IPS-1 polymorphisms in regulating interferon response in HBV infection

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Summary

Single nucleotide polymorphisms (SNP) influence the outcome of antiviral therapy in chronic hepatitis B patients. Interferon β promoter stimulator 1 polymorphisms (IPS-1) regulate interferon (IFN) mediated viral clearance in hepatitis B virus (HBV) infection. In our study, HepG2 and HepG2.2.15 were transfected with different SNP genotype expression vectors of IPS-1 (wild-type, rs17857295, rs7262903 and rs7269320). The production of IPS-1 and IFN were evaluated in these transfected cells. IPS-1 in the HepG2.2.15 cells transfected with rs17857295 or rs7262903 was 37% or 31% lower than that with wild-type transfection (p < 0.001). IFN-β in rs17857295 or rs7262903 transfected HepG2.2.15 cells was 5.4 or 3.7 fold higher than that of wild-type transfection (p < 0.0001). IPS-1 in rs7269320 SNP transfected HepG2.2.15 cells was 40% lower than that of wild-type transfection (p < 0.0001); no significantly different IFN-β was observed between rs7269320 SNP and wild-type transfections. IFN-β expression was > 2 fold higher in rs17857295 transfected HepG2.2.15 cells than HepG2 cells (p < 0.001). The data suggests that host HBV viral clearance is stronger in IPS-1 rs17857295 or rs7262903 SNP genotype patients than wild-type patients. Relatively weak inducible IFN-β production in HBV infected patients with IPS-1 rs7269320 SNP or wild-type may contribute to chronic virus infection.

Keywords: IPS-1, single nucleotide polymorphism, hepatitis B virus, interferon response, chronic HBV infection

1. Introduction

Chronic hepatitis B (CHB) is still a major challenge to clinicians, due to its serious clinical complications with huge financial and psychological burdens (1,2).

The precise pathogenesis of CHB is not fully clear, especially the host immunity in CHB viral clearance, and subsequent chronicity, despite decades of extensive research (3).

Single nucleotide polymorphism (SNP) is a polymorphism at the genome level, due to a single nucleotide missense mutation (4). Missense mutation of a single nucleotide in genetic code leads to the change of the sequence of bases, and transforms the amino acid sequence of the corresponding protein, influencing the structure and function of proteins. SNP is an important biological genetic marker, causing a biological genetic trait change. SNPs determine the effect of drug therapy and/or disease susceptibility in different genetic backgrounds, which may also contribute to different host immunities, especially antiviral immunity (5).

Host antiviral innate immunity is initiated via viral
pathogen-associated molecular patterns (PAMP), such as viral double-stranded RNA (dsRNA). All RNA and most DNA viruses, including HBV (6), generate dsRNA molecules at the transcription or replication level. Retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are very important pattern recognition receptors (PRRs) in vivo (7). RIG-I/MDA5 signaling pathway plays an important role in host recognition and clearance of the virus infection process (8). After binding to dsRNA, RIG-I transforms into an open conformation, translocates onto mitochondria, and interacts with the downstream adaptor to induce the production of interferon (IFN) and inflammatory factors via IRF3/7 and NF-κB pathways. IFN-β promoter stimulator 1 (IPS-1), an essential adaptor protein in the RIG-I/MDA5 anti-viral pathway, plays an important role in the cell recognition and the induce process of IFN-β during viral infection (9-12). Upregulation of IPS-1 results in increased production of IFN-β, which finally prevents viral entry (9,12). RIG-I mediated antiviral immunity and its downstream signaling pathway have been well illustrated in hepatitis C virus (HCV) infection (13). Following HBV stimulation, MDA5 activates innate immune signaling to suppress hepatitis B virus (HBV) replication (14). IFN inhibits HBV replication via the MDA5/IPS-1 pathway, but patients display different prognosis to HBV infection.

