Promoter polymorphism in \textit{IL12B} and serum levels of IL-12p40 in healthy Bulgarians

ABSTRACT

Interleukin 12 (IL-12) is an important immunoregulatory cytokine that provides a link between the innate and adaptive immune responses and promotes the development of Th1 responses. IL-12 is a proinflammatory heterodimeric cytokine, which consists of two subunits, IL-12p35 and IL-12p40, encoded by the \textit{IL12A} and the \textit{IL12B} genes, respectively. The \textit{IL12B} gene contains several polymorphisms, including a complex polymorphism -6415CTCTAA/GC in the promoter region (\textit{IL12Bpro}). In this study we assessed the relationship between the \textit{IL12Bpro} polymorphism and the serum levels of IL-12p40 in healthy 18-year-old Bulgarians from the Stara Zagora region. The IL-12p40 levels of 140 subjects were determined by enzyme-linked immunosorbent assay (ELISA). Genotyping for the \textit{IL12Bpro} polymorphism was performed using amplification refractory mutation system (ARMS). The allele frequencies were 0.464 for the \textit{IL12Bpro-1} allele (CTCTAA) and 0.536 for the \textit{IL12Bpro-2} allele (GC). The genotype frequencies were 0.221 for \textit{IL12Bpro-11}, 0.293 for \textit{IL12Bpro-22} and 0.486 for \textit{IL12Bpro-12}. The obtained IL-12p40 serum levels were as follows: 83.68 ± 36.94 pg/ml for \textit{IL12Bpro-11}, 85.32 ± 32.46 pg/ml for \textit{IL12Bpro-22} and 90.16 ± 43.63 pg/ml for \textit{IL12Bpro-12}. A higher IL-12p40 level was found in subjects with the \textit{IL12Bpro-12} genotype compared to the other genotypes but the difference was not statistically significant (p>0.05, t-test). Although our data do not show a significant influence of the \textit{IL12Bpro} polymorphism on the serum levels of IL-12p40 in healthy Bulgarian subjects, the possibility remains that such an effect exists for patients with certain immunomediated diseases.

Key words: \textit{IL12B}, polymorphism, IL-12p40 serum levels, Bulgarians

Introduction

Interleukin 12 (IL-12) is an immunoregulatory cytokine, produced by macrophages and dendritic cells in response to antigenic stimulation. It is a key factor driving naive T cells into Th1 differentiation and plays an important role in interferon gamma (INFγ) production by T cells and NK cells (Hoelscher, 2004). Human IL-12 (IL-12p70) is a heterodimeric proinflammatory cytokine, which consists of two disulfide-linked polypeptide chains, p35 and p40 encoded by the \textit{IL12A} and \textit{IL12B} genes, respectively (Sieburth et al, 1992). In addition the IL-12p40 subunit can be secreted as a monomer, as a dimer (IL-12p80) and can form another heterodimeric proinflammatory cytokine, IL-23, with the p19 subunit. In humans the \textit{IL12B} genomic sequence is located on chromosome 5 at 5q33. The complete genomic sequence of \textit{IL12B} is characterized by the presence of several polymorphisms, including a complex insertion/deletion polymorphism CTCTAA/GC at position -6415 in the promoter region (\textit{IL12Bpro}), resulting from a 4-base-pair microdeletion combined with an AA/GC transition (rs17860508). As this promoter polymorphism could influence the expression of the \textit{IL12B} gene and the production of IL-12p40 and IL-12p40-related immunoregulatory cytokines, it could be relevant to the development of a number of immunomediated diseases. Several studies have been conducted on the pathological relevance of the \textit{IL12Bpro} polymorphism and have shown association of this polymorphism with the risk, progression and severity of diseases as diverse as asthma (Morahan et al,
2002a; Tatebayashi et al, 2004), systemic lupus erythematosus (Miteva et al, 2012), silicosis (Stanilova et al, 2008) and cerebral malaria (Morahan et al, 2002b). Much less studied is the association of the IL12Bpro polymorphism with gene expression and production of IL-12p40 in apparently healthy subjects with respect to age and gender. One study (Morahan et al. 2002a) which determined the production of IL-12p40 mRNA in cultured peripheral blood mononuclear cells from asthmatic children found that the IL12Bpro-12 genotype was associated with significantly reduced IL-12p40 gene transcription and decreased IL-12p70 production. It has been demonstrated in one of our previous studies that the IL-12p40 levels can influence the development and progression of colorectal carcinoma (CRC) independently of the IL12Bpro polymorphism, so that whereas IL-12p40 levels were associated with CRC, the IL12Bpro genotype was not (Miteva et al, 2009). The aim of this study was to determine the association of the IL12Bpro polymorphism with the IL-12p40 levels in healthy subjects.

