



The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population

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1. Introduction

The incidence of allergic rhinitis (AR) is currently increasing worldwide. One of the most noteworthy environmental antigens provoking the disease in Hungary is the pollen of ragweed (*Ambrosia artemisiifolia*). Ragweed was brought to Hungary in cereal shipments after World War I and has spread extremely rapidly ever since. During its bloom time from July until October, ragweed induces AR, affecting the quality of life of hundreds of thousands in Hungary, and has a significant social and economic impact.

Regulatory T cells (Treg) play a key role in regulating immune responses through their ability to suppress T helper cells, and their functional abnormalities in the dysregulated peripheral tolerance seen in autoimmune and allergic diseases are well known [1–3]. Their role in limiting immune responses to allergens and preventing allergic disease has already been demonstrated [4,5]. CD4⁺CD25⁺ Treg cells were demonstrated to be involved in the induction of tolerance against food allergens in children [6] and administration of oral antigen dramatically induces antigen-specific Treg numbers and function in mice [7]. Treg cells are capable of regulating airway allergic responses not only in animal models [8,9] but also in humans [10]. In addition, activation and expansion of Treg cells has been demonstrated in the mechanism of allergen-specific immunotherapy [11], which is proven to be effective in the management of allergies [12].

Forkhead box P3 (FOXP3) expression is a well-established marker of regulatory T cells and its role in the development and function of CD4⁺CD25⁺ T cells is well characterized [13–15]. The gene

coding for this forkhead box transcription factor is localized on chromosome X (Xp11.23). Mutations of FOXP3 in humans cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). IPEX is characterized by a high incidence of autoimmune diseases, including insulin-dependent diabetes mellitus and thyroiditis, severe refractory diarrhea, and eczema, typically correlated with elevated levels of immunoglobulin E in the serum [16–18]. Bacchetta et al. reported that FOXP3 mutations result in heterogeneous biologic abnormalities and diverse clinical phenotypes in IPEX patients [19]. FOXP3 polymorphisms that occur with high frequency in the general populations have been studied in common multifactorial human diseases such as type I diabetes [20], Crohn's disease [21], primary biliary cirrhosis [21], Graves' disease [22], autoimmune Addison disease [22], and atherosclerosis [23].

Although early data on regulatory T-cell dysfunctions in AR were published in 2004 [10], the first FOXP3 polymorphism study relating to this disease appeared only in 2009 [24]. In that study, on a Han Chinese population, 6 FOXP3 polymorphisms in promoter and intron regions were genotyped. The stratified analyses for homozygotes and heterozygotes revealed that Han Chinese heterozygotes for the rs3761548 single nucleotide polymorphism (SNP) are more susceptible to AR, but when the patients were further stratified for allergens, another association was apparent: between AR in response to house dust mites and the rs3060515 polymorphism. Furthermore, the authors reported a strong linkage disequilibrium (LD) for rs3761548 with another SNP, rs2280883, indicating that the AR-associated polymorphism is a determinant of a haplotype in the Han Chinese population.

In our study, we examined whether the haplotype detected in the Han Chinese population also exists in a European population and whether the association of the rs3761548 polymorphism with

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AR exists in the group of Hungarian Caucasian patients with ragweed pollen allergy.

2. Subjects and methods

2.1. Subjects

One hundred seventy-eight AR patients were enrolled in the study. All had been diagnosed with ragweed pollen allergy through the use of the skin prick test, 38 were allergic exclusively to ragweed, and 140 had an allergy to additional mix allergens, such as other pollens, house dust mites, and animal dander. The patients were diagnosed by allergologists according to the criteria of Allergic Rhinitis and its Impact on Asthma (ARIA 2001 and 2008) [25,26]. The healthy control individuals ($n = 217$), who volunteered to participate from among visitors to the Department of Dermatology and Allergology, were all asymptomatic and gave a negative reaction to ragweed and the most common aeroallergens in Hungary in the skin prick test. The demographic data on the enrolled AR patients and controls in Table 1 demonstrate that the 2 groups were age and gender matched. Informed consent approved by the Internal Review Board of the University of Szeged was obtained from all participating donors and the study was conducted in full accordance with the principles of the Declaration of Helsinki.