Studies on genetics determine susceptibility of HBV infection and subsequent chronicity has been well documented in families of segregation analysis or twin studies (15,16). SNP of IL28B is responsible for the therapeutic outcomes following PEG-IFN plus ribavirin treatment in chronic viral hepatitis C (CHC) (17). It has been demonstrated that three SNPs upstream of IL28B (rs12979860 CC, rs12980275 AA, and rs8099917 TT) are highly associated with HCV recovery, but inversely with HBV (18). Recent study of polymorphisms near IL28B (rs12980275 AA, rs12979860 CC) have also demonstrated an association with serologic response to PEG-IFN in HBeAg+ CHB patients (19). Interestingly, IL28B rs12979860 CC genotype do not contribute to the outcome of HBV infection (20). HLA-DPA1 rs3077G/G is associated with HBeAg seroconversion and combined response rates at 6 months of the reply in HBeAg+ CHB patients with IFN, suggesting HLA-DPA1 contributes to IFN-induced HBeAg seroconversion (21). Our previously published data demonstrated that polymorphisms in IPS-1 (rs2464 CC) are independently associated with response to PEG-IFN among Chinese HBeAg+ CHB patients (22). The data from ourselves and others illustrates that different proteins regulated by SNPs contribute to HBV viral clearance and subsequent disease outcome via host antiviral immunity in response of interferon. However, it was still unclear whether SNP of IPS-1 polymorphisms influences IFN response in HBV infection.

2. Materials and Methods

2.1. Cell lines and Culture

HepG2 and HBV-replication-stable HepG2.2.15 cell lines were maintained in our laboratory, which were obtained from the Chinese Academy of Sciences (23-25). HEK293T cells, obtained from American Type Culture Collection, were used as a transfection positive control (26). HepG2, HepG2.2.15 and HEK293T cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum at 37°C (Life Technologies, Carlsbad, CA), in a humidified atmosphere containing 5% CO₂.

2.2. Site-directed Mutagenesis

The sequences containing IPS-1 cDNA (FL18489) based on a human cDNA library were inserted into pCMV-SPORT6 plasmid, and then transfected into E. coli for amplification. The correct clone was confirmed with sequencing, and used as positive control. Primers were designed including the whole ORF sequences of IPS-1, joining enzyme loci Bgl II and Cal I at the both ends, respectively. In addition, KOZAK was also added as protection. The sequence of these two strains of primers were as follows: IPS-1seq-sense: ACCAGATCTGCCACCATTGCGTTGTCTGAAGACAAGACC; IPS-1seq-antisense: ATTTGTCGAGCTGTAGTCAGACGCGCCGCGGTAGA. IPS-1 cDNA fragment, inserted into pRRL-cPPT-PGK-eGFP-WPRE plasmid (Addgene#12252), was amplified for final identification.

Three point mutations, identified from each sequence of IPS-1 cDNA, were localized at the position of the SNPs of interest (Supplement Figure S1, http://www.biosciencetrends.com/action/getSupplementalData.php?id=40). We used site-directed mutagenesis to obtain the wild-type vector of IPS-1 and other different expression vectors of SNP genotype. The primers for site-directed mutagenesis are listed in Supplement Table S1 (http://www.biosciencetrends.com/action/getSupplementalData.php?id=40). PCR was performed as follows: 94°C for 2 minutes, then 25 cycles at 94°C for 15 seconds, 56°C for 30 seconds, 68°C for 1 minute, and 68°C for 9 minutes.

2.3. qRT-PCR

HEK293T, HepG2 and HepG2.2.15 cells were transfected with human wild-type IPS-1 or different IPS-1 SNP genotypes (rs17857295, rs7262903 or rs7269320), respectively in triplicate plates. RNA was extracted from individual treated cells, using Trizol (Life Technologies, Carlsbad, CA). cDNA was generated from 1μg total RNA, using the RevertAid™ Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, MA). The forward and reverse primers for pgRNA are listed in Supplement...
rabbit-HRP, Amersham Pharmacia Biotech, Saclay, France). The signal was developed and imaged using ImageQuant™ LAS 4000 (Fujifilm, Tokyo, Japan).

