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Original Contribution

GENOTYPE AND ALLELIC FREQUENCIES OF A Taq1 POLYMORPHISM IN THE 3'-UTR OF THE IL-12 p40 GENE IN BULGARIANS

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ABSTRACT

Interleukin-12 (IL-12) is a 75-kDa heterodimer composed of two disulfide-linked subunits, designated p35 and p40, and encoded by separate genes on chromosomes 3p12-3q13.2 and 5q31-33 respectively. A complete genomic sequence analysis of the IL-12 gene encoding its p40 subunit identified several intronic polymorphisms and a Taq1 (A/C) single nucleotide polymorphism (SNP) in the 3'-untranslated region (UTR) at position +16974.
The aim of our study, the first to be performed on Bulgarian citizens, was to determine the genotype and allelic frequencies of a Taq1 polymorphism in 3'-UTR of the IL-12 p40 gene in two ethnic groups – Bulgarians (n=58) and Turkish minority (n=33) – in Bulgaria. Genomic DNA was PCR amplified using primers that covered the Taq1 restriction site. The 1046-bp amplified fragment was cut with the Taq1 restriction enzyme. The 16974 C allele yielded restriction site for the enzyme. Our results showed that Bulgarians and Turkish minority in Bulgaria have no differences in genotype and allelic frequencies of SNP in the 3'-UTR of the IL-12 p40 gene. Genotype distribution in the group of Bulgarian citizens was: AA (61%), CA (32%) and CC (7%). The observed allelic frequency of "A" - 0.77 for Bulgarian citizens- was similar to frequencies reported for other Caucasian populations.

Keywords: IL-12p40; Polymorphism; Bulgarians

INTRODUCTION

The capacity of immune cells to respond to different stimuli by expression of cell surface molecules, proliferation or cytokine secretion is, at least in part, genetically predetermined.
Cytokines play a pivotal role in the regulation of the type and magnitude of the immune response (1). The polymorphic nature of the cytokine genes may confer flexibility on the immune response

Interleukin-12 (IL-12) is a heterodimeric pro-inflammatory cytokine that is critical in the orchestration of cell-mediated immune responses in both the innate and adaptive immune systems. It is a key factor in the induction of T cell-dependent and independent activation of macrophages, generation of Th1 and cytotoxic T cells (2). IL-12 is now known to be mainly a product of activated inflammatory cells (monocytes, macrophages, neutrophils, microglia and DCs). Biologically active IL-12 is composed of two disulphide-bounded polypeptide chains- p35 and p40 (3). While many cell types express the p35 subunits, the activated macrophages and B cells mainly express the p40 chain of IL-12.

Recently, a complete genomic sequence analysis of the IL-12 gene encoding its p40 subunit, identified several intronic polymorphisms and a single nucleotide polymorphism (SNP) (+16974 A/C) at position +16974 in the 3'-untranslated region (UTR) of IL-12 p40 subunit gene (IL-12B) (3, 4). IL-12B is located at chromosome 5q31-33, with Genebank accession number: AY008847.

Also, the 3'-UTR region plays an important role in the expression of many eukaryotic genes by governing mRNA stability, localizing mRNA, and regulating translation efficiency. Any polymorphism in this region of the gene might affect gene expression.

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IL-12B is highly conserved among humans. Huang D et al. have investigated a large group of unrelated individuals of diverse European descent, and have reported the “A” allele having a frequency of 0.82 (3). Other research groups have reported similar frequencies ranging from 0.77 through 0.82 in Caucasian populations from The Netherlands, Northern Ireland and UK. However, the allelic frequency of rarer “C” allele in some populations is higher. For example, Tsunami et al. had reported 0.50 allelic frequency of “C” allele in 100 healthy donors from the Japanese population (5).

Against this background, we examined for the first time the genotype and allelic frequencies of Taq1 polymorphism in 3'-UTR of the IL-12 p40 gene in two ethnic subgroups from Bulgaria, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. In this paper we present our preliminary results which show that the allelic frequency of this SNP for Bulgarians and their Turkish minority was similar to frequencies reported for other Caucasian populations.

MATERIALS AND METHODS

Subjects

A total group of 91 healthy volunteers was included in the study of the frequency distribution of the Taq1 IL-12 p40 gene polymorphism. The mean age of the group was 34.4 years (range 18–71 years) and contained 28.57% males and 71.43 % females. This group was divided in two subgroups, according to the ethnic affiliations – Bulgarians (n=58) and the Turkish minority in Bulgaria (n= 33). The mean age of the subgroup of Bulgarians was 37.1years (27.59 % males and 72.41 % females), and the mean age of the subgroup of Turkish minority in Bulgaria was 30.0 years (33.33 % males and 66.67 % females).

Informed consent was obtained from all subjects and authorisation was given by the Ethics Review Board of the Faculty of Medicine, Trakia University.

DNA extraction and TaqI RFLP analysis

Peripheral blood specimens were collected in tripotassium EDTA sterile tubes. Genomic DNA was extracted using standard phenol/chloroform extraction procedure or salting out method according to Miller *et al.* (6, 7). Isolated DNA samples were subsequently stored at -20°C for use in further analyses.

