Association of SNP in the IL-4, IL-18 and eotaxin genes with asthma in a Jordanian population

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Abstract
The association of polymorphisms in IL-4 (Th2 cytokine), IL-18 (Th1 cytokine) and eotaxin (eosinophil chemoattractant) with asthma and allergic diseases genes is thoroughly investigated. This study aimed to reveal the single nucleotide polymorphisms (SNPs) in IL-4 (-590C>T), IL-18 (-137G>C), and eotaxin (-384A>G) genes and their possible association with asthma in Jordanian population. Results showed that GG homozygous genotype of the eotaxin gene promoter at -384 position as well as the frequency of the mutant allele were significantly higher among asthma patients compared to control subjects. However, no significant differences in either the genotype distribution or the allelic frequencies of the IL-4 and IL-18 gene polymorphism were observed between asthma patients and controls. From the genotypes of IL-4, IL-18 and eotaxin polymorphisms, 27 genotypes were deduced, of which only 13 combinations appeared in the study. The genotype combination frequencies of asthma patients are quite similar to those observed in healthy controls. Only the genotype combination CT GG GG which is composed of the heterozygous genotype of IL-4 CT, the normal genotype of IL-18 GG and the homozygous genotype of eotaxin GG was preferentially transmitted to the asthmatic children. The results of this study suggest a possible association of asthma with the eotaxin -384A>G. Moreover, the genotype combination CT GG GG, may be one of the factors that participate in the pathogenesis of asthma or its intermediary phenotypes.

Keywords: IL-4; IL-8; eotaxin; asthma.

INTRODUCTION

Asthma is one of the most common chronic disorders affecting children and young adults in industrialized countries (Palmer et al., 2001). There has been a dramatic increase in the prevalence of asthma over the last few decades (Arshad, 2005). It has become an epidemic affecting 155 million individuals in the world. The cost of this disease is substantial, and the market for the pharmaceutical industry is estimated at 5.5 billion/year (Palmer and Cookson, 2000). Both environmental and genetic factors contribute to the inception and evolution of asthma (Renaulds 2001). Although environmental factors are known to contribute to development of the disease, epidemiological studies point towards a strong genetic influence (Busse and Lemanske, 2001).

It is a commonly held view that asthma is caused by multiple interacting genes, where some have a protective effect and others contribute to the pathogenesis of disease, however, each gene has its own tendency to be influenced by the environment. Thus, the complex nature of the asthma phenotype, together with substantial locus heterogeneity and environmental influence has made it difficult to unravel the genetic factors that underline asthma (Hakonarson et al., 2002). More than 100 linkage and association studies have reported more than 500 atopy and asthma loci throughout the genome. However, none of the linkage studies found loci that meet reasonable criteria for significance and only a few of the association studies reported, qualify for possible significance (Hakonarson et al., 2001). At least five regions in the human genome (chromosomes 5, 6, 11, 12 and 13) contain genes consistently found to be associated with asthma, but several other regions (chromosomes 2, 7, 14, 19, 17 and 21) could also contain candidate genes (Hakonarson et al., 2001).
Asthma and allergic diseases are thought to be due to an imbalance between Th1 and Th2 branches of the immune system, which are responsible for allergic inflammatory reactions and delayed hypersensitivity reactions respectively (Peters, 2003). Cytokines play a crucial role in the balance between Th1 and Th2 immune responses (Renaudls, 2001). In recent years polymorphisms in various cytokine genes have been identified and an indication of functional relevance exists for some of them. They have been associated with atopic disorders such as hay fever, asthma, eczema or elevated IgE level (Liang et al., 2005; Imboden, 2006a; Raby, et al., 2006).

Numerous studies have shown the association of polymorphisms in IL-4 (Th2 cytokine), IL-18 (Th1 cytokine) and eotaxin (eosinophil chemotactrant) genes with asthma and allergic diseases in many populations (Bagheri et al., 2006), but no previous study has reported the association between these polymorphisms and asthma in Jordan. Because of that, the aim of this work was to study the status of IL-4 (-590C>T), IL-18 (-137G>C), and eotaxin (-384A>G) polymorphisms and their potential association with asthma in Jordanian population.