2.5. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Two-tailed Student's *t*-test and one-way ANOVA were used for data analysis. All statistical analyses were performed using the SPSS for Windows, Version 19.0 (SPSS Inc., Chicago, IL). Differences were considered statistically significant at *P* < 0.05.

3. Results

3.1. Construction of recombinant mammalian expressing vector with different IPS-1 SNP genotypes

It was confirmed that constructions of recombinant mammalian expressing vector carried IPS-1 wild-type or other SNP genotypes (rs17857295, rs7262903 or rs7269320), by sequencing (Figure 1). Furthermore, to verify the ability to express IPS-1 properly, HEK293T cells (GFP gene contained in pRRL-cPPT-PGK-eGFP-WPRE plasmid) were transfected with the vectors, which carried wild-type or different IPS-1 SNPs. Thus GFP production in the cells indicates successful and stable transfection. IPS-1 mRNA from the wild-type or SNP mutated gene type transfected cells were significantly higher than that of mock transfected cells (*p* < 0.05) (Figure 2), confirming the vector plasmids were successfully generated with correct sequence.

3.2. IPS-1 polymorphisms in HBV infection

Subsequently, to determine the relationship between SNPs and IPS-1 expression during HBV infection in hepatocytes, HepG2 or HepG2.2.15 cells were transfected with the vectors containing IPS-1 SNPs or wild-type genotypes, respectively. IPS-1 gene

![Figure 1. Construction of recombinant mammalian expressing vector with different IPS-1 SNP genotypes. (A). Incision enzyme digestion of different IPS-1 genotype plasmids; (B). Sequencing of different IPS-1 genotype plasmids (wt: IPS-1 wildtype, SNP1: rs17857295, SNP2: rs7269320, SNP3: rs7262903).](http://www.biosciencetrends.com/action/getSupplementalData.php?ID=40)

![Figure 2. The expression of IPS-1 mRNA in different IPS-1 SNP genotype plasmids transfected 293T cells.](http://www.biosciencetrends.com行動)
was significantly higher in the cells transfected with these three different IPS-1 SNPs or wild-type than the mock transfected group ($p < 0.05$). IPS-1 mRNA level in HepG2.2.15 cells transfected with wild-type, rs17857295, rs7262903, rs7269320 was 11, 14, 8 or 4 fold higher than HepG2 cells ($p < 0.05$). In contrast, IPS-1 mRNA level was 37%, 31%, 40% lower in the HepG2.2.15 cells transfected with IPS-1 SNP genotypes than wild genotype transfection group (Figure 3A).

3.3. IPS-1 SNP polymorphisms regulate interferon response in HBV infection

To determine the role of IPS-1 SNP polymorphisms in regulating interferon response in HBV infection, IFN-β mRNA level was detected after the vectors containing these three different IPS-1 SNPs or wild-type genotypes transfected into HepG2 or HepG2.2.15 cells. In HepG2.2.15 cells, IFN-β expression in IPS-1 wild-type vector transfected group had no obvious change compared to pEGFP empty control group. IFN-β mRNA level was 5.4 or 3.7 fold higher in the transfected HepG2.2.15 cells with IPS-1 rs17857295 or rs7262903 SNP genotypes than wild genotype transfected group ($p < 0.0001$). In IPS-1 rs7269320 SNP genotypes transfected groups, IFN-β expression had no obvious change compared to wild-type group. In addition, in IPS-1 rs7269320 SNP genotypes transfected groups, IFN-β mRNA level in HepG2.2.15 cells was > 2 fold higher than that in HepG2 cells ($p < 0.001$) (Figure 4).

4. Discussion

Following transfection of rs17857295 or rs7262903 in the HBV carried hepatocytes, IPS-1 was suppressed, but IFN-β was up-regulated compared to that with
wild-type transfection. IPS-1, but not IFN-β, was suppressed in the HBV carried hepatocytes transfected with rs7269320 SNP compared to that with wild-type transfection. IFN-β was higher in rs17857295 transfected HBV carried hepatocytes than in non-HBV carried hepatocytes.

