Huzhou Science and Technology Bureau, China (Project no: 2017GY67).

References