SUBJECTS AND METHODS

Subjects

A total of 40 diagnosed asthmatic children (22 males and 18 females) and a total of 40 age matched control subjects (25 males and 15 females) were included in this study. All children were chosen from those visiting the pediatric clinic at Princess Rahmah hospital in northern Jordan. The asthmatic children are patients who suffer from cough, wheezing and shortness of breath, and they are visiting the emergency unit for nebulization with typical symptoms of asthma. The control subjects are healthy children who don’t have any history of asthma and do not suffer from any diseases at the time of visit and blood collection. Families were informed about the study and gave consent to be involved in the study.

Blood collection

Two to 5 milliliters of peripheral blood samples were collected by specialized nurses in the children clinic and stored at -20°C until DNA extraction.

Genomic DNA extraction

The genomic DNA from the blood subjects was extracted using a genomic DNA extraction kits; Generation Capture Column Kit (Gentra, USA) as per manufacturer procedure. The quality of the extracted DNA was verified by running on 1.5% agarose gel and detected by standard ethidium bromide staining. The DNA samples were then stored at 4°C until needed.

Polymerase chain reaction with sequence-specific primers (PCR-SSP)

IL-4 (-590C>T) and IL-18 (-137G>C) genotyping was performed using polymerase chain reaction with specific primers (PCR-SSP) assay, which uses identical amplification and detection conditions, enabling rapid and cost-efficient analysis of polymorphisms (Bagheri et al., 2006). This technique utilizes sequence specific primers with 3'-end mismatches and identifies the presence of specific allelic variants through the PCR amplifications (Janssen, et al., 2004). For the IL-4 (-590C>T) SNP, PCR was performed using a common reverse primer, 5'-TTT GCA TAG AAG GGA GAG GCC-3', and two sequence specific forward primers, 5'-CTA AAC TTG GGA GAA CAT TGT CC-3' and 5'-CTA AAC TTG GGA GAA CAT TGT CT-3'. PCR gives an amplification product of 251 bp. A control forward primer, 5'-AAC TAG GCC TCA CCT GAT ACG-3', was used to amplify a 291 bp fragment covering the polymorphic site to serve as an internal positive amplification control (Mullighan., 1999). PCR for the polymorphism IL-18 (-137G>C) was performed using a common reverse primer, 5'-AGG AGG GCA AAA TGC ACT GG-3' and two sequence-specific forward primers, 5'-CCC CAA CT TTA CGG AAG AAA AG-3' and 5'-CCC CAA CT TTA CGG AAG AAA AAC-3' were used to amplify a 261 bp PCR product. A control forward primer, 5'-CCA ATAGG CTG ATT CCG CA-3', was used to amplify a 446 bp fragment covering the polymorphic site as an internal positive amplification control (Boraska et al., 2006). All PCR reactions were performed in a total volume of 25 μl, containing 2 μl genomic DNA, 12.5 μl 2X PCR Master Mix (Go Taq® Green Master mix, Promega, USA ) , 0.25 μM of the control forward primer and each sequence specific primer and 0.5 μM of common reverse primer. PCR reactions were performed in thermocycler (Biometr, Germany) according to the following cycling conditions:

For IL-4 (-590C>T) SNP were 1 min at 95 °C, followed by five cycles for 25 s at 95 °C, 45 s at 70 °C, 45 s at 72 °C, and 21 cycles of 25 s at 95 °C, 45 s at 65 °C, and 50 s at 72 °C, followed by four cycles of 95 °C for 25 s, 55 °C for 60 s and 72 °C for 2 min. The cycling conditions for IL-18 (-137G>C) SNP were 2 min at 94 °C, followed by five cycles for 20 s at 94 °C, 1 min at 68 °C and 25 cycles of 20 s at 94 °C, 40 s at 62 °C, 40 s at 72 °C and a final elongation at 72 °C for 5 min. All PCR products generated for the two polymorphisms were separated in 1.5% agarose gels stained with ethidium bromide. Gels were visualized under UV transillumination and documented by BioDocAnalyze (Biometr, Germany) imaging system.
Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

