Original article

Preliminary identification and analysis of point mutations correlated with response to interferonα in hepatitis B virus post-transcriptional regulatory elements

XING Tong-jing, LUO Kang-xian and HOU Jir-lin

Keywords: hepatitis B virus · post-transcriptional regulatory elements · point mutations · cytokine

Background: It is still unclear whether viral genetic variability influences response to interferonα (IFNα) treatment. Recent reports suggest that IFNα effects may be associated with hepatitis B virus (HBV) post-transcriptional regulation. This study was designed to explore the heterogeneity of HBV post-transcriptional regulatory elements (HPRE) and the relationship between the diversity of HPRE and the response to IFNα treatment.

Methods: The HPRE sequences from 31 Chinese patients infected with HBV were determined by directly sequencing of polymerase chain reaction (PCR) product, and comparing them to those from Caucasian patients. Subsequently, eukaryotic expression vectors containing HPRE at various points were constructed and transfected into HepG2 cells, which were then exposed to recombinant human cytokines.

Results: The T to C point mutation at nt 1504 and the C to T (G) at nt 1508 in HPRE were found in 21 and 19 patients with chronic hepatitis B, respectively; the C to T point mutation at nt 1509 was found in 17 patients. These point mutations did not exist in the HPRE of the Caucasian patients. The activity of the CAT gene obviously increased in the case of T to C point mutation at nt 1504, but did not change in the case of the C to T (G) mutations at nt 1508 and 1509. The activity of the CAT gene at these point mutations of HPRE could be inhibited by IFNα/γ and tumor necrosis factor (TNF)α except for the point mutations at nt 1508 of HPRE which may escape the suppression role of IFNα on HPRE.

Conclusions: There are point mutations between the HPRE of Chinese and Caucasian HBV patients, which might be correlated with response to IFNα. The variation of HPRE might affect the function of HPRE and influence the regulative function of IFNα other than that of IFNγ or TNFα on HPRE.


Therapy for chronic hepatitis B with interferon-α (IFNα) may result in viral clearance and hepatitis B seroconversion in 30% - 40% of patients. The efficacy is lower in Chinese patients than in Caucasian patients. However, it is still unclear whether viral genetic variability influences response rates. Several studies have recently reported that certain mutations are associated with the response to IFNα, but the pivotal mutations influencing IFNα response to hepatitis B remain unclear. Recent studies have reported that hepatitis B virus (HBV) replication is inhibited in a non-cytopathic manner by IFNα and IFNγ. Gordien and colleagues have demonstrated that MxA antiviral action against HBV occurs, partly at least, at a post-transcriptional level, by inhibiting the nuclear export of viral RNAs. These reports suggest that IFNα effects may be associated with HBV post-transcriptional regulation.

Department of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China (Xing TJ)
Department of Infectious Diseases, Nanfang Hospital, Guangzhou 510515, China (Luo KX, Hou JL)
Correspondence to: Dr. XING Tong-jing, Department of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, China (Tel: 86-571-87236759. Email: xingtj @21cn.com)
HBV is a partially double-stranded circular DNA virus which replicates via reverse transcription. The viral RNAs are intronless and are exported from the nucleus unspliced. Several studies have identified an orientation-dependent RNA element that is required for the efficient expression of the HBV proteins. This element was named HBV post-transcriptional regulatory element (HPRE). The function of the HPRE is mediated through secondary structure elements (stem-loops), which may act as binding sites for the cellular RNA-proteins that are necessary for the function of HPRE.

In vitro, cellular proteins (p30 and p45 that interact with HPRE) have been identified and might be part of an HBV RNA export mechanism. Some studies have shown that HBV RNA is downregulated by inflammatory cytokines produced in the liver of HBV transgenic mice by adoptively transferred HBV-specific cytotoxic T lymphocytes (CTLS). The disappearance of HBV RNA is tightly associated with the cytokine-induced proteolytic cleavage of an already defined HBV RNA-binding protein known as La autoantigen. La binds to a predicted stem-loop structure at the 5' end of the post-transcriptional regulatory element of HBV RNA between nucleotides 1243 and 1333. Since the non-cytopathic mechanism associated with the function of HPRE plays an important role in the response to IFNα, we speculate that variations in HPRE sequences might influence the response to treatment with IFNα. In order to test this hypothesis, we investigated variations of the HPRE between Chinese patients and Caucasian patients. Subsequently, eukaryotic expression vectors containing HPRE segments at various points were constructed. The effect of cytokines on HPRE transfected into HepG2 cells was observed.

