The relationship between HLA-G and viral loads in non-responder HCV-infected patients after combined therapy with IFN-α2α and ribavirin

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A B S T R A C T

Hepatitis C disease is a virus mediated infection causing major health problem worldwide. Conversions of immune surveillance play an important role in response to virus clearance. Immune modulating molecules such as HLA-G and IL-10 that convert immune response toward Th2 may play a role to inhibit response from combined therapy with IFN-α2α and ribavirin. The objective of this study was to investigate the expression of HLA-G and IL-10 in responder and non-responder HCV positive patients. In this study, characteristics of the virus and 48 responder and non-responder patients in response to the combined therapy with IFN-α2α and ribavirin were analyzed. The expression levels of HLA-G and IL-10 were conducted using real-time PCR. Also, soluble HLA-G in both groups of patients and healthy individuals were determined by enzyme-linked immunosorbent assay. According to the obtained data, HCV 1a was the predominant genotype in responder and non-responder patients. Expression levels of HLA-G and IL-10 in non-responder group was significantly more than responder and control groups (P < 0.001). Additionally, expression levels of HLA-G and IL-10 were remarkably higher compared to healthy individuals at the beginning of treatment. Soluble HLA-G in non-responder patients was noticeably increased in comparison to responder patients after treatment (P < 0.05). These findings suggest that elevation of HLA-G and IL-10 in HCV infected patients may play an important role in response to combined therapy with IFN-α2α and ribavirin.

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1. Introduction

Infection with hepatitis C virus is considered to be a major public health issue worldwide with more than 150 million people infected [1]. Prevalence of anti-positive HCV infected individuals in Iran are ranged 0.08–1.3% [2]. Large numbers of infected Iranians are hemophilia and thalassemia patients [3]. Chronic hepatitis often occurs following chronic HCV infection which may be develop to liver cirrhosis and hepatocellular carcinoma (HCC). Moreover, HCC may progress to malignant neoplasm and leads to patients death [4]. Viral eradication is an important approach to diminish the incidence of HCC. The most prevalent treatment in HCV positive patients is therapy with IFN-α2α and ribavirin (RBV) leads to reduce of viral loads to undetectable levels while improving prognosis. Sustained virological response (SVR) is the primary goal of treatment in HCV patients. Despite recent advances, some HCV positive patients do not respond to the current antiviral therapy and 45–50% of patients cannot successfully clear the virus due to many factors including virological and host factors [5,6].

Cytokines and serum soluble factors play critical roles in immune response and treatment outcome of HCV infection. TGF-β, IL-4 and IL-13 known as Th2 cytokines induce the production of extracellular matrix proteins and promote fibrosis in cases of chronic patients [7]. Bias of immune reaction from Th1 to Th2 response plays an important role in chronic HCV hepatitis [8]. HLA-G is a non-classical human leukocyte antigen (HLA) showing

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major suppressive properties including inhibit cytotoxic NK and T cells, induce regulatory T cells and Th2 cytokines profile. As a result of its immunomodulatory function, involvement of HLA-G is reported in various pathologies including malignancies and viral infection [9]. In various types of viral infection such as human cytomegalovirus (HCMV), neurotropic viruses (herpes virus and rabies virus), influenza A virus, human immunodeficiency virus (HIV) and hepatitis B virus enhanced levels of HLA-G is accounted [8]. Recent studies reported elevated levels of some cytokines and soluble molecules such as IL-10, IL-12, IL-18 and HLA-E in non-responder (non-SVR) patients [4,10], but relation between levels of HLA-G and response to combined therapy in HCV patients have not been investigated.

The aim of this study was to evaluate the relation between expression of HLA-G and IL-10 and response to treatment with IFN-α2x and RBV in HCV positive patients.

2. Materials and methods

2.1. Study population

Blood samples were collected from 48 HCV-positive patients from the Digestive Diseases Research Center (DDRC), Shariati Hospital, Tehran, Iran. The control group was consisted of 50 age- and gender-matched and healthy subjects. Eligible patients were negative for hepatitis B surface antigen (HBsAg) and HIV and never treated with interferon. Liver biopsies were performed before therapy showing histological evidence of chronic hepatitis. Liver histology was staged according to the hepatitis activity index (HAI) [11]. The study protocol was approved by the Institutional Review Board and Ethics Committee of the Digestive Disease Research Center of Tehran University of Medical Sciences.