2.2. Genotyping

Genomic DNA was isolated from venous blood of the AR patients and controls using a Biorobot EZ (Qiagen, Hilden, Germany) with the EZ1 DNA blood kit. Genotyping of the rs3761548 (C/A) and rs2280883 (T/C) polymorphism was carried out with the polymerase chain reaction (PCR)-based Assay-on-Demand method (Applied Biosystems, Foster City, CA) with kits C__27476877_10 and C__15874601_20, respectively, following the manufacturer's instructions. PCR reactions were run and end-point detection was performed using an iQ5 real-time PCR machine (Bio-Rad, Hercules, CA).

2.3. Statistical analysis

Data analysis was carried out separately for males and females. Because FOXP3 is localized on human chromosome X, differences between the controls and the AR patients in the distribution of the rs3761548 polymorphism were calculated by two-tailed Fisher's exact test; $p < 0.05$ was regarded as statistically significant. Odds ratios (OR) with 95% confidence intervals (95% CI) were also calculated. Statistical analysis was performed using SPSS 15.0 (SPSS, Inc., Chicago, IL).

Table 1
Demographic and clinical characteristics of the study population

Characteristic	AR patients ($n = 178$)	Controls ($n = 217$)
Age, mean (range) (years)	42.71 $\pm$ 12 (22–68)	42.45 $\pm$ 14 (10–84)
Number of males/females (N, [%])	72 (40.45)/106 (59.55)	73 (33.64)/144 (66.36)
Number of patients with AR (N, [%])	178 (100)	—
With AR and asthma (N, [%])	12 (6.74)	—
With AR and atopic dermatitis (N, [%])	12 (6.74)	—
With AR, asthma and atopic dermatitis (N, [%])	3 (1.68)	—
Allergen category	—	—
Ragweed (N, [%])	178 (100)	—
Ragweed and mixed (N, [%])	140 (78.65)	—

Table 2

Numbers of individuals with the genotypes of the FOXP3 rs3761548 (C/A) single nucleotide polymorphism in the female and male subgroups of the AR patients and the controls

	Females ($n = 250$)	
	AR patients ($n = 106$)	Controls ($n = 144$)
CC	43	56
CA	47	49
AA	16	39
	Males ($n = 145$)	
	AR patients ($n = 72$)	Controls ($n = 73$)
C	36	37
A	36	36

2.4. In silico LD analysis

The LD plot for the rs3761548 and rs2280883 polymorphisms was generated by the SHEsis program (<http://analysis.bio-x.cn/myAnalysis.php>) and the data sets of HapMap for Europeans (HapMap-CEU) and for sub-Saharan Africans (HapMap-YRI) were used for analysis.

3. Results

The genotypic distributions of the FOXP3 rs3761548 (C/A) polymorphism in the male and female cohorts of AR patients and healthy controls are presented in Table 2.

To establish genotype–phenotype connections, data processing was performed from 5 aspects: distribution of genotypes, differences in allele frequency, and carriers of the rare allele: those heterozygous for the rare allele and those carrying the rare allele in homozygous form; the latter 2 analyses could implicitly be performed only for females. The first 4 modes of analysis did not indicate any significant differences between the AR and control groups, either for males or for females (data not shown). However, when the numbers of homozygous females (A/A) were compared in the diseased and healthy groups, a significant ($p = 0.0351$) difference was detected: the proportion of females carrying the rare allele in homozygous form was significantly higher in the control group than that of homozygotes identified in the AR group (Fig. 1). This suggests that females homozygous for the rare FOXP3 rs3761548 allele (A/A) are somehow protected against AR; otherwise, females who are either wild types (C/C) or heterozygote

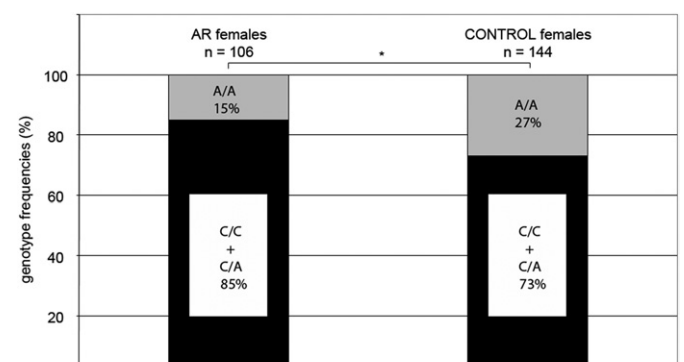


Fig. 1. The proportion of females homozygous for the rs3761548 (A/A) FOXP3 polymorphism was significantly lower ($p = 0.0351$) among the allergic rhinitis (AR) patients compared with controls. The results suggest that the allele is a protective genetic factor against AR in the Hungarian population; otherwise, females who are either wild types (C/C) or heterozygote carriers (C/A) of the rare allele are more susceptible to AR (odds ratio [95% confidence interval] = 2.089 [1.095; 3.988]).