**METHODS**

**Patients**

Thirty-one patients diagnosed with hepatitis B at Southern Hospital from April 2001 to December 2001 were included in this study. Those patients treated with IFNα in the most recent six months were excluded. This group consisted of 28 patients with chronic HBV infection and 3 patients with acute hepatitis B. All patients were seronegative for markers of hepatitis C, D and E viruses. Twenty-eight patients with chronic HBV infection had been seropositive for HBsAg for at least 6 months, including 21 patients who were also HBeAg positive. The three patients with acute hepatitis B tested positive for HBsAb about 1 - 2 months after testing positive for HBsAg. All the markers of HBV were tested by commercial immunoassays (Abbott Laboratories, USA). Serum HBV DNA was identified using the standard hybridization techniques.

**DNA extraction and amplification**

HBV DNA was extracted using a DNA extraction kit (Promega, USA) from the serum of patients. The sequences of primers that were used were as follows: sense, 5'-TGC CAA GTG TTT CTT GAC GC C-3' (bp 1175 - 1195); antisense, 5'-ACA AAC AGT CTT TG A AGT AT-3' (bp 1724 - 1704). The length of the amplified fragment was 547 bp. The PCR conditions were: initial heating at 94 ºC (5 minutes), denaturing at 94 ºC (40 seconds), annealing at 56 ºC (40 seconds), and extension at 72 ºC (50 seconds). A total of 35 cycles were performed, followed by a final extension at 72 ºC (5 minutes). Distilled water was used as the template for each patient as a negative control. Ten μl of PCR products were electrophoresed on 2 % agarose gel stained with ethidium bromide, and then visualized under ultraviolet light.

**Sequence analysis**

All the products of the PCR were analyzed by TaKaRa Biotechnology Co., Ltd., USA, using the ABI PRISM™ 377 XL DNA sequencer. The resulting sequences were analyzed by DNASIS software and compared with published HBV DNA sequences. Sequence numbering used is based on the following GenBank sequences: HBV, M38454; subtype: adr; genotype: C type. HBV sequences of 30 Caucasian patients were collected from the GenBank, and included 8 strains of A type (GenBank accession numbers: L13994, M57663, Z35717, E00010, X70185, X51970, Z72748, X02763); 6 strains of B type (GenBank accession numbers: D00329, D23677, D50521, M54923, D23679, D23678); 8 strains of C type (GenBank accession numbers: D00630, D12980, M38454, X04615, D23680, D23683, D50517, M38636); 8 strains of D type (GenBank accession numbers: L27106, M32138, J02203, X02496, X80926, Y07587, X72702, X80924). No patient selected for this study had received treatment with IFN-α or any other antiviral drugs.

**Plasmid construction**

The reporter plasmids prepared for these experiments were cloned using standard techniques. The chloramphenicol acetyltransferase (CAT) reporter
plasmid pDM138, which has been previously described, was derived from the second intron of HIV-1, into which the CAT gene was inserted to occupy the position of the envelope. It was a versatile and well-characterized reporter system that has been utilized to study the RNA export elements of complex retroviruses. When the pDM138 reporter was transiently transfected, RNAs transcribed from the reporter were either spliced, removing the CAT coding region, or exported from the nucleus unspliced. The appearance of unspliced RNAs in the cytoplasm was dependent upon the presence of an RNA export element. When the unspliced RNAs were exported from the nucleus, CAT was translated and accurately quantified.

pDM138-HPRE, containing the nucleotides 1219 - 1584 of HBV, was constructed by ligating a GeneClean (Bio101) purified Cla I digested PCR product into the unique Cla I site located within the intron of pDM138, 3’ of the CAT reporter gene. The primers used are shown in Table 1. The fragments tested in this study were amplified by 30 cycles of PCR with the P3.8 II (1219 - 1584) plasmid as a template. The P3.8 II plasmid was recombined plasmid, in which whole HBV DNA (adr subtype) had been inserted into the pBS’ plasmid, including the full transcriptional units starting at the C gene basic promoter and ending at the poly A signal.

<table>
<thead>
<tr>
<th>Primer (5’-3’)</th>
<th>Position in HBV genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1: CAC GGA TCC AICGK’ C tga tgc gcc atc</td>
<td>1219 - 1266</td>
</tr>
<tr>
<td>Primer 2: CAC GGA TCC AICGK’ gca cac ggt ceg</td>
<td>1584 - 1567</td>
</tr>
<tr>
<td>G506 mutant primer</td>
<td></td>
</tr>
<tr>
<td>P1: CCC CTT CTT CAT CAA CCG TTC CG</td>
<td>1491 - 1513</td>
</tr>
<tr>
<td>P2: GCC GAA CCG TAG GTC AAG AAG GGG</td>
<td>1513 - 1491</td>
</tr>
<tr>
<td>T506 + 1506 mutant primer</td>
<td></td>
</tr>
<tr>
<td>P1: TTC TTC ATC TAC TAT TGC TCC GCC CGA</td>
<td>1495 - 1518</td>
</tr>
<tr>
<td>P2: TCC GGC GCC ACG ACA AAG GAC GAA</td>
<td>1518 - 1495</td>
</tr>
<tr>
<td>C506 + T506 + 1506 mutant primer</td>
<td></td>
</tr>
<tr>
<td>P1: TCC CTT TCT TCA CCT ATT GTT CCG GCC GAC</td>
<td>1490 - 1519</td>
</tr>
<tr>
<td>P2: GCG GCC CGG AAC AAT AAG TGA AGA AAG</td>
<td>1519 - 1490</td>
</tr>
</tbody>
</table>