2.2. Treatment

Patients were treated with 180 μg pegylated interferon α2a weekly plus 800–1200 mg ribavirin (RBV) daily for 24 weeks. Dose of RBV was based on virus genotype and patient weight. Viral response and adverse effects were recorded.

2.3. Virological and genotype analysis

Plasma HCV RNA levels were measured using COBAS TaqMan HCV RNA assay (Roche COBAS Amplicor HCV Monitor v 2.0, Roche Diagnostics, Mannheim, Germany). HCV RNA levels were measured for all subjects at three different stages: before, at the end and 24 weeks after the treatment completed. Patients with undetectable viral RNA 24 weeks after the completion of the treatment were classified as responders (sustained virologic response (SVR)) and those with detectable viral RNA 24 weeks after the end of treatment were classified as non-responders. HCV genotypes were determined by TaqMan transcription-polymerase chain reaction (RT-PCR) using amplification of the core region with genotype-specific primers and probes by COBAS® TaqMan® HCV Test v2.0 (COBAS® TaqMan® 48 Analyzer).

2.4. RNA isolation and real-time PCR

Total RNA was extracted from PBMC using RNA extraction kit (High pure RNA isolation Kit, QIAGEN, Hilden, Germany) according to the manufacturer’s protocol on Roche light cycler version 3.5. The following set of specific primers for HLA-G (88 bp) was used: Forward primer: 5’-CAGAGCGAGGCCAGCTT-3’ Reverse primer: 5’-CCATCGTAGGCTACTGTGC-3’ And the primer set for IL-10 (105 bp) was as follows: Forward primer: 5’-CTGGACATTTAAGGTTAC-3’ Reverse primer: 5’-CGGTCGTCTGTTGCCTTGG-3’ as an internal control for the normalization of HLA-G and IL-10, hyoxanthine guanine phosphoribosyltransferase (HGPRT, 99 bp) was used with the following primers: Forward primer: 5’-CTGGCGCTCCTAGATGT-3’ Reverse primer (R): 5’ TCAGTCTGGTTGCTGCC-3’. PCR amplification was conducted for 40 cycles with the following conditions: denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 1 min at 70°C.

2.5. sHLA-G enzyme-linked immunosorbent assay

Plasma sHLA-G levels from 48 HCV patients and 50 age-matched normal healthy individuals were determined with the sHLA-G specific ELISA kit (sHLA-G kit, Biovender, R&D, USA). Each sample was measured in duplicate. The optical densities were measured at 450 nm (StatFax 2500, USA). The final concentration was determined by optical density according to the standard curves. The detection limit of the kit was 1 U/ml. Details of the procedure were as instructed by the manufacturer.

2.6. Statistical analysis

Data are presented as means ± standard deviation of the mean (SD). The paired T test was used to analyze the expression levels of HLA-G and IL-10 between control and patients groups. Differences of sHLA-G between groups were analyzed by one-way ANOVA. Pearson regression test was used to access correlation between different variables. Statistical analysis was performed using SPSS 16.00. The Values of $P < 0.05$ were considered as significant level.

3. Results

3.1. Characteristics of HCV positive-patients and normal controls in responder and non-responder groups

Characteristics of the HCV positive-patients in responder and non-responder groups are shown in Table 1. Healthy individuals were age and sex matched with patients and levels of their liver enzymes were normal. In the present research, 48 HCV-positive patients and 50 characteristic matched, unrelated, healthy individuals were included.

Among the both responder and non-responder patients, genotype 1a was the most prevalent (54.16% and 45.84% respectively). The mean of HCV virus levels in responder group was 5.45 x 10^5 copies/ml. Conversely, HCV virus levels in non-responder group were significantly higher (46.1 x 10^5 copies/ml) ($P < 0.0001$).

3.2. Levels of plasma sHLA-G

In overall, concentration of sHLA-G in patients was higher than healthy individuals before treatment (Fig. 1). sHLA-G expression in non-responder group in comparison to responder groups was significantly increased after treatment by IFN-α2x and RBV showing in Fig. 1 ($P < 0.05$). Mean of sHLA-G in non-responder group after treatment was 108.8 U/ml versus 80.5 at the beginning of combined therapy. Also, mean of sHLA-G in responder group before and after treatment was 93.69 and 85.88 respectively. Concentration of sHLA-G in normal control was 11.38 U/ml.

