Association of Leukotriene C4 Synthase A-444C Polymorphism with Asthma and Asthma Phenotypes in Romanian Population

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ABSTRACT

Introduction: Leukotriene C4 synthase (LTC4S) gene –444A/C polymorphism has been implicated in susceptibility to asthma, but a large number of studies have reported inconclusive results. The aim of this study was to investigate the association between the –444A/C polymorphism of LTC4S gene and asthma, asthma phenotypes (aspirin intolerant/tolerant asthma) and different characteristics of the patients.

Material and methods: We included 106 patients with asthma (60 with aspirin tolerant asthma - ATA, 46 with aspirin intolerant asthma - AIA) and 103 controls. All the subjects were genotyped for LTC4S-444 A/C by Real-Time PCR. We assessed the association of LTC4S promoter polymorphism with asthma and its phenotypes and with clinical and biological characteristics of asthmatic patients.

Results: We did not find a significant association between the studied polymorphism and asthma, but the minor allele tended to be more frequent in AIA patients. We found a significant association between the minor allele C and lower levels of serum total immunoglobulin E and eosinophils, suggesting a possible role of –444A/C LTC4S polymorphism as modulating factor of allergic inflammation in asthma.

Conclusion: The results show that LTC4S -444A/C SNP is not associated with susceptibility to asthma in Romanian patients, but could influence asthma phenotype, namely aspirin intolerant asthma.

Keywords: asthma, aspirin intolerance, leukotrienes, single nucleotide polymorphisms.
INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways influenced by genetic and environmental factors, characterized by respiratory symptoms such as dyspnea, wheezing, cough, chest tightness and sputum production. Cysteinyl-leukotrienes (cysLTs) are pro-inflammatory mediators involved in asthma pathogenesis, with an important role in the induction of bronchoconstriction, airway edema, increased mucus secretion and eosinophil infiltration (1). Leukotriene C4 synthase (LTC4S) is the rate limiting enzyme for the synthesis of cysLTs and it has been shown that the expression of LTC4S mRNA is higher in blood eosinophils from asthmatic patients than in control subjects (2). In addition, aspirin-intolerant asthma (AIA), a specific subtype of asthma affecting around 3% to 20% from adult subjects with asthma, is characterised by cysLT overproduction (3) and over-expression of LTC4S enzyme at the level of bronchial mucosa, compared with aspirin tolerant asthma (ATA) and controls (4). In AIA patients, aspirin and other nonsteroidal anti-inflammatory drugs (NSAID), non-selective inhibitors of cyclooxygenase (COX), shunts arachidonic acid metabolism toward lipo-oxygenase pathway resulting in an enhanced release of cysLTs into airways inducing bronchospasm (5). For these reasons LTC4S gene is considered as an important candidate gene for asthma.

The human LTC4S gene is located on the long arm of chromosome 5 (5q35) and spans ~2.5 kilobases. It contains five exons and four introns (3). By linkage analysis was shown that the gene is located in a region implicated in asthma, near region 5q31-33 that includes several other candidate genes for asthma (6). One important single nucleotide polymorphism (SNP) named −444A/C (rs730012) has been identified in the promoter region of LTC4S gene and it was reported as a strong risk factor for aspirin intolerant asthma in Polish population (7). Functional studies have demonstrated an increased transcription rate of this gene in case of vectors carrying the mutant allele C of the SNP (2,7). An association between −444C allele and susceptibility to asthma and/or asthma severity or other phenotypes has been described by some authors (1,8-13), but not by others (14-25). The role of the LTC4S promoter polymorphisms in AIA and ATA is still unclear, the connection of this polymorphism with asthma inflammatory process still need to be researched.

In the present study, we have evaluated the LTC4S gene polymorphism −444A/C in Romanian patients with asthma regarding its association with susceptibility to asthma, asthma phenotypes and in relation with clinical and biological features of asthma.

METHODS

Subjects and clinical assessment

We have performed a case-control association study of LTC4S gene single nucleotide polymorphism −444A/C (rs730012) in a group of Romanian asthmatic patients versus ethnically matched controls. We enrolled a total number of 106 Romanian unrelated non-smoking adult patients with a current physician diagnosis of asthma who were taking asthma medication on a regular basis, and 103 healthy controls. The asthma lot was divided in patients with aspirin tolerant asthma – ATA (60 subjects, age 42.75±15.08 years; 23 men, 37 women) and patients with aspirin intolerant asthma – AIA (46 subjects, age 44.17±15.05 years; 15 men, 31 women). The diagnosis of AIA was made on the basis of a documented history of asthma attack precipitated by NSAID administration. Patients with ATA and healthy control subjects had no history of aspirin intolerance, they took aspirin or other NSAIDs without any adverse symptoms. Asthma severity was evaluated depending on the degree of control and treatment step according to the guidelines recommendations (26).