carriers (C/A) of the rare allele are more susceptible to AR (OR [95% CI] = 2.089 [1.095; 3.988]; power = 0.6235). Because we could detect this association only in female AR patients, we hypothesize that there might be minor differences in immunologic mechanisms in males and females leading to the multifactorial disease AR.

After confirming the association of the FOXP3 rs3761548 SNP with AR in Hungarians, we set out to investigate whether this polymorphism is a marker of a haplotype in Europeans and in sub-Saharan Africans, as detected in the Han Chinese population [24]. Plots were generated for both Europeans and sub-Saharan Africans for the LD of the rs3761548 and rs2280883 polymorphisms of FOXP3. Analysis of the HapMap-CEU dataset and haplotype analysis of our Hungarian Caucasian samples for these 2 polymorphisms resulted in perfect LD ($r^2 = 1$), whereas the same analysis on the HapMap-YRI dataset resulted in a lower LD value ($r^2 = 0.34$). These results indicated that the AR-associated rs3761548 SNP of FOXP3 is a marker of a haplotype in both the Han Chinese and the European populations, but not in sub-Saharan Africans.

4. Discussion

Gene polymorphism–disease association studies are of increasing importance from the aspect of understanding multifactorial diseases such as AR [27]. The value of such studies can be enhanced by independent multiple investigations in ethnically and geographically distinct populations. The topic of this article is closely related to that of an article recently published in this journal by Zhang et al. [24]. Those authors studied several polymorphisms of the FOXP3 gene in Han Chinese AR patients and identified the rs3761548 (C/A) SNP as being associated with AR in heterozygous form and the rs3060515 (ATA-) polymorphism as being associated with AR, especially in response to house dust mite, indicating that different polymorphisms may contribute to sensitization mechanisms of different antigens.

In our present investigation, we were able to confirm the findings of Zhang et al. [24] in a geographically and ethnically distinct population, the Hungarians: the rs3761548 (C/A) polymorphism of the FOXP3 gene is associated with AR. The mode of association of this polymorphism with AR differed slightly in the Chinese and Hungarian studies: in Han Chinese the heterozygous carrier status proved to be a susceptibility factor for the disease, whereas in Hungarians the rare allele in homozygous form provides protection against the disease. The explanation for this difference may lie in the compositions of the AR groups in the Chinese and Hungarian studies: whereas the Hungarian patients' group was homogeneous in the sense that beside other allergens they were all allergic to ragweed pollen, the Han Chinese AR patients were less homogeneous, mainly comprising patients allergic to house dust mites, with a minority who exhibited AR in response to nondefined pollens and mixed allergens. As emphasized by Zhang et al. [24], polymorphisms may contribute differentially to the sensitization processes of different antigens. Another explanation for the above difference between the 2 ethnically and demographically distinct populations is that the rs3761548 (C/A) FOXP3 SNP displays different genotype distributions and minor allele frequencies in the general Han Chinese and European populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3761548).

Nonetheless, we consider it noteworthy that the association of a FOXP3 gene polymorphism, rs3761548 (C/A), with AR could be demonstrated in 2 independent examinations. Our data indicate that it is a protective allele against AR in Europeans. Whether it is a marker of an extant protective effect or whether the polymorphism itself exerts a protective effect against AR by an as yet undiscovered molecular mechanism demands further investigation. During the past decade, a number of papers have strongly suggested that polymorphisms of immune regulatory genes play regulatory roles

in the pathogenesis of immune mediated multifactorial diseases [28–30].

Our *in silico* analysis additionally demonstrated that the rs3761548 (C/A) FOXP3 SNP that is associated with AR in Han Chinese and in Hungarians is a marker of a haplotype in these populations, but not in sub-Saharan Africans, suggesting that this haplotype was fixed after early modern humans left Africa some 70,000 years ago.

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