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پروپوزال نویسی
Decreased Expressions of STING but not IRF3 Molecules in Chronic HBV Infected Patients

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Abstract

Context: The stimulator of interferon genes (STING) induces the activation of interferon regulatory factor 3 (IRF3) in response to intracellular viral double-stranded (ds) DNA. The aim of this study was to evaluate mRNA levels of STING and its downstream transcription factor, IRF3, in the isolated peripheral blood mononuclear cells (PBMCs) of patients with chronic HBV (CHB) infection.

Methods: This study was performed on 60 healthy controls and 60 CHB patients. The mRNA levels of STING and IRF3 were determined using Real-Time polymerase chain reaction (PCR) techniques. The SPSS software version 18 was used to analyze raw data.

Results: The results revealed that mRNA levels of STING were significantly decreased in CHB patients in comparison to healthy controls ($P = 0.013$). Our results also revealed that expression levels of IRF3 were not different between CHB patients and healthy controls ($P = 0.828$).

Conclusions: In the present study, we found that CHB patients were unable to express appropriate levels of STING. Thus, it may result in impairment of HBV-DNA recognition and subsequently disruption of immune responses. These results suggest a plausible mechanism which may partially define the fact that immune responses are impaired in CHB patients.

Keywords: Chronic HBV infection, IRF3, STING


Introduction

It has been documented that patients with chronic HBV (CBH) infection are unable to eradicate HBV in either hepatocytes or sera completely.1 The reason for the defective immune response in CHB patients has yet to be clarified. It has been hypothesized that immunological factors of hosts play crucial roles to determine eradication or persistence of the virus.2–4 Pattern recognition receptors (PRRs) play a critical role in induction of appropriate immune responses against microbes including viruses, to stimulate several immune cell functions.5 The stimulator of interferon genes (STING) recognizes intracellular viral double-stranded (ds) DNA which leads to activation of interferon regulatory factor 3 (IRF3).6 IRF3 transcripts from genes of inflammatory cytokines including type I interferons (IFNs) which are the first immune responses against viral infections.7 Thus, downregulation of these molecules may result in impaired immune responses against viral infection. Based on the fact that CHB patients suffer from attenuated immune responses which are unable to completely remove HBV from hepatocytes,8 it appears that some parameters of their immune systems is impaired in comparison to clearance individuals. STING and IRF3 play a crucial role for recognition and stimulation of immune responses against viral infection. Therefore, it appears that these molecules may be involved in the pathogenesis of CHB. Therefore, the main aim of this study was to investigate the levels of mRNA of STING and its downstream transcription factor, IRF3, in the peripheral blood mononuclear cells (PBMCs) of patients with chronic HBV (CHB) infection.

Furthermore, it has been reported that depression and anxiety are two important factors which can modulate immune responses; hence, the other aim of this study was to examine the effects of depression and anxiety on the expression of STING and IRF3.

Materials and Methods

Samples

In this cross-sectional study, 5 mL peripheral blood was obtained from 60 healthy controls and 60 CHB patients and the sera were separated and stored at -70ºC. The including and excluding criteria for the CHB patients and healthy controls were defined in our previous study which was performed on the same population.2 The CHB patients were diagnosed according to the “Guideline for Prevention and Treatment of Viral Hepatitis” by an expert internal medicine specialist.9 Depression and anxiety in the CHB patients were examined by an expert psychologist based on responses to the standard Beck questionnaire.10 This project was approved by the ethical committee of the Kerman University of Medical Sciences and written informed consent was signed by all participants prior to sample collection.

Detection of HBV markers

HBsAg and hepatitis B e antigen (HBeAg) were examined using ELISA (Behring, Marburg, Germany) technique. HBV-DNA

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was extracted from 200 μL of plasma using a commercial kit from Cinnaphoton Company, Tehran, Iran, according to the manufacturer’s guidelines. Quantification of HBV-DNA was also performed using a commercial kit (Primer Design, UK) based on the manufacturer’s instructions.

