



## ASSOCIATION OF CYTOKINE GENE POLYMORPHISMS WITH ATOPIC DERMATITIS

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### ABSTRACT

**PURPOSE:** The present study examine the influence of *IL12Bpro* polymorphism, A/C substitution in 3'UTR of *IL12B* and A/G substitution in the promoter of *IL10* on susceptibility to atopic dermatitis (AD) among Bulgarian patients. **METHODS:** Fifty three patients with AD and 206 healthy controls were included in the present study. Genotyping of the *IL12B* A/C polymorphism in 3'UTR was performed by restriction fragment length polymorphisms-PCR assay and for the *IL12Bpro* polymorphism and -1082A/G polymorphism in *IL10* gene by allele specific- PCR. **RESULTS:** The genotype distribution of all investigated polymorphisms (*IL12Bpro* polymorphism, A/C substitution in 3'UTR of *IL12B* and -1082A/G substitution in promoter of *IL10*) among AD patients was similar to that observed in the control group. Respectively, the allele frequencies did not show significant difference between cases and controls. Lack of association between the genotypes of three studied polymorphisms and susceptibility to AD in investigated Bulgarian population was observed. **CONCLUSIONS:** In our present case-control study, we report for the first time that the *IL12Bpro* polymorphism was not associated with AD development. Also, our study suggests that the analyzed genetic polymorphisms in cytokine genes do not appear to be associated with AD susceptibility in our Bulgarian population.

**Key words:** atopic dermatitis; cytokine polymorphism, *IL12B*, *IL10*

### INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease resulting from a complex interaction of environmental factors, genetic predisposition and immunological pathogenesis. One potential immunological mechanism for the development of AD is that the regulation of Th1/Th2 response may be insufficiently balanced. In addition to the effector Th cell subsets (Th1 and Th2), T cells with immunoregulatory properties exist and these are broadly referred as Treg. Treg have the ability to control and modify the development of allergic diseases altering the

ongoing sensitization and effector phases via several major pathways including secretion of cytokine IL-10 and TGF- $\beta$  (1). Many other cells also produce IL-10, including helper and cytotoxic T cells, B cells, and keratinocytes (2). Overexpression of IL-10 in atopic dermatitis has been long known (3; 4) and functional polymorphisms in *IL10* could have a great impact on the development of AD. In the promoter region of *IL10* have been described several single nucleotide polymorphisms (SNP) including a substitution of A to G at -1082 position (rs1800896). In this region of *IL10* promoter is located a binding motif of transcription factors PU.1, Spi-B and Sp1. Also, it was shown that binding of PU.1 transcription factor leads to a decreased transcriptional activity of -1082A (5), and Sp1 transcription factor binds only to the -1087 position in the presence of a G allele, enhances transcription activity and promotes

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IL-10 mRNA transcription and protein production (6). Although the functional significance of this polymorphism is well known, the studies investigate the association of -1082A/G polymorphism and AD are limited. Most of them investigated the effect of haplotypes of several polymorphisms in 5'UTR of *IL10* (7, 8) on the development and clinical phenotype in AD. In contrast, the individual effect of -1082A/G polymorphism on susceptibility to AD was explored in limited number of studies (9; 10).

IL-12 is a heterodimeric cytokine consists of two disulfide-linked subunits designated as p35 and p40, encoded from *IL12A* and *IL12B* genes, respectively. IL-12p70 regulates Th1 differentiation, promotes cell-mediated immune response and antagonizes Th2 differentiation including production of Th2 cytokines. In addition, IL-12p40 subunit can be secreted as monomer, homodimer (IL-12p80) or can form other heterodimeric pro-inflammatory cytokine IL-23, with p19 subunit. IL-12p40 containing cytokines possess related, but distinct biological activities (11; 12). Two functional polymorphisms in the *IL12B* gene have been described. A single-nucleotide polymorphism (SNP) in 3'-untranslated region (UTR) of *IL12B* with number rs3212227 and a complex polymorphism in promoter region of the *IL12B* (*IL12Bpro*), resulting from 4bp microinsertion combined with an AA/GC transition (rs17860508). Although, many studies have demonstrated the functional role of these polymorphisms and their effect on IL-12p40 production (13-15), so far only one study has explored the role of 3'UTR polymorphism in *IL12B* on AD susceptibility among patients from Asian population (16). The role of other functional polymorphism in promoter region of the same gene *IL12Bpro* for pathogenesis of AD was not investigated.