**Materials and Methods**

**Subjects**

A group of 140 healthy donors from the Stara Zagora region in Bulgaria was included in the study. The group consisted of 60 (42.9%) males and 80 (57.1%) females, all of which were 18 years old. Informed consent was obtained from all subjects and authorization was given by the Ethics Review Board of the Faculty of Medicine, Trakia University.

**DNA extraction and genotyping**

Peripheral blood samples were collected in sterile tripotassium EDTA tubes. Genomic DNA was extracted using the illustra blood genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Pittsburgh, PA). Genotyping for the -6415CTCTAA/GC polymorphism in the promoter region of IL12B (IL12Bpro) was performed using amplification refractory mutation system (ARMS) polymerase chain reaction (PCR). For the IL12Bpro-1 allele (CTCTAA) a 196 bp fragment was amplified using the primer 5’-TGTCTCCGAGAGGCCTCTAA-3’ and for the IL12Bpro-2 allele (GC) a 192 bp fragment was amplified using the primer 5’-TGTCTCCGAGAGGCCGTGT-3’, in combination with a generic primer, 5’-TGTCTCCGAGAGGCCTCTAA-3’. The PCR reactions were performed in a total volume of 20 µl containing 1-3.6 ng/µl genomic DNA template, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.4 pM/µl of each primer and 1U/rxn Taq polymerase. The cycling parameters for the IL12Bpro polymorphism were as follows: an initial incubation step of 15 minutes at 95°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 65°C and 30 seconds at 72°C, followed by a final extension step of 7 minutes at 72°C.

All reagents for the PCR reactions were supplied by ThermoScientific (Waltham, MA). Amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The PCR products were visualized on a 2% agarose gel, stained with ethidium bromide (0.5 mg/ml). In each PCR run a heterozygous control template was used to ensure accuracy.

**IL-12p40 determination**

The quantitative determination of IL-12p40 was performed by enzyme-linked immunosorbent assay (ELISA) in serum samples according to the manufacturer's protocol (Invitrogen Corporation, Frederick, MD). The color reaction was measured as optical density units at 450 nm. The IL-12p40 concentration was determined using a standard curve constructed with the kit's standards and expressed in pg/ml. The minimum detectable concentration of the ELISA kit for IL-12p40 was less than 2 pg/ml.

**Statistical analysis**

The allele and genotype frequencies were calculated by direct counting. The goodness of fit to Hardy-Weinberg equilibrium was determined by calculating the expected frequencies for each genotype and comparing them to the observed values using a χ² test.

Differences in the serum levels of IL-12p40 between the genotypes were analyzed using a Student's t-test for independent variables using the genotypes for the IL12Bpro polymorphism as independent variables.

Statistical analysis was performed with StatSoft 6. In all cases a p value of less than 0.05 was considered significant.

**Results**

The allele frequencies for the IL12Bpro polymorphism were calculated by direct counting and were 0.464 for the IL12Bpro-1 allele and 0.536 for the IL12Bpro-2 allele, giving an approximately one to one allele ratio (IL12Bpro-1:IL12Bpro-2=1.15:1). The frequencies of the IL12Bpro-11,
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IL12Bpro-12 and IL12Bpro-22 genotypes were also determined by direct counting and are given in Table 1 for the total number of subjects, for males and for females. Of the 140 subjects, which were genotyped, 31 (0.221) had the IL12Bpro-11 genotype, 41 (0.293) had IL12Bpro-22 and 68 (0.486) had the IL12Bpro-12. The genotype frequencies did not show a significant departure from the Hardy-Weinberg equilibrium ($\chi^2=0.0779$, $p<0.9618$, $\chi^2$ test) and the obtained genotype ratio of approximately 1 to 2 to 1 ($IL12Bpro-11:IL12Bpro-12:IL12Bpro-22=1:2:2:1.3$) was expected from the allele ratio.

Table 1. Genotype frequencies for the IL12Bpro polymorphism, total and by gender (male or female).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of subjects (N) (male/female)</th>
<th>Genotype frequency (male/female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL12Bpro-11</td>
<td>31 (14/17)</td>
<td>0.221 (0.233/0.213)</td>
</tr>
<tr>
<td>IL12Bpro-12</td>
<td>68 (33/35)</td>
<td>0.486 (0.55/0.438)</td>
</tr>
<tr>
<td>IL12Bpro-22</td>
<td>41 (13/28)</td>
<td>0.293 (0.217/0.35)</td>
</tr>
</tbody>
</table>

There was also no significant difference in the levels between the IL12Bpro-11, IL12Bpro-12 and IL12Bpro-22 genotypes in either males or females ($p>0.2$). A higher IL-12p40 level was found in females with the IL12Bpro-12 genotype compared to the other genotypes but the difference was not statistically significant ($p=0.237$ for IL12Bpro-11 vs. IL12Bpro-12 and $p=0.246$ for IL12Bpro-22 vs. IL12Bpro-12).