RNA extraction, reverse transcription and quantitative real-time PCR
Total RNA was purified from whole blood using RXN extraction kit (Cinnalon, Iran). The quality of extracted RNA was identified with measuring absorption at 260/280 nm by spectrophotometer. cDNA was synthesized from purified RNA using a commercial cDNA synthesis kit (Pars Touss, Tehran, Iran) containing oligo (dT) primers. The reverse transcription process and Real-time PCR condition are described elsewhere, except for the applied primers (Table 1). Real-Time PCR was performed and the β-Actin was used as housekeeping gene to normalize the amplification signals of target genes. The relative expressions of target genes were determined using the 2^DDCT formula.

Data analysis and statistical methods
In order to analyze the raw data, parametric statistical analyses were performed using t-test with the SPSS software version 18.  P value less than 0.05 was considered as significant.

Results
Detection of HBV markers
The results revealed that all patients carried HBsAg, while 16 out of 60 patients (26.6%) were HBcAg positive. Our results demonstrated that HBV-DNA was detectable in all patients. Accordingly, HBV-DNA copy numbers/mL in 28, 14 and 18 of the patients were less than 20,000, between 20,000 and 100,000 and greater than 100,000, respectively.

Expression levels of target genes
Compared to healthy controls, mRNA levels of STING were 4.7-fold decreased in the PBMCs of CHB patients (Figure 1). There was a significant difference between CHB and healthy controls (P = 0.013). The results also showed that relative expression levels of IRF3 were 0.77 ± 0.34 in CHB patients and 0.90 ± 0.44 in healthy controls. The statistical analysis demonstrated that the difference between groups regarding expression levels of IRF3 was not significant (P = 0.828). There were no significant difference between expression levels of STING (P = 0.478) and IRF3 (P = 0.406) of HBeAg positive and negative CHB patients (Figure 2). The results revealed that mRNA levels of STING in CHB patients with HBV-DNA viral loads less than 20,000, between 20,000 and 100,000, and higher than 100,000 copy numbers/mL were 0.019 ± 0.14, 0.09 ± 0.03 and 0.29 ± 0.18, respectively (Figure 3). The statistical analysis demonstrated that expressions of IRF3 were not also different among CHB patients with various HBV-DNA copy numbers (P = 0.561) (Figure 3). The statistical analysis demonstrated that mRNA levels of STING were significantly increased (P = 0.005) in CHB patients with moderate depression in comparison to CHB patients with no and mild depression (Figure 4). The statistical

Table 1. Sequences of primers applied for STING and IRF3 analysis in Real-Time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>F</th>
<th>R</th>
</tr>
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<tbody>
<tr>
<td>IRF3</td>
<td>5'- CAGATTATCAATCAACCCCT-3'</td>
<td>5'- AAATTCATCACTTTCTTCG-3'</td>
</tr>
<tr>
<td>STING</td>
<td>5'-GAGAGCCACCAGAGCACA-3'</td>
<td>5'- TAGATGGAAGCAGGAACAG -3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AATCCCATCACCAGTCCTCAAC-3'</td>
<td>5'-AAATGAGCAGCTCAGGCTT-3'</td>
</tr>
</tbody>
</table>

Figure 1. Expression levels of STING and IRF3 (P = 0.828) in CHB patients in comparison to healthy controls. The figure reveals that mRNA levels of STING (P = 0.013) were significantly decreased, while IRF3 (P = 0.828) were not different in CHB patients in comparison to healthy controls.
Relative expression of STING

**Figure 2.** Expression levels of STING and IRF3 in HBeAg positive and negative CHB patients. The results showed that the mRNA levels of STING \( P = 0.478 \) and IRF3 \( P = 0.406 \) were not significantly different in HBeAg positive compared to HBeAg negative CHB patients.

Relative expression of IRF3

**Figure 3.** mRNA levels of STING and IRF3 in CHB patients with various HBV-DNA copy numbers/mL. The figure reveals that expression levels of STING \( P = 0.751 \) and IRF3 \( P = 0.561 \) were not different between patients carrying various HBV-DNA copy numbers/mL.

