A Functional Polymorphism of the Granulocyte Macrophage Colony Stimulating Factor is not Associated with the Outcome of HTLV-I Infection

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Abstract

Introduction
Genetic background has known to be associated with the outcome of human T cell lymphotropic virus (HTLV) type 1 infection. In the present study we investigate the association between GM-CSF gene polymorphisms with the outcome of HTLV-I infection.

Materials and Methods
We analyzed 3 single-nucleotide polymorphisms in the promoter region of granulocyte macrophage colony stimulating factor (GM-CSF) at positions -677*A/C, -1440*A/G and -1916*T/C in 68 patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and 77 HTLV-I-seropositive asymptomatic carriers and 175 healthy controls from an area in Iran, Mashhad, where HTLV-I is endemic.

Results
No significant differences were observed in the distribution of GM-CSF polymorphisms between HAM/TSP patients, HTLV-I carriers and healthy controls (P > 0.05). The -677*A/C polymorphism fall within the transcriptional enhancer factor-2 (TEF-2) binding site, so an electrophoretic mobility shift assay (EMSA) was performed to determine the effects of polymorphisms on protein binding to the GM-CSF promoter. The result showed a significantly higher binding efficiency of nuclear protein to the A allele compared with the C allele.

Conclusion
Our study suggests that polymorphisms in the GM-CSF promoter is not associated with the outcome of HTLV-I infection, however, GM-CSF polymorphism at position -677 could indeed influence gene expression.

Keywords: Electrophoretic Mobility Shift Assay, Gene Polymorphisms, Granulocyte-Macrophage Colony-Stimulating Factor, Human T-lymphotropic virus 1

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Introduction

Human T cell lymphotropic virus type I (HTLV-I) is an exogenous retroviruses which is associated with adult T cell leukaemia (ATL) and a progressive neurologic disorder named HAM/TSP (1, 2). This virus is endemic in South-West Japan, parts of Africa and South America (3-6). The North-East part of Iran (Mashhad) has been recognized as an endemic area for HTLV-I infection. The prevalence of HTLV-I infection in the whole population of Mashhad and blood donors is 3% and 0.44%, respectively (7, 8).

It is poorly understood why the majority (95%) of HTLV-I-infected individuals remain asymptomatic carriers of the virus and less than 5% develop HTLV-I-associated diseases. Viral variation and host genetic factors, both may contribute to diseases susceptibility in HTLV-I infected-individuals (9, 10). It has been shown that there is no association between HTLV-I variants and susceptibility to HAM/TSP (11). However, Furukawa et al., demonstrated that a variant of the Tax gene is more frequently observed in patients with HTLV-I asymptomatic carriers (9), which may suggest infection with HTLV-I alone is not sufficient to cause HAM/TSP.

More recently Birmann et al., showed that the dysregulation of host immune response contributes to differences in natural history of HTLV-I infection in Japanese and Jamaican population. (12). Therefore, host genetic background may have a pivotal role in the efficiency of immune response to HTLV-I and pathogenesis of HTLV-I progression. A number of possible candidate genes such as human leukocyte antigen (HLA) class I and II, and cytokine gene polymorphisms have been implicated in HTLV-I infection (10, 13-18). Studies have shown that both cytokine gene polymorphisms and HTLV-I tax protein up-regulate cytokine production level. The promoter region of many genes including interleukin 2 (IL-2), transforming growth factor β (TGB-β), IL-15, IL-6 and GM-CSF contain nuclear factor kappa binding (NF-κB) sites and transactivitated by HTLV-I tax protein (19).

GM-CSF is a growth factor known to be a potential factor in stimulating, proliferation, maturation and function of hematopoietic cells (20). GM-CSF has a not only variety effects on regulation, differentiation and proliferation of hematopoietic progenitors cells, but also it regulates the function of mature cells such as monocytes, macrophages, DCs cells and NK cells. The 5'-flanking region of human GM-CSF contain many transcription factors binding sites (21). We have previously detected novel polymorphisms in the human GM-CSF gene at positions −677*A/C and -1916. There was no significant association between GM-CSF gene polymorphisms at position -677*A/C and –1916*T/C and GM-CSF production on stimulated peripheral blood mononuclear (PBMC) by enzyme linked immunosorbent assay (ELISA). We found association between -677 gene polymorphism and the frequency and severity of atopic dermatitis (22). The -677 polymorphism seem to fall within the transcriptional enhancer factor-2 (TEF-2) binding site. TEF-2 binds to the enhancer of simian virus 40 (SV40) and to the CACCC box of the human β-globin promoter (23, 24).