Genotyping for the Taq1 polymorphism in the 3’-UTR of the IL-12 p40 gene was performed as previously described with modifications (8). Briefly, a 1046-bp fragment containing + 16974 A/C SNP at the 3’-UTR of IL-12p40 was PCR-amplified using the forward primer: 5’-ATTTGGAGGAAAAGTGGAAGA - 3’ and the reverse primer: 5’ – AATTTCATGTCCTTAGCCATA – 3’. The PCR was carried out in 20 μL volumes containing GeneAmp 10x PCR buffer, 0.25 U of AmpliTaq Gold polymerase, 3.0 mM $MgCl_2$, 100 μM of each dNTP, 4 pmol of each primer (*Applied Biosystems, USA*), and 0.1- 0.5 mg of genomic DNA. Amplifications were performed in a GeneAmp PCR System 9700 (*Applied Biosystems*). After initial 10-min incubation at 95°C, PCR was performed for 30 cycles at 94°C for 1 min, at 54.3 °C for 1 min, at 72 °C for 2 min. A final extension step of 7 min at 72 °C completed the reaction. The successful amplification was confirmed using gel electrophoresis on a 2% agarose gel stained with ethidium bromide (0.5 mg/ml). Amplified products (10 μL) were digested for 4 h at 65°C using 10 units of Taq1 (*Amersham-Pharmacia, Amersham, U.K*) per reaction. Digested products were electrophoresed on a 2% agarose gel and visualised using ethidium bromide. The 16974 C allele yielded two fragments, 906 bp and 140 bp, respectively. Ethidium bromide and agarose were supplied by *Sigma Chemical Co*. The100 bp DNA marker ladder was supplied by *Amersham-Pharmacia, Biotech*.

Statistical analysis

Statistical analyses were performed using StatSoft version 6. Comparison of the distribution of the genotypes for the IL12 p40 SNP were performed using the chi-square test. A p-value < 0.05 was considered statistically significant.

RESULTS

PCR amplification products, 1046 bp fragments, were digested by Taq1 restriction enzyme and produced two fragments containing allele “C”. Electrophoresis pattern of three genotypes: AA, AC and CC are presented in **Figure 1**. Heterozygous individuals show three bands: 1046bp, 906bp and 140bp. (**Figure 1**).

The genotype and allelic frequencies of the IL-12 p40 gene polymorphism in Bulgarian citizens are shown on **Tables 1** and **2**, respectively.

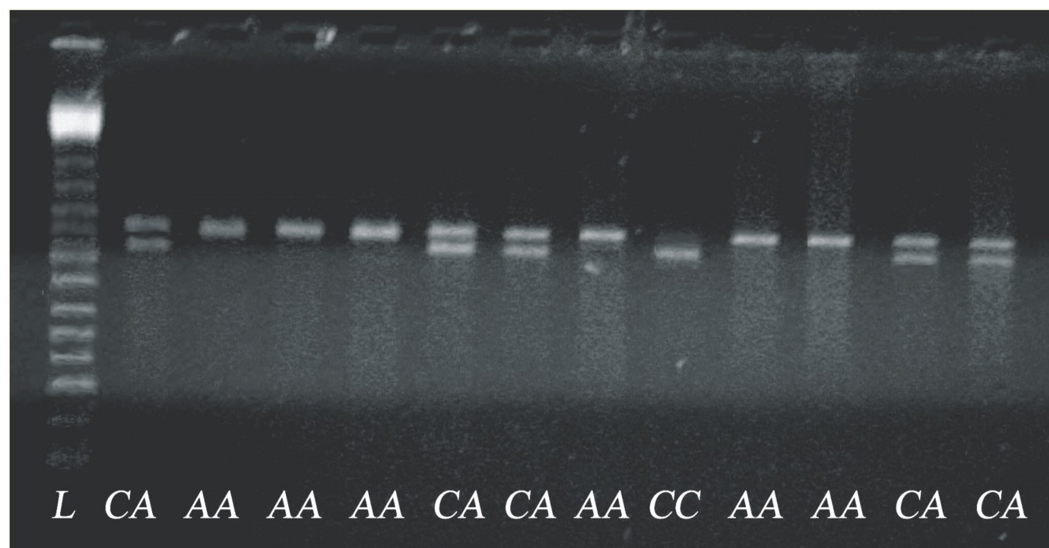

Figure1. Representative result of PCR-RFLP analysis of Taq1 SNP in the 3'-UTR of the IL-12B.
The PCR products were digested by Taq1 and electrophoresed on 2% agarose gels.
AA indicates absence of the Taq1 restriction site; CC indicates homozygosity for the presence of the Taq1 restriction site, and CA indicates heterozygosity. L- indicates the100 bp DNA marker ladder

Table 1: Genotype frequencies of the IL-12 p40 gene polymorphism in Bulgarian citizens

Genotype	Bulgarians, n = 58	Turkish minority, n = 33	Total group of Bulgarian citizens, n = 91
AA, n (%)	36 (62)	20 (61)	56 (61)
CA, n (%)	19 (33)	10 (30)	29 (32)
CC, n (%)	3 (5)	3 (9)	6 (7)