Relative expression of STING

**Figure 4.** Expression levels of STING and IRF3 in CHB patients without depression, mild depression and moderate depression. The figure shows that expression levels of STING were significantly increased in moderately depressed CHB patients \( P = 0.005 \), while, IRF3 expressions were not different \( P = 0.926 \) among patients with various stages of depression.

Relative expression of IRF3

**Figure 5.** Expression levels of STING and IRF3 in CHB patients with no, mild, moderate and severe anxiety. The figure shows that mRNA levels of STING \( P = 0.152 \) and IRF3 \( P = 0.823 \) were not differ among patients.
analysis also demonstrated that expression levels of STING were not different among CHB patients with various stages of anxiety (P = 0.152) (Figure 5). Our results also showed that mRNA levels of IRF3 were not significantly different among CHB patients with various stages of depression (P = 0.926) and anxiety (P = 0.823) (Figures 4 and 5).

**Discussion**

Our results revealed that the mRNA levels of STING were significantly decreased in the PBMCs from CHB patients compared to healthy controls. It has been reported that STING plays a key role as an intracellular microbial DNA sensor. In the present study, we found that CHB patients may be unable to promote expression of several inflammatory factors which are required for clearance of the HBV in a STING-dependent manner. These results suggest that the STING pathway may be defective in CHB patients. Thus, STING impaired expression may be considered as an important mechanism that can explain how HBV infection is persistent in CHB patients. In the present study, for the first time, we showed the expression levels of STING molecule in PBMCs from CHB patients, however, other studies have reported expression levels of other PRRs in CHB patients. It has been reported that expression levels of toll like receptor 9 (TLR9), as another PRR which recognizes microbial dsDNA, were decreased in CHB patients. The results presented clearly demonstrated that CHB patients were unable to express suitable rates of PRRs which recognize microbial dsDNA, such as STING and TLR9 and deficiency of reorganization HBV-DNA by immune cells of CHB patients may lead to sustention of infection in the CHB patients. Additionally, we found that there is not a significant difference between the levels of expression of IRF3 in CHB patients and healthy control groups. This observation suggests that down-regulation of STING in CHB patients may lead to impairment of activation (phosphorylation) of IRF3 but its expression is normal. Recently, we reported that compared to the healthy control subjects, the expression of NF-κB, another pro-inflammatory transcription factor, was stable in CHB patients. On the other hand, mRNA levels of IRF7 were significantly decreased in the patients. Therefore, HBV infection targets expressions of IRF7 and activation of IRF3 and NF-κB in CHB patients. Jiang and colleagues (2010) demonstrated that HBx antigen suppresses IRF3 phosphorylation. It has been reported that HBV polymerase suppresses phosphorylation, dimerization and nuclear translocation of IRF3. In agreement with the aforementioned and our previous studies, we found that STING pathway may play an important role in the HBV recognition and subsequently activation immune response which are disrupted in CHB patients. Interestingly, we found that various HBV-DNA copy numbers did not alter the expression levels of STING and IRF3 in CHB patients. Thus, our hypothesis is confirmed because higher HBV-DNA copy numbers need more expression of dsDNA sensors like STING molecule. Additionally, our results showed that the expression levels of STING molecule were increased in parallel with accelerating of depression (Figure 4). It has been reported that depression induces inflammation, so, increased expression of STING in the PBMCs isolated from moderately depressed CHB patients (Figure 4) may be related to higher degree of depression. We also found that various stages of anxiety did not alter the expression of the STING and IRF3 molecules in CHB patients; thus, we suggest that anxiety is unable to alter the expression of the STING and IRF3 molecules. There is no significant difference between the expression of STING and IRF3 in HBeAg positive and negative CHB patients (P = 0.478 and P = 0.406, respectively). This result suggests that HBeAg also is unable to affect the expression of STING and IRF3.

In conclusion, in the present study, we found that expression levels of STING were impaired in CHB patients. The results suggest that future therapy could focus on up-regulation of STING to improve HBV-DNA recognition leading to activation of the immune system and facilitating the eradication of HBV in CHB patients.

**Acknowledgment**

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**References**


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