The role of GM-CSF in HTLV-I infection has not been studied very well. In other retroviral infection such as HIV, it is demonstrated that GM-CSF stimulates monocytes and monocytes derived macrophages to produce chemokines including RANTES, MIP-1α and MIP-1β which leads these chemokines bind to chemokine receptor 5 (CCR5) and modulate human immunodeficiency virus (HIV) infection in T cells and macrophages (25, 26). Furthermore, GM-CSF augments the immune response to vaccines directly against both infectious agents and various cancers (27, 28). Coimmunization with GM-CSF enhances both the antibody and T cell proliferation response (29, 30).

In the present study we aimed to assess the effect of GM-CSF –677*A/C gene polymorphism on TEF-2 binding site and also...
investigate the association of GM-CSF gene polymorphisms at positions -677*A/C, -1440*A/G and -1916*T/C with the outcome of HTLV-I infection in the North-east of Iran.

Materials and Methods

Samples collection
All participant enrolled in the study attended Ghaem Hospital, Mashhad University of Medical Sciences, Iran. The populations studied were 155 healthy controls, 77 randomly selected HTLV-I asymptomatic carriers and 68 HAM/TSP patients who fulfilled the accepted criteria for HAM/TSP (18). The mean age of HAM/TSP patients was 45±9.8 years (range 24-74), and the female to male ratio was 1.5. The clinical and laboratory findings of HAM/TSP patients have been described previously (18). Blood samples from HTLV-I carriers and healthy controls were obtained from subjects attending the Mashhad blood transfusion centre. The mean age of the asymptomatic carriers was 37.6±10.2 years, and 23 of the 77 carriers were women (36.5%). The mean age of the healthy controls was 34.4±8.1 years. Permission to conduct the study was obtained from the Ethics Committees of Mashhad University of Medical Sciences and the Mashhad blood transfusion centre and informed consent was obtained from all cases and healthy controls.

GM-CSF genotyping
DNA was extracted from 6 ml EDTA whole blood samples using a standard phenolchloroform method. Amplification refractory mutation system (ARMS)- polymerase chain reaction (PCR) was used for genotyping of single nucleotide substitution at positions -677*A/C and -1916*T/C of GM-CSF (22). PCR- Single-Stranded Conformational Polymorphism (SSCP) method was used for genotyping of -1440*A/G polymorphism. The details of method have been described previously (18).

Electrophoretic mobility gel shift assay (EMSA)
Oligonucleotide probes (27 mer) were designed to cover the region surrounding the A to C –677 GM-CSF polymorphism. Consensus and mutant TEF-2 competitor single-stranded oligonucleotides (20 mer) were also designed. The sequences of oligonucleotide probes are shown in Table 1, polymorphic sites are in bold and the site of the mutant region is underlined.

Oligonucleotides were annealed to form double stranded probes. In this case, each single stranded nucleotide was made up at 1 µg/µl concentration with dH2O, then 10 µl of each sense and antisense complementary strand of DNA were combined with 0.5 µl 5 M NaCl, 4.5 µl 10 mM tris HCl (pH 8.5). The contents were incubated in a PCR machine as follow: 95 °C for 5 min, 65 °C for 10 min and 37 °C for 10 min and then cooling down for 1 hr at room temperature. Then 3.5 µl of 5 M NaCl and 75 µl of 10 mM Tris-HCl (pH 8.5) were added to make up a final concentration of 0.1 µg/µl oligonucleotide probe. Then 5 µl (5 pmoles) of oligonucleotide probe was transferred to an Eppendorf tube and 5 µl of Kinase reaction buffer, 1 µl of T4-polynucleotide kinase (Gibco BRL, UK), 2.5 µl [γ-32 P]-ATP and 11.5 µl dH2O were added to end-label double stranded DNA. The contents were briefly centrifuged to collect the solution and incubated at room temperature for 1 hr. The reaction was stopped by adding of 75 µl of Tris-EDTA (TE) buffer (5 mM). Then the labelled probes were separated from unlabelled probe by using a prepacked spin column containing Sephadex G-50 (Amersham Pharmacia Biotech, UK). The materials and method of EMSA reaction and electrophoresis have been described previously (31).