The serum levels of the IL-12p40 subunit of IL-12 were determined for each subject and compared between the different IL12Bpro genotypes. Figure 1 shows the obtained levels for IL12Bpro-11, IL12Bpro-12 and IL12Bpro-22. The IL-12p40 serum levels were $83.68\pm36.94$ pg/ml for IL12Bpro-11, $90.16\pm43.63$ pg/ml for IL12Bpro-12 and $85.32\pm32.46$ pg/ml for IL12Bpro-22. A higher IL-12p40 level was found in subjects with the IL12Bpro-12 genotype compared to the other genotypes but the difference was not statistically significant ($p=0.842$ for IL12Bpro-11 vs. IL12Bpro-22, $p=0.475$ for IL12Bpro-11 vs. IL12Bpro-12 and $p=0.54$ for IL12Bpro-22 vs. IL12Bpro-12, Student’s t-test for independent variables).

The IL-12p40 serum levels were also compared between males and females of different IL12Bpro genotypes. Figure 2 shows the obtained levels for IL12Bpro-11, IL12Bpro-12, IL12Bpro-22 and all genotypes (total) in males and females. There was a tendency for higher IL-12p40 serum levels in females compared to males but no significant difference was found (females: $91.613$ pg/ml vs. males: $81.567$ pg/ml; $p=0.132$).

Figure 1. IL-12p40 serum levels in healthy subjects with each IL12Bpro genotype. The values are expressed as the mean (bars) and standard deviation (whiskers).

Figure 2. IL-12p40 serum levels in healthy subjects by gender (male or female) and IL12Bpro genotype. The values are expressed as the mean (bars) and standard deviation (whiskers).
Discussion

In this study the allele and genotype frequencies for the IL12Bpro polymorphism were determined for 140 healthy Bulgarians from the Stara Zagora region. The two alleles, IL12Bpro-1 and IL12Bpro-2, had an approximately equal frequency and the heterozygous IL12Bpro-12 genotype was the most common genotype, with an IL12Bpro-11:IL12Bpro-22:IL12Bpro-12 ratio of 1:1.3:2.2. These genotype frequencies were similar to those reported in healthy controls from other Caucasian populations (Morahan et al, 2002a; Mueller al, 2004; Glas et al, 2012), as well as in healthy Indians (Sam et al, 2014). However, they differed from those reported in Japanese, Chinese and Malay individuals in which IL12Bpro-22 was about twice as common as IL12Bpro-11 (Tatebayashi et al, 2004; Sam et al, 2014), and from those in a Turkish cohort, in which IL12Bpro-22 occurred over 10 times as frequently as IL12Bpro-11 (Ozbeý et al, 2008). These findings illustrate the well-known fact that genotype distribution depends on race and ethnicity. The distribution of the IL12Bpro genotype frequencies was in agreement with the Hardy-Weinberg equilibrium, which was not unexpected since healthy subjects were included in the study. However, in patients with certain medical conditions high-risk alleles and genotypes can be overrepresented and the genotype frequencies can deviate significantly from the Hardy-Weinberg equilibrium as has been demonstrated to a dramatic extent for children with severe asthma (Morahan et al, 2002a).

Comparison of the serum levels of IL-12p40 between the IL12Bpro-11, IL12Bpro-12 and IL12Bpro-22 genotypes revealed that there was no significant relationship between the IL12Bpro polymorphism and the IL-12p40 levels in the study group. There was also no significant difference in the IL-12p40 serum level between males and females. Although the level was slightly higher in heterozygotes, especially in heterozygous females, the difference did not reach statistical significance. Therefore, at least in healthy 18-year-old Bulgarians the IL12Bpro polymorphism, as well as gender, does not seem to have a great influence on IL-12p40 production. Nevertheless, the regulation of the basal IL-12p40 expression in healthy subjects is different from the induced one in the context of an inflammatory response. Studies have shown a relationship of the IL12Bpro polymorphism with IL-12p40 cytokine or mRNA production in severe childhood asthma (Morahan et al, 2002a) and silicosis (Stanilova et al, 2008). Further studies involving different populations and patients with different medical conditions are needed to examine the possibility of an effect of the IL12Bpro polymorphism on cytokine production.

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References