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3.3. IL-10 and HLA-G fold change

HLA-G fold changes were conducted using real-time PCR. IL-10 and HLA-G expression in patients was considerably higher in contrast to control groups before treatment (Fig. 2; \( P < 0.001 \)). Expression of HLA-G was significantly higher when compared to responder group after treatment (Fig. 2; \( P < 0.001 \)). sHLA-G fold changes pattern was observed for IL-10 too (Fig. 3). In addition, data showed that the elevated expression in IL-10 and HLA-G in HCV patients was unrelated to the HCV genotypes (Fig. 4A and B). On the other hand, expression levels of IL-10 and HLA-G were correlated with viral RNA load in HCV-infected patients (Fig. 5A and B). Interestingly, clear correlation between IL-10 and HLA-G was observed in HCV positive patients (Fig. 6).

4. Discussion

In this study, the levels of IL-10 and HLA-G were measured in HCV-positive patients and their association with the outcome from

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HCV patients N = 48</th>
<th>Responders N = 24</th>
<th>Non-responders N = 24</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>37/11</td>
<td>18/6</td>
<td>19/5</td>
<td>1.00</td>
</tr>
<tr>
<td>Age (range)</td>
<td>37.27 (21–74)</td>
<td>35 (21–57)</td>
<td>39.54 (21–74)</td>
<td>0.26</td>
</tr>
<tr>
<td>Liver function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (IU/L) (mean ± S.E)</td>
<td>40.52 ± 24.15</td>
<td>31.91 ± 15.87</td>
<td>49.12 ± 28.03</td>
<td>0.012</td>
</tr>
<tr>
<td>AST (IU/L) (mean ± S.E)</td>
<td>39.37 ± 21.55</td>
<td>32.08 ± 12.40</td>
<td>46.66 ± 26.15</td>
<td>0.017</td>
</tr>
<tr>
<td>AIP (IU/L) (mean ± S.E)</td>
<td>238.04 ± 112.67</td>
<td>189.00 ± 57.21</td>
<td>287.08 ± 132.85</td>
<td>0.002</td>
</tr>
<tr>
<td>AFP (IU/L) (mean ± S.E)</td>
<td>2.27 ± 2.56</td>
<td>1.85 ± 1.15</td>
<td>2.68 ± 3.42</td>
<td>0.268</td>
</tr>
<tr>
<td>HCV-RNA level × ( 10^5 ) (range)</td>
<td>25.8 (0.028–160)</td>
<td>5.45 (0.028–35.9)</td>
<td>46.1 (2.74–160)</td>
<td>0.000</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a (%)</td>
<td>24 (50)</td>
<td>13 (54.2)</td>
<td>11 (45.8)</td>
<td>0.77</td>
</tr>
<tr>
<td>1a/1b (%)</td>
<td>9 (18.8)</td>
<td>2 (8.3)</td>
<td>7 (29.2)</td>
<td>0.056</td>
</tr>
<tr>
<td>3a (%)</td>
<td>15 (31.2)</td>
<td>9 (37.5)</td>
<td>6 (25)</td>
<td>0.46</td>
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<tr>
<td>Histopathological stages</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 0 (%)</td>
<td>4 (8.3)</td>
<td>3 (12.5)</td>
<td>1 (4.2)</td>
<td>0.224</td>
</tr>
<tr>
<td>Stage 1 (%)</td>
<td>8 (16.7)</td>
<td>6 (25)</td>
<td>2 (8.3)</td>
<td></td>
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<tr>
<td>Stage 2 (%)</td>
<td>8 (16.7)</td>
<td>4 (16.7)</td>
<td>4 (16.7)</td>
<td></td>
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<tr>
<td>Stage 3 (%)</td>
<td>18 (37.5)</td>
<td>9 (37.5)</td>
<td>9 (37.5)</td>
<td></td>
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<tr>
<td>Stage 4 (%)</td>
<td>3 (6.2)</td>
<td>0 (0)</td>
<td>3 (12.5)</td>
<td></td>
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<tr>
<td>Stage 5 (%)</td>
<td>5 (10.4)</td>
<td>2 (8.3)</td>
<td>3 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Stage 6 (%)</td>
<td>2 (4.2)</td>
<td>0 (0)</td>
<td>2 (4.2)</td>
<td></td>
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</table>
treatment by IFN-α2α and RBV were analyzed. Expression levels of IL-10 and HLA-G in HCV-positive patients was significantly higher in comparison to healthy individuals. In addition, expression levels of IL-10 and HLA-G as well as serum levels of HLA-G in non-responder (non-SVR) patients to combination therapy were remarkably higher than responder patients (SVR). On the other hand, IL-10 and HLA-G levels in responder patients decreased during treatment and sustained low. These data suggest that IL-10 and HLA-G especially HLA-G may regulate host immune response to the virus.