Serum total IgE levels and eosinophils were screened. Skin prick testing was completed for the common allergens (Dermatophagoides pteronyssinus, Dermatophagoides farinae, Alternaria alternata, Aspergillus fumigatus, Penicillium notatum, Cladosporium cladosporioides, cat dander, dog dander, Blattella germanica, pollens of Betula verucosa, salix, grasses, Ambrosia artemisiifolia, Artemisia vulgaris) (Alyostal, Stallergenes) and atopy was defined as one or more positive reaction to the skin prick test. A wheal diameter of at least 3 mm larger than that of the negative control 15 minutes after the puncture was considered a positive result. The control subjects had no evidence of personal history of asthma and other allergic diseases. When the patients were in a
clinically stable condition, we collected blood samples and baseline pulmonary function (forced expiratory volume in 1 second - FEV1, forced vital capacity - FVC, forced expiratory flow, mid expiratory phase - FEF25-75, and peak expiratory flow - PEF) were monitored by spirometry using a Vitalograph spirometer which was calibrated daily. All studies were approved by the local ethics committees. The details were explained to all patients and controls and consent for genetic and biologic screening were obtained.

Genotyping of LTC4S -444A/C Promoter Polymorphism

The genomic DNA was extracted from venous blood with the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. Patients and controls were genotyped for LTC4S -444A/C (rs730012) promoter polymorphism by allelic discriminating TaqMan Real-Time PCR with TaqMan SNP Genotyping Assay (C_644967_10) according to manufacturer protocol (Applied Biosystems, USA) on a 7300 Real Time PCR System (Applied Biosystems, USA).

Statistical Analysis

Alleles and genotypes frequencies of the studied SNP were obtained by direct counting. The Hardy-Weinberg equilibrium was tested using the Chi-square test. The association tests and HWE tests were performed with the software package PLINK v 1.07 and p values ≤ 0.05 were considered statistically significant (27).

For comparison of the distribution of categorical variables, T-Student Test and Pearson correlation were applied. IgE levels were transformed to log10 values to provide a normal distribution for statistical analysis. A P-value of less than ≤ 0.05 was considered statistically significant.

RESULTS

Patients and characteristics

The phenotypic data of the study participants are shown in Table 1. The proportion of male and female participants was equivalent in the study groups, median age was not significantly different. The percentage of atopic subjects and of symptomatic allergic rhinitis were significantly higher in the ATA group than in the AIA group (65% vs 34.7%, p=0.02 for atopy, 40.5% vs 19.8%, p=0.001 for allergic rhinitis). As we expected, the nasal polyposis was more frequent diagnosed in patients with AIA compared to ATA group (82.6% vs 11.7%, p=0.0001). No significant differences were detected for eosinophil levels in the two groups. The serum level of total IgE tended to be higher in ATA patients group, but did not reach statistical significance.

Association of LTC4S promoter polymorphism with asthma and asthma phenotypes

Genotyping success rate was 100% for the control group and the group ATA, 95.7% for AIA group. 2 patients were excluded from ge-

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AIA</th>
<th>ATA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>46</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Mean age (±SD)</td>
<td>44.17 (±15.05)</td>
<td>42.75 (±15.08)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex ratio (male/female)</td>
<td>15/31</td>
<td>23/37</td>
<td>NS</td>
</tr>
<tr>
<td>Atopy</td>
<td>16 (34.7%)</td>
<td>39 (65%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Allergic rhinitis</td>
<td>21 (19.8%)</td>
<td>43 (40.5%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Nasal polyposis</td>
<td>38 (82.6%)</td>
<td>7 (11.7%)</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

TABLE 1. Demographic characteristics of the subjects.