Table 2: Allelic frequencies of the IL-12 p40 gene polymorphism in Bulgarian citizens

Allele	Bulgarians, n = 58	Turkish minority, n = 33	Total group of Bulgarian citizens, n = 91
A	0.78	0.76	0.77
C	0.22	0.24	0.23

No obvious genotype frequency differences were observed between Bulgarians and their Turkish minority. However, 62 % of Bulgarians had the genotype AA *vs*. 61% of their Turkish minority (p=0.8903), heterozygous genotype CA 33% *vs*. 30% respectively (p-value 0.8090). Three subjects from each ethnic subgroups had the genotype CC and the genotype distribution was 5% *vs*. 9%; p-value = 0.4690 (**Table 1**).

Comparisons on **Table 2** show that the allelic frequencies did not show significant differences between the two subgroups for the IL-12 p40 polymorphism (p=0.7368).

We compared the genotype and allelic distribution depending on the sex in both subgroups, but no significant deference was observed in each (data not shown).

DISCUSSION

Post-transcriptional regulation and the formation of mRNA 3’ ends are crucial for gene expression in eukaryotes. Interspecies conservation of many sequences within the 3’-UTRs reveals selective constraints due to similar function. Several SNPs in the 3’-UTR have been reported recently, one of which is SNP studied in this work - C/A polymorphism at position +16974 in the 3'-UTR of the IL-12p40 gene (9, 10).

A wide array of studies has further demonstrated differences in genotype and allelic frequencies of cytokine gene polymorphisms depending on ethnicity and race (11, 12, 13, 14).

Our study group comprised 91 healthy volunteers from two ethnic subgroups - Turkish minority in Bulgaria and Bulgarians. Bulgaria had been under Turkish rule for five centuries. In 1878, Bulgaria became an independent country and, expectedly, many Turkish families continued to live in Bulgaria, especially in South Bulgaria that formed source of our Turkish minority in this study. Against this background, it became a matter of interest to determine whether there could be differences in genotype and allelic frequencies of Taq1 polymorphism in the 3'-UTR of the IL-12 p40 between Bulgarians and their Turkish minority. Our results, however, did not support this hypothesis. Comparisons of genotype distribution, as well as allelic distribution using chi square test, showed no significant difference between these two subgroups. Our findings are somewhat close to the results obtained by Yilmaz and colleagues, who had demonstrated that the frequency of the AA genotype of IL-12 p40 was 55.3% in 101 healthy Turkish donors (15). However, we observed a higher frequency of the AA genotype (61%) in the studied group of Turkish minority in Bulgaria. These differences might be due to limited number of investigated groups and to the fact that Turkish and Bulgarian populations in Bulgaria have intermarried over time. These circumstances could lead to equation of genotype and allelic frequencies at the IL-12 p40 +16974 polymorphic site between Bulgarians and Turkish minority in Bulgaria. Perhaps, further parallel genetic studies are required in both populations. Based on our results, the groups of Turkish minority in Bulgaria and Bulgarians can be included in a total group from Bulgarian population.

The genotype and allelic frequencies of SNP in the 3'-UTR of the IL-12 p40 gene have been investigated in different populations and races. Huang et. al. have investigated a large group of unrelated individuals of diverse European descent, and reported the “A” allele having a frequency of 0.82 (3). Hall et. al have reported 0.80 frequency of the “A” allele (4). Others have reported similar frequencies of “A” allele – 0.81, from 145 healthy donors matched for age, sex and ethnicity in The Netherlands, and from 237 healthy donors from The Denmark (16, 17). In one hundred normal healthy Caucasian individuals from the Northern Ireland population the same frequency of “A” allele was also reported (18). In the group of 157 Caucasian UK donors Panagiota Latsi et al. observed that the allelic frequency of “A” allele in the IL-12 p40 gene was 0.77.

Considering our present results, the second conclusion to be drawn is that the allelic frequency of the “A” allele in 91 Bulgarian citizens (0.77) was similar to frequencies reported for other populations from the Caucasian race (0.77 - 0.82).

However, the remaining Bulgarian cytokine allelic frequency was distinctive when compared to other populations. Tsunemi et.al reported 0.50 allelic frequency of rarer “C” allele among 100 healthy controls from the Japanese population (5). This great difference was not surprising. The allele, genotype and haplotype frequencies in the Japanese population of many polymorphisms are remarkably different from those in the Caucasian population. For example, the frequency of 0.21-0.23 of the ATA haplotype of IL-10 promoter polymorphism has been described in population from the UK and Poland (20, 21). On the other hand, a higher frequency of the ATA haplotype (0.71) has been reported in the Japanese population (22).

In conclusion, we have evaluated the distribution of single nucleotide polymorphism in the 3'-UTR region of IL-12 p40 gene in two ethnic subgroups of Bulgaria. Our results seem to suggest that Bulgarians and their Turkish minority have no genetic difference in the 3'-UTR region of IL-12 p40 gene polymorphism. The data may facilitate the investigations of cytokine polymorphisms and differences in immune response between ethnic groups and populations.

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