Statistical analysis
Differences in GM-CSF alleles and genotype between patients with HAM/TSP, HTLV-I asymptomatic carriers and healthy controls were performed, using STATA version 11 (STATA Corp.,). The significance of differences in allele and genotype frequencies among all groups were established, using the Fisher exact test and by calculation of odds ratios (OR) with 95% confidence intervals (95% CI). The significance of genotype frequency differences between any of the three groups were examined by the Chi-squared test.
Results

Association between GM-CSF gene polymorphisms and HTLV-I infection

The allele and genotype frequencies of the three GM-CSF polymorphisms at position -677*A/C, -1440*A/G and -1916*T/C were determined to compare any differences between HAM/TSP patients, HTLV-I carriers and healthy controls.

The GM-CSF -677*A allele was more frequent in HAM/TSP patients (82.3%) and HTLV-I carriers (82.5%) compared with healthy controls (76.8%), but no statistical significant difference was observed between HAM/TSP patients and healthy controls ($P=0.18$), HTLV-I carriers and healthy controls ($P=0.15$), or HAM/TSP patients and HTLV-I carriers ($P=0.97$) (Table 1).

The distribution of alleles and genotypes of the GM-CSF -1440*A/G polymorphism was the same in HAM/TSP patients, healthy controls and HTLV-I carriers (Table 3-16). No significant differences in allele and genotype frequencies in the GM-CSF -1440*A/G polymorphism were identified between any of the three groups ($P>0.05$).

Genotype and allele frequency analysis of healthy controls, HTLV-I carriers and HAM/TSP patients was performed for the T to C substitution at position –1916 of the GM-CSF gene (Table 1). The distribution of the allele T was the same between all of the three groups and no significant differences were observed between any of the three groups ($P>0.05$).

Effects of GM-CSF –677 gene polymorphism on transcription factor binding

EMSA was used to assess the effect of GM-CSF -677*A/C gene polymorphism on transcription factor binding. For the binding assay, HeLa nuclear extract was used as a source of transcription factor. Two double-stranded oligonucleotides probe (27 bp) each representing either the -677*AA or -677 *CC genotype were made and labelled with $\gamma^{32}$ATP. Both probes cover the region of GM-CSF from –689 to –663. HeLa nuclear protein extract was incubated with either the labeled-677*A or the –677*C probe using consensus and mutant TEF-2 oligonucleotides competitor. Then, electrophoresis was performed using 4% acrylamide gel. Incubation of nuclear extract from HeLa cell with radio labelled GM-CSF -677*A probe gave a protein/DNA complex (lane 6) (Figure 1).

No band was apparent when oligonucleotide was incubated without HeLa cell extract (lane 1). When the nuclear protein extracts from HeLa cell was incubated with either unlabelled TEF-2 consensus or -677*A allelic oligonucleotides inhibited the formation of the DNA/protein complex (lanes 3 and 5, respectively) compared with lane 2. By contrast, a scrambled TEF-2 oligonucleotide or the -677*C sequence did not inhibit formation of the DNA/protein complex (lanes 4 and 6, respectively). Similar experiments with a radio labelled -677*C oligonucleotide gave similar results (data not shown).

Figure 1. EMSA assay of specific binding of A probe of the GM-CSF –677 gene by nuclear protein extract from HeLa cell. Nuclear extract from Hela cells was incubated with radiolabelled GM-CSF -6778*A oligonucleotide. The protein/DNA complex can be seen in lane 2. The oligonucleotide without HeLa cell extract was run in lane 1 (negative control) and there was no band apparent. Preincubation of the HeLa cell extract for 20 min with either unlabelled TEF-2 consensus or -677*A allelic oligonucleotides inhibited the formation of the DNA/protein complex (lanes 3 and 5, respectively) compared with lane 2. By contrast, a scrambled TEF-2 oligonucleotide or the -677*C sequence did not inhibit formation of the DNA/protein complex (lanes 4 and 6, respectively). Similar experiments with a radio labelled -677*C oligonucleotide gave similar results (data not shown).
Table 1. Sequences of oligonucleotide probes of GM-CSF.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequencing of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF “C” sense</td>
<td>5’-CTT GAC CCAGCCACCCCCTCTGAAG-3’</td>
</tr>
<tr>
<td>GM-CSF “C” anti-sense</td>
<td>5’-CTTCAGAGGGGGTGTTGGCTGGAAG-5’</td>
</tr>
<tr>
<td>GM-CSF “A” sense</td>
<td>5’- CTT GAC CCAGCCACCCCCTCTGAAG-3’</td>
</tr>
<tr>
<td>GM-CSF “A” anti-sense</td>
<td>5’- CTTCAAGGGGGTGTTGGCTGGAAG-3’</td>
</tr>
<tr>
<td>TEF-2 (consensus) sense</td>
<td>5’-GATCCTTAGGGTGATC-3’</td>
</tr>
<tr>
<td>TEF-2 (consensus) anti-sense</td>
<td>5’-GATCCTTAGGGTGATC-3’</td>
</tr>
<tr>
<td>TEF-2 (mutant) sense</td>
<td>5’-GATCCTTACCCACTGGACCA-3’</td>
</tr>
<tr>
<td>TEF-2 (mutant) anti-sense</td>
<td>5’-TGGTCCAAGGGTGATGACCA-3’</td>
</tr>
</tbody>
</table>