HPRE point mutants were generated by PCR-based site-directed mutagenesis in vitro. The PCR-based site-directed mutagenesis kit was purchased from Stratagene Co., USA. The mutant eukaryotic expression vectors were constructed by molecular cloning.

Cell culture and transfections
The human hepatoblastoma cell line Hep G2 was transfected in all assays. Hep G2 cells were cultured in DMEM (Gibco, USA) with 10% fetal calf serum (HyClone, USA) and grown at 37 °C in a 5% CO2-95% air mixture in 24-well plates. Transfections were performed using a lipofectamine (Gibco, USA) mediated method at 50% - 80% confluence, with each confluence condition performed in duplicate. Approximately 0.35 μg of pDM138 vector was used per well. Differences in transfection efficiency in each well were normalized to CAT activity in samples cotransfected with 0.05 μg of pSEAP (secretory alkaline phosphatase) vector (ClonTech, USA). The cells remained in the Lipofectamine-DNA mixture for 16 hours, and were then washed two times with media without serum. Fresh medium was added, and transfected cells were exposed to cytokines. Two days after cytokine exposure, the cells and the supernatant were harvested.

Human recombinant cytokines were obtained from commercial sources. These cytokines included IFNY, TNFα, and IFNα (PeproTech, UK).

CAT and pSEAP assays
CAT assays were performed using an ELISA kit (Roche, Switzerland). Cell lysates were prepared with the lytic solution provided from the kit. CAT activity was quantified according to the manufacturer’s protocol. An aliquot of each supernatant was assayed for pSEAP activity, which was detected using a pSEAP kit (ClonTech, USA). Typically, two different preparations of each test plasmid were assayed.

Statistical analysis
All values are expressed as mean ± SD. Data analysis was performed using software SPSS 11.0. One-way analysis of variance (ANOVA) after the SNK q test was performed among the groups. Student’s t test was used between the groups. A P value less than 0.05 was considered statistically significant.

RESULTS

Comparison of HPRE between Chinese and Caucasian patients
The T to C point mutation at nt 1504 and C to T (G) mutation at nt 1508 were found in HPRE in 21 and 19 patients with chronic hepatitis B, respectively, and the C to T point mutation at nt 1509 was found in 17 patients (Fig. 1). These point mutations were not found in HPRE of the Caucasian patients. These mutations were also not found in the 3 patients with acute hepatitis B.

Fig. 1. Comparison of sequences of HPRE in part isolated from the Chinese and Caucasian patients. The sequence of the wild-type genome (PADR1 DNA) served as a reference. The upper sequences from the Caucasian patients, and the lower sequences were from Chinese patients. Nucleotide differences to the patients HPRE are indicated in boldface and underlined.

Construction of plasmid vectors containing HPRE various point mutations
Three different pDM138 reporter vectors, containing the 1504 nt, 1508 (9) nt, or 1504 + 1508 (9) nt HPRE point mutations, were constructed. Recombinant pDM138 vector digested with Cla I and the HPRE segment were detected on 1% agarose gel. All fragments were confirmed by sequencing after insertion into the pDM138 vector.

Effect of the three point mutations on the function of HPRE
The activity of the CAT gene was significantly different among wild strains and strains containing one of the point mutations (F = 5.22, P < 0.05) (Fig. 2). Compared with the wild strain, the activity of the CAT gene increased in the case of T to C point mutation at nt 1504 (q = 4.31, P < 0.05).

Comaprison of the effects of IFNγ and TNFα on CAT activity of recombinant pDM138 vectors containing mutated HPRE
Recombinant pDM138 vector containing HPRE point

Table 2. Effect of IFNγ and TNFα on CAT activity of recombinant pDM138 vector containing HPRE point mutations (pg/ml)

<table>
<thead>
<tr>
<th>Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1286.4 ±245.2</td>
<td>793.5 ±161.7</td>
<td>1043.2 ±138.9</td>
</tr>
<tr>
<td>IFNγ</td>
<td>742.5 ±113.4</td>
<td>386.5 ±97.8</td>
<td>604.2 ±107.5</td>
</tr>
<tr>
<td>TNFα</td>
<td>457.5 ±57.6</td>
<td>212.7 ±86.7</td>
<td>353.3 ±38.4</td>
</tr>
<tr>
<td>IFNγ + TNFα</td>
<td>1149.2 ±194.4</td>
<td>644.9 ±371.4</td>
<td>1172.2 ±153.4</td>
</tr>
<tr>
<td>F value</td>
<td>48.75</td>
<td>37.02</td>
<td>77.06</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Compared with control group, P < 0.01; compared with IFNγ and TNFα group, P < 0.01; A: mutant type at nt 1504; B: mutant type at nt 1508 and 1509; C: mutant type at nt 1504 and nt 1508 + 1509.