IPS-1 plays a critical role in the RIG-I signaling pathway, leading to IFN-β activation in cells following viral stimulation (9-11). However, viruses may still circumvent IFN initiated host response via an unknown mechanism in susceptible individuals (14,27), resulting in chronic viral infection. Lin et al. (28) demonstrated that HCV protease NS3-4A inhibits IFN-β production via degrading IPS-1 protein. However, the effect of IPS-1 SNP polymorphism during HBV infection is still unclear. Among 29314bp DNA sequences within IPS-1 gene, 229 SNPs loci have been discovered. Considering the effects on the protein structure and function, we focused on the missense coding SNP (cSNP) sites within the IPS-1 gene. Three out of 12 missense cSNP sites show a mutation frequency which is > 1%, including rs17857295 in exon 3 (446 g>C, Q93E), rs7269320 in exon 5 (761 a>C, Q198K) and rs7269320 in exon 7 (1395 t>C, S409F). The encoding amino acids of rs17857295 loci is in the connection position between CARD structure domain and a proline rich area (Pro-rich), suggesting this SNP is closely related to the IPS-1 function (29).

In the current study, IPS-1 was significantly higher in the wild type or SNPs transfected HBV carried hepatocytes (HepG2.2.15 cells) than that in non-HBV carried hepatocytes (HepG2 cells), suggesting that IPS-1 production is responding to HBV infection in the hepatocytes. Our data is in line with our previously published data in macrophages, IPS-1 in macrophages from CHB patients is significantly higher than that of good health controls following vesicular stomatitis virus stimulation (26).

Furthermore, IPS-1 was suppressed in any of the SNPs transfected (rs17857295, rs7269320 or rs7269320) HBV carried hepatocytes compared to that transfected with wild type. In contrast, IFN-β was substantially higher in the rs17857295 or rs7269320 SNP transfection HBV carried hepatocytes than that of the wild type transfected group. This is consistent with our previous finding that IPS-1 in macrophages from CHB patients is significantly higher than that of good health controls or acute hepatitis B, following vesicular stomatitis virus stimulation, inversely correlated with IFN-β production (26). Our current finding suggests that increased IPS-1 production from the virus stimulation in HBV carried hepatocytes is due to compensation for compromised IPS-1 function, and possibly due to interference from HBV infection. This is supported by the finding that HBx protein of HBV interferes the ubiquitination of IPS-1 to restrain the RIG-I pathway of innate immunity (30). However the underlying mechanisms leading to compromised IPS-1 function still remain to be explored.

Such findings invite speculation that the viral infection signals are delivered more effectively in rs17857295 and/or rs7269320 SNP transfected HBV viral carried hepatocytes compared with wild-type transfected ones, contributing to IFN-β mediated antiviral immunity. In addition, IFN-β from rs17857295 transfected HBV carried hepatocytes was significantly higher than from non-HBV carried hepatocytes. No significantly different IFN-β production was detected between HBV carried and non-HBV carried hepatocytes following wild-type transfection, suggesting IPS-1 rs17857295 SNP may play an important role in host resistance to HBV challenge. Moreover, there was no significantly different IFN-β between wild type and rs7269320 SNP transfection, suggesting rs7269320 mutation may not be a critical factor or may be compensated by other factors in the viral host. The precise underlying mechanism will be determined in the future.

There are some limitations in our current study: First, the current study was mainly focused on basic virology. Thus future experiments may focus more on clinical research. Second, more downstream pathways of IPS-1 in RIG-I will be explored in the future. Third, in our current experiment there was correlated up-regulation of IPS-1 and IFN-β, but the viral load wasn't determined at the same time. However it is well known that IFN-β is responsible for viral clearance. Thus it invites speculation that IPS-1 rs17857295 or rs7269320 SNP genotype might contribute to viral clearance. The precise correlation between IPS-1 and viral clearance will be determined in our future study.

In conclusion, rs17957295 and rs7269320 IPS-1 SNP genotypes may contribute to viral clearance, especially rs17857295 SNP genotypes, in comparison with the wild-type or rs7269320 SNP genotypes in HBV infected patients.

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