Polymorphic sites are in bold and the site of the mutant region is underlined.

Discussion

It is well known that GM-CSF has ability to induce proliferation and differentiation of myeloid progenitor’s cells. The capacity of cytotoxicity and antigen presenting by granulocytes and macrophages are increased by GM-CSF (20).

Production of many cytokines is under genetic control and GM-CSF may not be excluded from this phenomenon. In the present study we compared the allele and genotype frequencies of three GM-CSF polymorphisms at positions -677*A/C, -1440*A/G and -1916*T/C among HAM/TSP patients, HTLV-I asymptomatic carriers and healthy controls. The analysis of genotype and allele frequencies of these polymorphisms showed there is no significant difference between HAM/TSP and HTLV-I carriers, HAM/TSP and healthy controls and HTLV-I carriers and healthy controls (Table 2). Although, the frequencies of -677*C, -1440*G and -1916*C alleles were slightly increased in HAM/TSP patients and HTLV-I carriers compared with healthy controls, however, the result did not reach significant levels. This may be due to the small numbers of HAM/TSP patients and HTLV-I carriers. Recently, we found that there is a association between GM-CSF gene polymorphisms at position -677*A/C and -1916*T/C in patients with severity of atopic dermatitis (22). The results identified that the frequencies of -677*A and -1916*T allele are greater in children with atopic dermatitis than in control group (P< 0.05) (22). Inheritance of a GM-CSF -1916*T allele was associated with doubling of the frequency of atopic dermatitis (P< 0.001). This result is consistent with the previous observations that suggest alteration in GM-CSF levels in the skin is associated with variation in the severity of atopic dermatitis (32). Although, activated T cells and B cells are the major source of GM-CSF production, other cells such as astrocytes and microglial cells are able to produce GM-CSF (33). The production of GM-CSF is not detectable under normal conditions. Furthermore, the GM-CSF gene is under the regulation of transcription factors such as NF-κB and nuclear factor-activated T cells (NF-AT) (34). Therefore, it seems that stimulation of relevant cells by suitable induction signals may lead to up regulation of GM-CSF and polymorphism in the promoter region may act in a cell specific-manner.

There has been considerable interest in the role of GM-CSF in other retroviral infections such as HIV. Stimulation of peripheral blood monocytes and monocyte-derived macrophages by GM-CSF leads to induction of chemokines such as regulation of activation of normal T cell expressed and secreted (RANTES) F, Macrophage-inflammatory protein (MIP)-1α and MIP-1β. These inflammatory chemokines bind to HIV coreceptor, CCR5 and modulate HIV infection in T cells and macrophages (26). In vitro studies have shown that GM-CSF induced monocytes to differentiate to macrophages and also suppressed the mRNA expression of CXCNR4 and CCR5 which leading to decreased HIV entry to these cells (25). In contrast to this effect, Wang et al reported that addition of GM-CSF to monocyte culture before and during infection with HIV increased both the extent of HIV entry and replication as well as CCR5 expression (35).
**Table 2. Genotype and allele frequency in HAM/TSP patients, HTLV-I carriers and healthy controls.**