The subjects were genotyped for eotaxin (-384 A>G) polymorphism by means of PCR-RFLP. This involved a combination of PCR amplification and digestion with restriction endonuclease Taq I (Promega, USA) followed by gel electrophoresis analysis.

The region of interest was amplified with a primer pair (5'-GGT TTC CTT GCT CCT TTC CTC-3' (forward), and 5'-GCA GAA CAG AAG AGA GAG GCA A-3' (reverse)) and the resulting PCR product was 204bp (Tsunemi et al., 2002). The original sequence at position -410–-387 was introduced in the upstream primer in order to obtain the restriction site for Taq I, which can cleave the PCR product into two fragments: 187bp and 17bp. The presence of polymorphism (G allele) abolishes the cleavage site for Taq I. The restriction digestion was then allowed to proceed for 3 h at 65 °C. The generated fragments were separated on 15% acrylamide gel with ethidium bromide staining and visualized in UV light.

The PCR reactions were carried out in a total volume of 50 μl containing 4 μl of genomic DNA, 12.5 μl 2X PCR Master Mix and 0.2 μM of each primer. The cycle conditions consisted of an initial 5 min denaturation at 94°C, and then 40 cycles at 95°C, 1 min annealing at 55°C, and 1 min extension at 72°C followed by 7 min extension at 72°C. The resulted PCR product was verified using a 1.5% agarose gel after electrophoresis and ethidium bromide staining. The restriction digestion was then allowed to proceed for 3 h at 65 °C. The generated fragments were separated on 15% acrylamide gel with ethidium bromide staining and visualized in UV light.

Statistical analysis

Data were analyzed with SPSS 13.0 software. Comparison of allelic frequencies and genotypes among groups, and association of the three polymorphisms with asthma were examined for statistical significance with Chi square ($\chi^2$) test. The quantification of the relative risk, the odds ratio (OR) and the confidence interval (CI) were calculated at the 95% level. Statistical significance was assumed for $P$ values less than 0.05.

RESULTS

Detection of the IL-4 (-590C>T) polymorphism

The frequencies of the different genotypes for IL-4 (-590C>T) among asthma patients and healthy control subjects are summarized in Table 1 [Supplementary data]. As shown in the Table, the genotype frequencies for the -590 bp SNP in asthma patients were as follows: CC in 31 patients (77.5%) and CT in 9 patients (22.5%). Whereas in control subjects the genotype frequencies were: CC in 33 subjects (82.5%) and CT in 7 subjects (17.5%). The homozygous genotype TT was missing among the population under study. The frequency of the heterozygous genotype tended to be higher in asthma patients compared to control subjects but this was not statistically significant ($\chi^2 = 0.313, P = 0.390$). Distribution of allelic frequencies of IL-4 (-590C>T) among the asthma patients and the healthy control subjects are presented in Table 2 [Supplementary data]. The T allele frequencies were slightly higher in the asthmatic group (11.25%) than the control group (8.75%), which was not statistically significant ($\chi^2 = 0.278, P = 0.690$). Representative PCR products of the IL-4 gene at the position -590 is shown in figure 1.

![Figure 1](image)

**Figure 1:** 1.5% agarose gel electrophoresis of PCR products of IL-4 gene at the position -590. The 291 bp bands represent the PCR internal control amplified using the forward control primer and the common reverse primer. The 251 bp represent the PCR amplification product of the alleles C and T amplified using the sequence specific primers with the common reverse primers. M: 100bp DNA ladder; 1: heterozygous genotype CT; 2.3 and 4: normal genotype CC.