The aim of the present study was to examine the influence of *IL12Bpro* polymorphism, a A/C substitution in 3'UTR of *IL12B* and A/G substitution in promoter of *IL10* on susceptibility to AD among Bulgarian patients.

## MATERIALS AND METHODS

**Study Subjects:** Fifty three patients with AD were included in the present study. Patients, attending to the Clinic of dermatology and venerology, University Hospital, Faculty of Medicine, Trakia University, Stara Zagora,

Bulgaria, between October 2007 and December 2009 were selected. The diagnosis Atopic dermatitis was set by the criteria of Hanifin and Rajka (17). The group of AD patients was consisting of 19 (36%) male and 34 (64%) female with the mean age 29 years (range: 2÷80 years). Disease history and informed consent was taken from patients or their parents. Patients who received phototherapy or oral immunotherapy (e.g. corticosteroids) were excluded. Individuals with any other autoimmune, allergic, parasitic or dermatologic diseases were also excluded.

Two hundred and six unrelated, healthy individuals with no personal history of atopic disease were recruited as control subjects. The group of control subjects was consisting of 80 (39%) male and 126 (61%) female with the mean age 44 years (range: 7÷81 years). This study was conducted according to the ethical standards of the Ethics Review Board of the Faculty of Medicine, Trakia University, Stara Zagora

**DNA extraction:** Genomic DNA was extracted from EDTA-blood probes using illustra blood genomicPrep Mini Spin Kit (GE Healthcare, Austria) and stored at -20°C until use.

### Genotyping of -1082A/G SNP in *IL10*:

Genotyping of the -1082 G/A polymorphism in promoter region of the *IL10* was also performed with ARMS- PCR methodology. The sequences of used primers were: *IL10G* 5'-CCT ATC CCT ACT TCCC CC-3', *IL10A* 5'-CCT ATC CCT ACT TCCC CT-3' and *IL10* generic primer 5'-AGC AAC CAC TCC TCG TCG CAA C-3'. PCR amplification was carried out in 20 µL volumes containing 10x PCR buffer, 0.5 U Taq polymerase, 2.5 mmol/l MgCl<sub>2</sub>, 100 µmol/l of each dNTP, 0.4 µmol/l of each primer. The cycling parameters were as follows: initial denaturation step of 10 min at 95°C; 30 cycles of 30 sec at 94°C; 1 min at 60°C and 1 min at 72°C and final extension step of 7 min at 72°C.

### Genotyping of A/C SNP in 3'UTR of *IL12B*:

Genotyping of the *IL12B* A/C polymorphism in 3'UTR was performed by restriction fragment length polymorphisms (RFLP) - PCR assay after amplification of 1046bp fragment with forward primer: 5'-ATT TGG AGG AAA AGT GGA AGA-3' and reverse primer: 5'- AAT TTC ATG TCC TTA GCC ATA-3'. PCR amplification was carried out in 20µl

volumes containing 10xPCR buffer, 0.5U Taq polymerase, 3.0 mmol/l MgCl<sub>2</sub>, 100 μmol/l of each dNTP, 0.4 μmol/l of each primer. The cycling parameters for *IL12B* A/C SNP in 3'UTR were as follows: initial incubation step of 5 min at 95°C; 30 cycles: 1 min at 94°C, 1 min at 54.3°C, and 2 min at 72°C and a final extension step of 7 min at 72 °C completed the reaction. Amplified products (10μl) were digested using 10U TaqI (Fermentas) per reaction for 4h at 65°C. The +16974C allele yields two fragments, 906bp and 140bp, respectively.

**Genotyping of *IL12Bpro* polymorphism:** Genotyping of the *IL12Bpro* polymorphism was performed by amplification refractory mutation system (ARMS) - PCR. The sequences of used primers were: CTCTAA allele, marked as *IL12Bpro-1*: 5'-TGT CTC CGA GAG AGG CTC TAA -3'; GC allele, marked as *IL12Bpro-2*: 5'- TGT CTC CGA GAG AGG GCT GT-3' and *IL12Bpro* generic primer 5'-TGG AGG AAG TGG TTC TCG TAC-3'. PCR amplification was carried out in 20μl volumes containing 10xPCR buffer, 0.5U Taq polymerase, 1.5 mmol/l MgCl<sub>2</sub>, 100 μmol/l of each dNTP, 0.4 μmol/l of each primer. The cycling parameters for *IL12Bpro* polymorphism were as follows: initial denaturation step of 15min at 95°C; 30 cycles of 30 sec at 95°C; 30 sec at 65°C and 30 sec at 72°C and final extension step of 7 min at 72°C. PCR amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) using PCR reagents supplied by Fermentas (Lithuania). Primers were supplied by LKB Vetriebs GmbH (Vienna, Austria). PCR products and restriction fragments were visualized on an agarose gel stained with ethidium bromide (0.5 mg/ml) supplied by Sigma-Aldrich Inc., St Louis, USA (**Figure 1**). In each PCR run, heterozygous control template was used to