High levels of IL-10 in non-responder patients to IFN-α2α and RBV is consistent with previous studies [12]. Also, diminish levels

Fig. 3. Real-time PCR assay of HLA-G expression between responder, non-responder and healthy individuals groups before and after treatment. The highest fold change in HLA-G expression was belonged to non-responder patients (column 3) compared to responder and control groups after treatment. **P < 0.01, ***P < 0.001.

Fig. 4. Comparison of HLA-G (A) and IL-10 (B) expression levels among HCV genotypes.

Fig. 5. Correlation between HLA-G (A) and IL-10 (B) expression levels with HCV viral RNA copies using Pearson linear regression.
of IL-10 in responder patients after treatment is parallel to funding of Yoneda et al. [4] and could be considered as predictive marker. Key factors to protect from HCV infection are natural killer cell and Th1 cell-mediated immune response that inhibit their function by IL-10. Moreover, genetic component plays an important role in IL-10 production and increased levels of IL-10 while relation between IL-10 and inadequate clearances of HCV virus in non-responder patients are reported in –1082 G/G genotype [4,13]. Although, recent researches is reported that IL-10 may be prevents the pathologic effects of Th17 [14].

One of the important soluble factors that significantly increased in chronic HCV-positive patients was HLA-G. Large number of studies is reported the elevated levels of HLA-G in different pathological situation such as autoimmune diseases, tumors and viral infections [9]. Further, enhanced levels of HLA-G are reported in chronic hepatitis C virus infection [5], hepatitis B virus infection [6] and primary HCC lesions [7]. HLA-G regulated immune response through three categorized functions including direct inhibitory function, indirect inhibitory function and other functions with inhibitory consequence. HLA-G directly inhibit the immune effectors cells including NK cells, CD4+ Cells, antigen presenting cells (APC) and cytotoxic T cells through its receptors. Also, HLA-G induce regulatory T cells resulting modulate immune response [18]. These modulatory and inhibitory functions of HLA-G may be playing a critical role in deficient of virus clearance in non-responder patients in response to combined therapy with IFN-α2a and RBV. In this study, high-level expression of HLA-G was observed from PBMC in non-responder patients after treatment. Amiot et al. [8] reported no elevated levels of HLA-G in monocytes, dendritic cells and T lymphocytes in hepatitis C virus-induced liver fibrosis and reported that mast cells are responsible for the production of HLA-G. Recently, secretion of IL-10 was reported by murine mast cells [19]. In this study, enhanced expression level of IL-10 was observed in non-responder HCV positive patients that may induce HLA-G production through mast cells. On other hand, viral loads in non-responder patients were significantly higher than responder groups showing import ance of viral loads in treatment outcomes. Some studies showed that patients who had more than 400,000 IU/ml exhibited a higher non-response rate [20]. So, increased levels of IL-10, HLA-G and viral loads induced IFN-α2a and RBV therapy outcomes.

Fig. 6. Correlation between HLA-G and IL-10 expression levels using Pearson linear regression.

Effect of diverse classes of Interferon (IFN) in production of HLA-G is different. In contrast to Interferons classes II and III, class I interferons including IFN-α, β and γ induced HLA-G secretion [21]. HLA-G in soluble form is susceptible to split by metalloproteinase and may be cleavage of HLA-G in non-responder HCV positive patients are insufficient. Also, released IFN-α, β and class III increase following viral infection like HCV and may induce HLA-G production from mast cells. Furthermore, previous researches suggested autocrine loop in mast cells through HLA-G receptor, ILT2. Similarly, dendritic cells and monocytes showed this type of regulation with connected HLA-G and ILT-4 [22].

In conclusion, this study shows that elevated levels of HLA-G and IL-10 in HCV-positive patients may be considered as biomarkers to predict non-response to therapy by IFN-α2a and RBV. Our data, also suggested the significant increase in plasma HLA-G expression might play a role in non-response to combined therapy.

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References