Detection of the IL-18 (-137 G>C) polymorphism

The frequencies of the three genotypes for IL-18 (-137 G>C) among asthma patients were as follows: GG in 21 patients (52%), GC in 18 patients (45%) and CC in 1 patient (2.5%). Whereas the frequencies among control subjects were: GG in 26 patients (65%), GC in 14 patients (35%). The homozygous genotype for the mutant allele did not appear in this group (Table 1). Since only one asthma patient exhibited the
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The frequencies of the different genotypes among the group of asthmatics were AA in 4 patients (10%), AG in 12 patients (30%) and GG in 24 patients (60%). However in the group of controls the frequencies of the three genotypes were AA in 10 subjects (25%), AG in 14 subjects (35%) and GG in 16 subjects (40%) (Table 1).

The frequency of the homozygous genotype was significantly higher in asthma patients than in control subjects ($\chi^2 = 4.103, P = 0.043, OR = 3.750, 95\%$ confidence interval (CI) = 1.001-14.05). The heterozygous genotype AG tended to be slightly lower in asthma patient compared with the group of controls, but this was not statistically significant ($\chi^2 = 1.172, P = 0.230$). When the Eotaxin -384 allele frequencies were analyzed, the frequency of the G allele was found to be significantly higher in asthma patients than in control subjects ($\chi^2 = 5.479, P = 0.015, OR = 2.217, 95\%$ confidence interval (CI)=1.132-4.345). The distribution of allelic frequencies of eotaxin (-384 A>G) among asthma patients and the healthy control subjects is shown in Table 2.

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Genotype combinations of IL-4 (-590C>T), IL-18 (-137G>C) and eotaxin (-384A>G)

Each SNP had two alleles and three genotypes. When combining the genotypes of the three SNPs (IL-4 (-590C>T), IL-18 (-137G>C) and Eotaxin (-384A>G)), 27 genotype combinations were possible, from which only 13 combinations appeared in the study groups (Table 3 [Supplementary data]). The genotype combination frequencies of asthma patients are quite similar to those observed in healthy controls, no statistically significant differences are reported between subjects and asthma patients. One exception is for the genotypes combination CT GG GG, which was preferentially transmitted to the asthmatic children ($\chi^2 = 5.238, P = 0.045$). This genotype combination is composed of the heterozygous genotype of IL-4 CT, the normal genotype of IL-18 GG and the homozygous genotype of eotaxin GG.

DISCUSSION

In the current study, the association of three different promoter SNPs of the IL-18, IL-4 and eotaxin, which are respectively -137G/C, -590C/T and -384A/G with asthma in Jordanian population was investigated. These three SNPs were selected in this study for their frequent investigation worldwide.

To date there have been several reports about polymorphisms of the IL-4 gene and their association with asthma. So far, IL-4 promoter polymorphism C/T at position -590 counting from the first ATG codon is the only reported polymorphism of the IL-4 gene

homozygous genotype for the mutant allele (C allele) it was included in the group of heterozygous for purposes of statistical analysis and calculations of OR. The prevalence of heterozygous was higher in asthmatic patients compared to the control subjects but this difference did not reach the statistical significance ($\chi^2 = 2.032, P = 0.362$). The C allele was higher in asthma patients compared to controls, but it was not statistically significant ($\chi^2 = 1.345, P = 0.167$) (Table 2).

The PCR amplification products of the IL-18 gene at the position -137 from some representative samples are shown in figure 2.

Detection of the Eotaxin (-384 A>G) polymorphism

The PCR amplification of the eotaxin gene at the position -384 produced a product of 204 bp that is cleaved into 187 bp and 17 bp fragments after TaqI treatment in wild type allele (Figure 3).