AIA-aspirin intolerant asthma; ATA-aspirin tolerant asthma; IgE-immunoglobulin E/SD – standard deviation
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The data for controls were compared with data available in SNP database for samples of European descent (http://hapmap.ncbi.nlm.nih.gov/whatishapmap.html). The frequency of genotypes for LTC4S -444 A/C in the control group was different in Romanian population compared with the published data for European population: the carriers of the minor allele C (AC + CC genotypes) were less frequent in our control group compared with European data: 41.7% versus 53%, but only marginally significant (p=0.05, OR 1.58).

Clinical phenotyping and subtyping have become of crucial importance in investigations of complex diseases. Therefore we were interested whether disease phenotypes are influenced by the investigated polymorphism.

There were not significant differences regarding the genotype distribution between asthma group and controls, nor AIA/ATA groups and controls (Table 2). The minor C allele frequency was higher in asthma patients, especially in AIA group than controls, without statistical significance (Table 2).

**Association of studied SNP with clinical and biological characteristics of asthmatic patients**

We assessed the clinical characteristics of patients with asthma in relation to genotypes and alleles frequency (Table 3).

We found a higher frequency of minor allele C in women than in men (0.328 vs. 0.243) but the difference did not reach a statistical significance (Table 3). There were no significant differences in genotypes distribution in terms of atopy, associated nasal polyposis or allergic rhinitis in asthma group (see Table 3) or when analyzed in AIA and ATA subgroups (data not shown), but the minor allele C frequency tended to be higher in non-atopic patients (0.326) compared with atopic patients (0.281), similarly higher frequency of minor allele in patients without allergic rhinitis (0.350) compared with those with allergic rhinitis (0.276). We did not identify any association between the -444A/C LTC4S polymorphism and plasma eosinophils, plasma eosinophils levels in asthma patients (p=0.02) and AIA patients (p=0.003), but not significant association with eosinophils level in ATA group.

**DISCUSSION**

In our study we have analyzed the genetic association between LTC4S -444A/C SNP and asthma and its aspirin intolerant phenotype in Romanian patients. To our knowledge, this is

**Table 3. Genotype-phenotype correlations.**

<table>
<thead>
<tr>
<th></th>
<th>Genotype AA</th>
<th>AC</th>
<th>CC</th>
<th>Minor Allele Frequency</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma (n=104)</td>
<td>54 (51.9%)</td>
<td>38 (36.5%)</td>
<td>12 (11.5%)</td>
<td>0.302</td>
<td>0.22</td>
</tr>
<tr>
<td>Sex F (n=67)</td>
<td>33 (49.2%)</td>
<td>24 (35.8%)</td>
<td>10 (14.9%)</td>
<td>0.328</td>
<td>0.12</td>
</tr>
<tr>
<td>M (n=37)</td>
<td>21 (56.7%)</td>
<td>14 (37.8%)</td>
<td>2 (5.4%)</td>
<td>0.243</td>
<td></td>
</tr>
<tr>
<td>Atopic patients (N=55)</td>
<td>30 (53.5%)</td>
<td>21 (37.5%)</td>
<td>5 (9%)</td>
<td>0.281</td>
<td>0.48</td>
</tr>
<tr>
<td>Nonatopic patients (N=49)</td>
<td>24 (48.9%)</td>
<td>18 (36.7%)</td>
<td>7 (14.3%)</td>
<td>0.326</td>
<td></td>
</tr>
<tr>
<td>Nasal polyposis (N=44)</td>
<td>23 (52.2%)</td>
<td>17 (38.6%)</td>
<td>4 (9.3%)</td>
<td>0.290</td>
<td>0.74</td>
</tr>
<tr>
<td>Without nasal polyposis (N=60)</td>
<td>31 (51.7%)</td>
<td>21 (35%)</td>
<td>8 (13.3%)</td>
<td>0.311</td>
<td></td>
</tr>
<tr>
<td>Allergic rhinitis (n=65)</td>
<td>34 (52.3%)</td>
<td>24 (36.9%)</td>
<td>6 (9.2%)</td>
<td>0.276</td>
<td>0.49</td>
</tr>
<tr>
<td>Without allergic rhinitis (n=40)</td>
<td>18 (45%)</td>
<td>16 (40%)</td>
<td>6 (15%)</td>
<td>0.350</td>
<td></td>
</tr>
<tr>
<td>Serum IgE (IU/ml)</td>
<td>222.48±231.73</td>
<td>64.72±54.50</td>
<td>55.40±40.86</td>
<td>-</td>
<td>0.004</td>
</tr>
<tr>
<td>LogIgE</td>
<td>4.84±1.21</td>
<td>3.79±0.97</td>
<td>3.71±0.92</td>
<td>-</td>
<td>0.004</td>
</tr>
<tr>
<td>Eo %</td>
<td>7.31±3.7</td>
<td>3.87±3.24</td>
<td>5.36±1.58</td>
<td>-</td>
<td>0.02</td>
</tr>
</tbody>
</table>
the first association study of LTC4S gene polymorphisms in Romanian asthmatics.