ensure accuracy. For quality control, 10% of random selected samples containing both cases and controls were analyzed a second time without finding any discrepancies.

**Statistical analysis:** The differences in genotype distribution and allele frequency among cases and controls were analyzed using the  $\chi^2$  test. When 2x2 tables contain only small observed frequencies Yates' corrected p-value (noted as pc) were taken into account. StatPages.net web site (<http://statpages.org/index.html>) was used to estimate odds ratios (OR), expressed with their 95% confidence intervals (95%CI) for disease susceptibility in relation to investigated polymorphisms. The goodness of fit to Hardy-Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values for patients and healthy controls, was performed using a  $\chi^2$  test. In all cases, two-tailed p-values less than 0.05 were considered as significant.

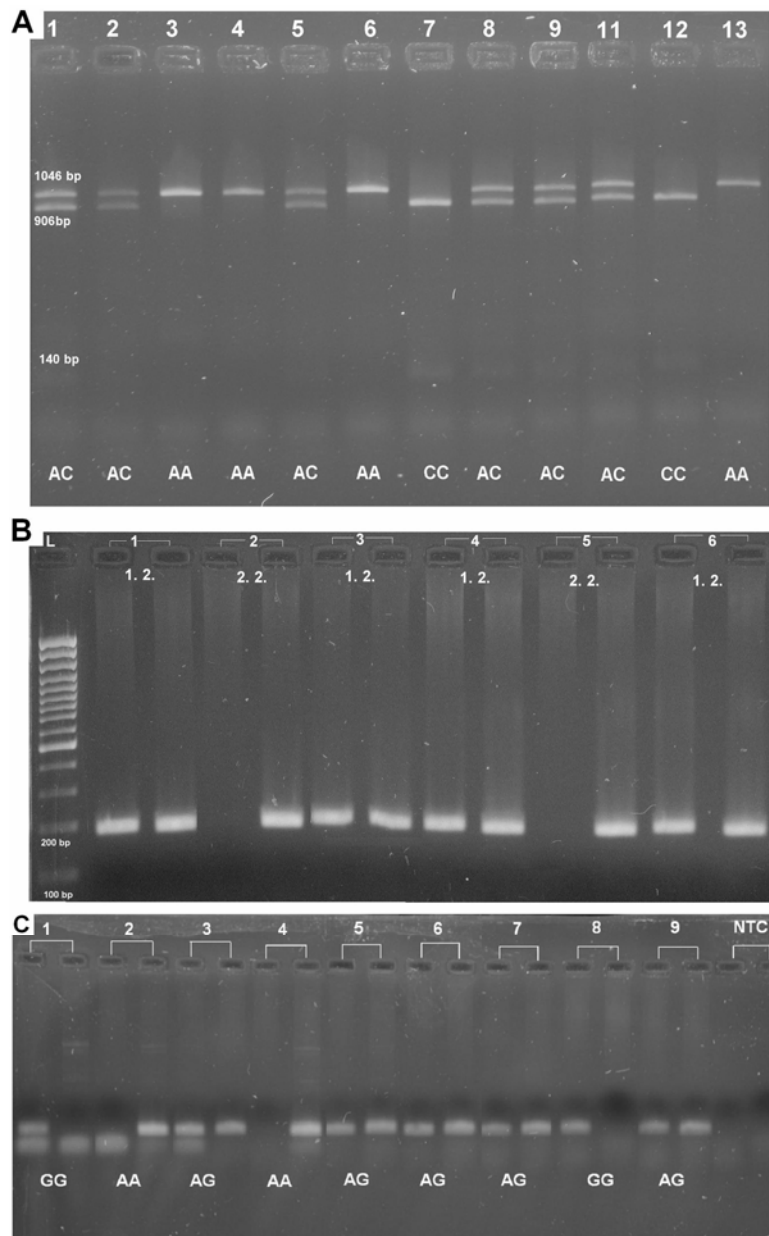
**RESULTS**

**Association of -1082A/G SNP in *IL10* with susceptibility to AD:** The genotype distribution of -1082A/G SNP in *IL10* among AD patients and healthy control was shown in **Table 1**. The observed genotypes in both groups did not deviate from the Hardy-Weinberg equilibrium (p>0.6). No significant differences in genotype frequency were detected between AD patients and healthy control ( $\chi^2 = 2.235$ ; df = 2; p =0.327).

In addition, the allele frequency of A-allele (65%) and G-allele (35%) in AD patients was similar to that observed in healthy controls (61% and 39%, respectively). The OR for the variant allele-G was 0.829 (95% CI: 0.518÷1.327; p=0.436).

**Table 1.** Genotype distribution of -1082A/G SNP in *IL10* among AD patients and healthy control

	AD patients n (%)	Healthy controls n (%)	OR (95 CI%)	p-value
<b>AA</b>	24 (45)	44 (35)	1.542 (0.806÷2.952)	0.192
<b>AG</b>	21 (40)	65 (51.5)	0.616 (0.322÷1.177)	0.144
<b>GG</b>	8 (15)	17 (12.3)	1.140 (0.469÷2.779)	0.778



**Figure 1.** A) 2% agarose gel electrophoresis of RFLP-PCR assay used for genotyping of A/C SNP in 3'UTR of *IL12B*; B) 1.5% agarose gel electrophoresis of PCR products after ARMS-PCR used for genotyping of *IL12Bpro* polymorphism C) 2% agarose gel electrophoresis of PCR products after ARMS-PCR used for genotyping of -1082A/G SNP in *IL10*. L- 100bp ladder, NTC- non template control

**Association of A/C SNP in 3'UTR of *IL12B* with susceptibility to AD:** The genotype distribution of A/C SNP in 3'UTR of *IL12B* among AD patients and healthy control was shown in **Table 2**. The observed genotype frequencies in both groups were similar to the expected according the Hardy-Weinberg equilibrium ( $p > 0.2$ ). There was no association between the genotypes of A/C SNP in 3'UTR of *IL12B* and susceptibility to AD in the investigated Bulgarian population. The

distribution of genotypes among patients was similar to that observed in the control group ( $\chi^2 = 3.442$ ;  $df = 2$ ;  $p = 0.179$ ).

Also, there were no significant differences between observed allelic frequencies in cases and controls ( $p = 0.975$ ). Compared the allelic frequencies between AD patients and healthy controls, the OR value was close to 1 (0.992 with 95% CI: 0.597÷1.649).

**Association of *IL12Bpro* polymorphism with susceptibility to AD:** The genotype distribution of *IL12Bpro* polymorphism among AD patients and healthy control was shown in **Table 3**. The observed *IL12Bpro* genotypes distribution was consistent with that expected under Hardy Weinberg equilibrium among AD patients ( $p=1$ ) and healthy controls ( $p=0.49$ ). The distribution and the frequencies of the *IL12Bpro* genotypes among the AD patients were the following: 14 (0.26) genotype-11, 26 (0.49) heterozygous genotype-12, and 13

(0.25) genotype-22. In comparison with genotype distribution in control group there was no significant differences ( $\chi^2 = 1.145$ ;  $df = 2$ ;  $p = 0.564$ ). Respectively, there were no significant differences between observed allelic frequencies in AD patients and healthy controls (OR=0.798 with 95% CI: 0.521÷1.222,  $p=0.3$ ).

	AD patients n (%)	Healthy controls n (%)	OR (95 CI%)	p-value
AA	30 (57)	124 (62)	0.779 (0.435÷1.469)	0.474
AC	22 (41)	62 (31)	1.580 (0.852÷2.932)	0.149
CC	1 (2)	14 (7)	0.255 (0.042÷1.565)	0.283 pc

**Table 2.** Genotype distribution of A/C SNP in 3'UTR of *IL12B* among AD patients and healthy control

**Table 3.** Genotype distribution of *IL12Bpro* polymorphism among AD patients and healthy control

	AD patients n (%)	Healthy controls n (%)	OR (95 CI%)	p-value
Genotype - 11	14 (26)	70 (34)	0.745 (0.382÷1.455)	0.392
Genotype - 12	26 (49)	93 (45)	1.001 (0.550÷1.823)	0.997
Genotype - 22	13 (25)	43 (21)	1.437 (0.708÷2.922)	0.321

## DISCUSSION

This is the first study which investigates the distribution of *IL12Bpro* polymorphism among patients with AD. Additionally, this study analyzed the associated of two other polymorphisms (SNPs in 3'UTR of *IL12B* and in promoter region of *IL10*) with susceptibility to disease among Bulgarian population.