<table>
<thead>
<tr>
<th>GM-CSF Polymorphisms</th>
<th>Controls Total =157 n (%)</th>
<th>HAM/TSP Total =68 n (%)</th>
<th>Carriers Total =77 n (%)</th>
<th>P value HAM/TSP vs carriers</th>
<th>P value HAM/TSP vs controls</th>
<th>P value Carriers vs controls</th>
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<tbody>
<tr>
<td><strong>-677</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Genotype</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>AA</td>
<td>93 (59.2)</td>
<td>48 (70.6)</td>
<td>53 (68.9)</td>
<td>0.77</td>
<td>0.22</td>
<td>NS*</td>
</tr>
<tr>
<td>AC</td>
<td>55 (35.0)</td>
<td>16 (23.5)</td>
<td>21 (27.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>9 (5.8)</td>
<td>4 (5.9)</td>
<td>3 (3.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele A</td>
<td>241 (76.8)</td>
<td>112 (82.3)</td>
<td>127 (82.5)</td>
<td>0.97</td>
<td>0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Allele C</td>
<td>73 (23.2)</td>
<td>24 (17.7)</td>
<td>27 (17.5)</td>
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<tr>
<td><strong>-1440</strong></td>
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<tr>
<td>Genotype</td>
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</tr>
<tr>
<td>AA</td>
<td>87 (55.4)</td>
<td>46 (67.6)</td>
<td>50 (64.9)</td>
<td>0.73</td>
<td>0.11</td>
<td>NS</td>
</tr>
<tr>
<td>AG</td>
<td>64 (40.8)</td>
<td>18 (26.5)</td>
<td>24 (31.2)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GG</td>
<td>6 (3.8)</td>
<td>4 (5.9)</td>
<td>3 (3.9)</td>
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<tr>
<td>Allele A</td>
<td>238 (75.8)</td>
<td>110 (80.9)</td>
<td>124 (80.5)</td>
<td>0.93</td>
<td>0.23</td>
<td>NS</td>
</tr>
<tr>
<td>Allele G</td>
<td>76 (24.2)</td>
<td>26 (19.1)</td>
<td>30 (19.5)</td>
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<td><strong>-1916</strong></td>
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<tr>
<td>TT</td>
<td>86 (55.0)</td>
<td>46 (67.6)</td>
<td>49 (63.6)</td>
<td>0.82</td>
<td>0.16</td>
<td>NS</td>
</tr>
<tr>
<td>CT</td>
<td>62 (39.0)</td>
<td>18 (26.5)</td>
<td>25 (32.5)</td>
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<tr>
<td>CC</td>
<td>9 (6.0)</td>
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<tr>
<td>Allele T</td>
<td>234 (75.0)</td>
<td>110 (80.9)</td>
<td>123 (79.9)</td>
<td>0.82</td>
<td>0.14</td>
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<tr>
<td>Allele C</td>
<td>80 (25.0)</td>
<td>26 (19.1)</td>
<td>31 (20.1)</td>
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</table>

No significant (NS)* differences were observed in allele and genotype frequencies between any of the three groups.

We proposed that the -677*C, -1916*C and -1440*G alleles are more common in patients infected with HTLV-I compared with controls; however, our results did not reach a significant level. It is likely that these polymorphisms may be a useful genetic marker for HIV, because no known role has been recognized for the entry of HTLV-I through CCR5.

Short oligonucleotides representing either the GM-CSF -677*A or the GM-CSF -677*C genotype of the GM-CSF promoter were designed to investigate whether the GM-CSF -677*A/C polymorphism affects transcription factor binding. The results identified the presence of a functional TEF-2 binding site in the promoter region of GM-CSF, with greater affinity of the allelic variant A than the C allele. The greater affinity of TEF-2 to GM-CSF -677*A may suggest that A to C substitution at position-677 weakens the binding of TEF-2 to the promoter of GM-CSF and may strengthen the promoter activity of GM-CSF when allele A is present at the polymorphic site. This experiment should be confirmed by super gel shift and reporter gene assays to investigate if -677*A/C polymorphism affects transcription of GM-CSF.

**Conclusion**

Our results showed that there is no association between GM-CSF gene polymorphisms and the outcome of HTLV-I individuals in the North east of Iran. Other studies in different population infected with HTLV-I may be useful to clarify the role of GM-CSF gene polymorphism in HTLV-I infection. Furthermore, the effect of another transcription
factor, TCF, which is located close to the –677*A/C polymorphism may be, involved in the induction of GM-CSF transcription.

Acknowledgment
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References
GM-CSF Gene Polymorphisms


کارگاه‌های آموزشی مرکز اطلاعات علمی چهار دانشگاهی

- پروپوزال
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