Our results did not show a definite association between the studied polymorphism and susceptibility to asthma and asthma subtypes, but there was a tendency of minor allele C to occur more frequent in the group of patients with aspirin intolerant asthma compared with aspirin tolerant asthma patients or controls. Without reaching a statistical significance, these results suggest a possible role of this SNP in the pathogenesis of aspirin intolerance in Romanian patients with asthma, in line with previous reports that described LTC4S -444A/C as a risk factor for aspirin intolerance in Polish (2,7) and Japanese (28) populations. The involvement of LTC4S gene polymorphisms in asthma pathogenesis is not completely elucidated and the results are controversial. A recent meta-analyses including 3042 cases and 1902 controls from 13 case-control studies, investigating the association between –444A/C polymorphism of the LTC4S gene and asthma risk, reported a significant association for Caucasians, but not for Asians and African-Americans, indicating the importance of the genetic background for this association (29). Regarding the asthma phenotypes, the authors found a significant association between the polymorphism and aspirin tolerant asthma. Further investigations in different populations, with large case-control cohorts, are needed to confirm the association.

In our study, the patients carrying the minor allele C (homozygous or heterozygous) had significantly lower levels of plasma total IgE and eosinophils. We also noted a tendency of minor allele C to have a higher frequency in patients without atopy and allergic rhinitis. The LTC4S gene is located on the 5q chromosome, region which was linked with atopy and bronchial hyperresponsiveness. Similar results were reported by Acevedo et al (30) which found slightly lower log-total IgE levels in patients with -444CC genotype and an association of CC genotype with low levels of specific IgE to D. pteronyssinus, suggesting that LTC4S could be involved in the regulation of IgE response to mite allergens. On the contrary, Choi et al (16) reported no association between minor allele C of LTC4S SNP and the level of total IgE.

LTC4S gene is one of the syntropic genes of allergic diseases identified on the basis of information from the HuGENet internet database (31). These genes include also: HLA genes of class II (HLADQB1, HLADRB1, HLADQA1), IL4 and IL13 (IL4 and IL13 are cytokines critical in IgE mediated reactions and Th2 immune reactions), IL4RA gene (it was reported that some of its polymorphisms have a considerable effect on the signal function of IL4 and IL13 by predisposing to increased production of IgE), MS4A2 gene (encodes the subunit of a high affinity IgE receptor responsible for initiation of an allergic response), IL10 and TGFβ genes (polymorphisms inducing decreased expression are associated with allergic diseases and their severity). By syntropic effect, LTC4S gene is an active component of allergic inflammation genes network. The reported results in favour of an association between LTC4S -444A/C SNP and levels of IgE, in addition to our results, suggest that the minor allele C of LTC4S gene SNP plays an important role in regulation of synthesis of IgE.

LTC4S gene is located on the long arm of the chromosome 5, the site at which many of the genes encoding growth factors, cytokines, and receptors relating to the asthmatic phenotype are located. These include IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor, all of them cytokines important in allergic inflammation (6). There are functional connections between LTC4S and Th2 type cytokines. Thus, it was reported that IL-4 induces the expression of LTC4S mRNA and protein in human mast cells (32), increasing the available activity of the enzyme for the synthesis of cysLT. The combination of either IL-3 or IL-5 with IL-4 markedly increases FcERI-mediated generation of cysLT by mast cells (33).

Interrelation between the genes involved in asthma and allergic diseases is extremely complex. It is expected that a gene variation produces changes regarding the risk or the severity of the diseases.

CONCLUSION

The results show that LTC4S -444A/C SNP is not associated with susceptibility to asthma in Romanian patients, but could influence asthma phenotype, namely aspirin intolerant asthma. The minor allele genotype was related to low levels of serum total immunoglobulin E and eosinophils in patients with asthma suggesting that LTC4S gene could be a modulating factor of allergic inflammation.

Conflict of interests: none declared.

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REFERENCES