The etiology of AD is complex and involves multiple immunological, genetics and environmental factors, and interaction among them. Various studies investigate the genetics to susceptibility to AD and many candidate genes were identified as risk factors. Some of these genes encoded proteins which forms a functional barrier at the skin surface (filaggrin), proteins involved in innate immune response (monocyte differentiation antigen CD14, Toll-like receptor 2), or adaptive immune response (GATA-binding protein 3, IL- 4, IL-10) (18). In our current study, we explored the hypothesis that three polymorphisms in genes of cytokines involved in innate and adaptive immune response could have a significant effect on susceptibility to AD. However, our results showed no

significant association of two polymorphisms in *IL12B* gene (A/C SNP in 3'UTR and *IL12Bpro* polymorphism) and one in *IL10* (-1082 A/G SNP) with susceptibility to AD.

The -1082A/G SNP in *IL10* gene is widely studied as a genetic factor involved in AD development (7-10; 19; 20). Our results are in accordance with some studies which were conducted among Germany (10) and British AD patients (9; 7) and no positive association between -1082A/G SNP and AD susceptibility was reported. The same polymorphism in *IL10* was not found to be associated with a predisposition to AD in Asian populations (20; 19). However, Sohn et al (20) have shown that different polymorphisms in the IL-10 gene at position -819 and -592 and the ATA haplotype are associated with AD phenotypes in Korean children. Respectively, we could not exclude the role of -1082A/G SNP in promoter region of *IL10* as a gene modifier of the severity and clinical phenotype of AD. In our present study, it is not possible to elucidate definitively whether carrying current genotype or allele of -1082A/G SNP in *IL10* correlates with clinical

phenotype of AD, and future study will be needed to investigate this association.

Additionally, our present results demonstrated that both studied *IL12B* gene polymorphisms in regulatory regions do not constitute a genetic risk factor for the susceptibility to AD. According to our knowledge, till now, there has been only one report in the literature which investigates A/C SNP in 3'UTR of *IL12B* in Asian population (16) and no study was focused on the *IL12Bpro* polymorphism as a genetic predisposition factor for AD. Tsunemi et al (16) have shown a significant elevation of A-allele of A/C SNP in 3'UTR of *IL12B* in AD patients compared to healthy controls or psoriasis patients. They have suggested that A-allele promotes enhanced IL-12p40 production which results in increased IL-12p70 production and affect Th1/Th2 cytokine balance. However, we failed to detect a significant association between A/C SNP in 3'UTR of *IL12B* and AD susceptibility. Although our results seem to be contradictory to those by Tsunemi et al (16) a proper explanation could be the well known racial/ethnic genetic differences. In support of this view is the fact that the reported frequency of genotype-CC (27%) among 100 control subjects investigated by Tsunemi et al (16) is quite different from that observed in our cohort of 200 controls (7%).

Also, we have present for the first time, that there was no significant difference between genotype distribution or allelic frequencies of other functional polymorphism in the same gene, *IL12Bpro* polymorphism, in AD patients and healthy controls. The lack of significant differences showed that *IL12Bpro* polymorphism does not contribute to AD susceptibility. However, the observation that certain genotypes of *IL12Bpro* polymorphism are seen in association with asthma susceptibility or severity (Khoo, 2004; Hirota 2005) indicates that this polymorphism could have an impact in immunopathogenesis of allergic diseases. Respectively, our present results need to be confirmed among AD patients from Caucasian population.

In conclusion, we reported for the first time, that the *IL12Bpro* polymorphism was not associated with AD development among Bulgarian patients. In addition, we could suggest that other two analyzed polymorphisms -1082A/G SNP in *IL10* and

A/C SNP in the 3'UTR of *IL12B* were also not associated with AD susceptibility and do not play an important role in the development of AD in Bulgarian population.

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