Effect of IFNα on CAT activity of three different recombinant pDM138 vectors containing HPRE point mutations
Recombinant pDM138 vectors containing HPRE point mutations were exposed to IFNα (500 U/ml) (Fig. 3). Compared with the untreated control, the activity of
CAT in recombinant pDM138 vectors containing either nt1504 or nt1504 + 1508 point mutations was markedly decreased by IFNα (t = 3.96, 6.18, P < 0.01). However, no significant decrease in CAT activity was detected in the case of the nt1508 point mutation (t = 1.85, P > 0.05).

Fig. 3. Effect of IFNα on CAT activity of recombinant pDM138 vectors containing HPRE point mutations. CAT activities ± SD in recombinant vector transfected HepG2 cells with: A, mutant type at nt 1504 (t = 3.96, P < 0.01); B, mutant type at nt 1508 and 1509 (t = 1.85, P > 0.05); C, mutant type at nt 1504 and nt 1508 + 1509 (t = 6.18, P < 0.01). 1: no treatment; 2: IFNα 500 U/ml.

DISCUSSION

IFNα is an effective drug in therapy of hepatitis B virus infections. The effect of IFNα is mediated both by direct antiviral activity and by enhanced host immune response. Recent studies have reported that the effectiveness of IFNα may result from direct and indirect effects on the HBV post-transcriptional regulation. Such a role of IFNα is achieved by inducing the product of IFNγ, which could downregulate HBV gene expression in the livers of transgenic mice and in human hepatocytes by a posttranscriptional mechanism that could contribute to viral clearance following HBV infection. 17,18

Some studies have shown that the nuclear export of HBV RNA may be mediated by a previously described PRE, located between nt 1239 and 1805, which targets the HBV RNA to a splicing-independent export pathway. 8,19 The minimal HPRE, encompassing nucleotides 1217 - 1582 of HBV, contains three distinct regions of activity (alpha, beta 1, and beta 2), two of which contain a highly conserved stem-loop structure, which is necessary for full HPRE activity. A series of HPRE mutants significantly reduce the nuclear export activity. 9 As a result of these studies, we are wondering if the diversity of HPRE can influence patients with HBV infection from various racial groups in their responses to IFNα. So HPRE sequences of Chinese or Caucasian patients with hepatitis B virus infection were compared in this study. The T to C point mutations at nt 1504 and C to T (G) at nt 1508 were found in HPRE in 21 and 19 Chinese patients with chronic hepatitis B, respectively, and the C to T point mutation at nt 1509 was found in 17 patients. None of the point mutations was found in HPRE of the Caucasian patients. Because the above point mutations occurred in region III of HPRE, we speculated that these point mutations might affect the function of HPRE, and consequently, patient responses to IFNα treatment. However, the positive correlation with different responses to IFNα needs further investigation.

The pDM138 CAT reporter vector containing the HPRE segment was constructed in our previous studies, and the role of HPRE was inhibited by IFNγ and TNF-α. 20 In the present study, mutant eukaryotic expression vector containing point mutations in HPRE was constructed, transfected into HepG2 cells, and exposed to three cytokine combinations. The activity of the CAT gene obviously increased in the case of T to C point mutation at nt 1504, but did not change in the case of the C to T (G) mutations at nt 1508 and 1509. These results suggest that the T to C point mutation at nt 1504 of HPRE may enhance the function of HPRE. Two of these point mutations of HPRE had no influence on the suppression role of IFNγ and TNFα on HPRE. However, the suppression role of IFNα on HPRE disappeared in the case of nt 1508 point mutation. These results suggest that point mutations in HPRE may not only affect the function of HPRE, but also the regulatory function of IFNα on HPRE.

In conclusion, the relationship was examined between the diversity of HPRE in various races and responses to IFNα. The results suggest that the diversity of HPRE may affect the patient responses to IFNα treatment. But the exact mechanism awaits elucidation.
Acknowledgements: We are grateful to Prof. Zhiming Huang, University of California School of Medicine, San Francisco for providing the pDM138 vector and technical assistance.

REFERENCES


(Received March 31, 2004)

Edited by Qian Shourchu and LIU Dongyun