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promoter (Lai et al., 2006). It is known that the specific genotype (C or T) at position -590 of the open reading frame of IL-4 gene is correlated with the expression of IL-4 as well as with IL-4 mediated activity as this polymorphism results in the transition from a weak binding site for transcription factor NF-AT1 (GGAGAA) to a strong binding site (GGAAAA) and thus mediates a transcriptional up-regulation of IL-4 (Nguyen et al., 2004). Such a functional polymorphism in the IL-4 gene may elevate IL-4 levels and thereby influences the IL-4 dependant events which determines disease progression. Several recent studies have demonstrated an association of the allele T at the position -590 from the open reading frame of the IL-4 gene with asthma and atopy in different populations and ethnic groups (Rosenwasser et al., 1995; Walley and Cookson, 1996; Zhu et al., 2000; Noguchi et al., 2001; Beghe et al., 2005).

Results from the present study showed that IL-4 promoter polymorphism at position -590 do not seem to be associated with asthma in the Jordanian population. Our findings are not in accordance with the previous reports. They support many studies on different populations which failed to find strong association of this polymorphism with asthma. In a Kuwaiti cohort, Hijazi and Haidar (2000) failed to find significant association of IL-4 -590 polymorphism with asthma. This SNP was not associated with asthma also in Australian population (Elliott et al., 2001) and in Mexican population (Lopez et al., 2006).

The IL-18 promoter polymorphism (-137G>C) was suggested in many studies to impair IL-18 expression (Jang et al., 2005; Xu et al., 2007). This SNP was predicted to fall within a potential histone H4 gene transcription factor 1 (H4TF-1) binding site, with the C allele possibly demolishing the binding site of H4TF-1. The functional consequence of the resulting -137 haplotype is a dramatic repression of the IL-18 promoter (Naeimi et al., 2006). Consistently, it was shown that this polymorphism has reached significant impact on asthma pathophysiology in German (Kruse et al., 2003) and in Japanese populations (Yoshizawa et al., 2002).

In the current study, we failed to detect significant associations between IL-18 (-137G>C) polymorphism and susceptibility to asthma. This is consistent with many previous studies that showed no association with asthma in many population as German (Heinzmann., 2004), Swiss (Imboden et al., 2006b), and Korean populations (Lee et al., 2006). Our negative association study cannot exclude the significance if IL-18 variants on the atopic diseases but might be just influenced by the opposing effects of IL-18. IL-18 represents a pleitropic cytokine with varying and sometimes opposing effects in the pathogenesis of asthma (Liang et al., 2005).

In the present study we found that the frequency of asthmatic patients with the homozygous genotype for eotaxin GG was significantly higher compared with that of the controls (P = 0.043, OR = 3.75), also frequency of the mutant allele was significantly higher in the asthma group than in controls (P = 0.015, OR = 2.217). These results are in agreement with previous studies (Miyamasu et al., 2001; Chang et al., 2005; Batra et al., 2007), and suggest that the G allele might be a risk factor for asthma in this population. However, since our sample size was relatively small, association or linkage study in larger sample size is needed to confirm the obtained results. Also, future studies should include the relatives of asthma patients to be certain the association between this SNP and the disease.

It has been previously suggested that these three SNPs (IL-4 -590C>T, IL-18 -137G>C and eotaxin A>G) contribute to susceptibility to asthma or atopic asthma. But no previous study has investigated the association of combination of these three SNPs with asthma and allergic disorders. In the present study we found some association of the genotypes combination CT GG GG with asthma in this Jordanian population. As long as this study was performed in a small population, more studies in other or larger population are needed to confirm these results.

In conclusion, the genetic study of cytokines is likely to provide relevant information on their polymorphisms and a possible relationship between these polymorphisms and diverse diseases including asthma. Our understanding of this relationship may help both to explain the biological events and to indicate to clinician’s ways of predicting, preventing or managing harmful situations in diseases with immunological components. Moreover, our findings are not only of interest to asthma, but may also be of relevance to other inflammatory and autoimmune diseases. Further examination of polymorphisms in cytokine genes should enhance our understanding of the cytokine network and provides clues to the pathogenesis of multiple diseases.
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