



Genetic association study of *FOXP3* polymorphisms in allergic rhinitis in a Chinese population

Luo Zhang^{a,b}, Yuan Zhang^a, Martin Desrosiers^{c,d}, Chengshuo Wang^b, Yan Zhao^b, Demin Han^{a,b,*}

^a Key Laboratory of Otolaryngology, Head and Neck Surgery, Ministry of Education of China, Beijing Institute of Otorhinolaryngology, Beijing 100005, People's Republic of China

^b Department of Otolaryngology, Head and Neck Surgery, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, People's Republic of China

^c Department of Otolaryngology, Hotel-Dieu Hospital, University of Montreal, Montreal, Quebec, Canada

^d Department of Otolaryngology, Head and Neck Surgery, McGill University, Montreal, Quebec, Canada

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ABSTRACT

The *FOXP3* gene encodes a transcription factor thought to be essential for the development and function of regulatory T cells, which are prevailing mediators of immunological tolerance via suppression/modulation of both T helper (Th)-1 and Th-2 mediated immune responses. Previous studies have demonstrated an association between common polymorphisms in *FOXP3* and a number of immune diseases. The aim of this study was to investigate whether genetic polymorphisms at the *FOXP3* locus predispose to allergic rhinitis (AR) in a Chinese Han population. Six polymorphisms in promoter and intron areas were genotyped in 193 AR subjects and 191 healthy controls. Twelve exons were also analyzed among cohorts of 157 AR patients and 118 healthy controls. Whole-population and gender strata analyses revealed that no single nucleotide polymorphisms in *FOXP3* were identified as significantly associated with AR. Regarding the stratified analysis for heterozygotes and homozygotes, the heterozygous allele in rs3761548 ($p = 0.020$, $OR_{Het} = 3.12$) appeared significant. Subgroup analysis for the presence of different allergen allergies also demonstrated a significant association for house dust mites (rs3060515, $p = 0.010$, odds ratio (OR) = 2.18; rs3761547, $p = 0.013$, OR = 2.00). Additionally, no polymorphisms in coding regions contributing to a susceptibility to AR were noted. Our study provides the first evidence for the association of the *FOXP3* polymorphism with AR in a Chinese population.

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1. Introduction

Regulatory T (T_{reg}) cells play a key role in balancing immune responses to maintain peripheral tolerance against antigens, including autoantigens and foreign allergens [1]. T_{reg} cells are characterized by their ability to suppress effector T cells of either T helper (Th)-1 or Th2 phenotypes involved in mediating inflammation in a cell-contact and antigen-specific manner [2]. Moreover, it is assumed that T_{reg} cells are crucial players in diseases characterized by dysregulated peripheral tolerance, including allergic and autoimmune diseases [3].

FOXP3 is a member of the forkhead or winged helix family of transcription factors that controls the development of T_{reg} cells [4,5]. The gene was first described as JM2 when its mutation was identified in an X-linked autoimmune and allergic dysregulation syndrome, which exhibits early-onset diabetes, enteropathy, endocrinopathy syndrome, and severe allergic inflammation including eczema, food allergy, high serum immunoglobulin E (IgE) levels, and eosinophil-dominated inflammation [6–8]. Currently, *FOXP3* has been identified as a master regulatory gene for cell-lineage commitment or developmental differentiation of $CD4^+CD25^+ T_{reg}$ cells [9–11]. Consequently,

normal *FOXP3* expression is crucial for maintaining $CD4^+CD25^+ T_{reg}$ function as well as the self-balance of the entire immune system. Allergic rhinitis (AR) is characterized by increased production of allergen-specific IgE and tissue eosinophilia, events that are under the regulation of Th2 T lymphocytes [12,13]. The development of an allergic response to common inhaled allergens has been postulated to occur as a consequence of impairment in the numbers, function, or both of allergen-specific T_{reg} cells [14,15]. Hence, polymorphisms in the *FOXP3* gene may be involved during the development and occurrence of AR, which is related to T_{reg} cells.

Given the evidence above, we hypothesized that *FOXP3* might be a strong candidate gene for AR and that SNPs in the *FOXP3* gene region might influence the risk of developing AR. Therefore, the aim of this study was to examine whether single nucleotide polymorphisms (SNPs) in the *FOXP3* gene are associated with susceptibility to developing AR. A population-based case–control association analysis was used to assess the risk of AR conferred by SNPs in the *FOXP3* gene region in our Chinese Han cohort.

2. Subjects and methods

2.1. Study subjects

A total of 193 affected individuals (100 males and 93 females) with AR were prospectively recruited from the rhinology clinic and

* Corresponding author.

E-mail address: dr.luozhang@gmail.com (D. Han).

ward of Beijing Tongren Hospital from February 2007 to December 2007. Individuals who presented with other allergic diseases such as asthma and atopic dermatitis were excluded from the study. A total of 191 controls (98 female, 51.3%) who were friends of the patients or healthy volunteers were recruited from an ethnically similar local population to determine background population allele frequencies. The controls had no clinical features or family history of AR and exhibited negative serum phadiatop determination. All subjects were of Chinese Han ethnic origin and all were from the Beijing region. The study was approved by the Beijing Tongren Hospital Ethics Committee, and written informed consent was obtained from all participants.

All AR patients were diagnosed based on symptoms, local signs, and allergen-related examinations, including a skin prick test (SPT) and serum-specific IgE measurement, according to the criteria of Allergic Rhinitis and its Impact on Asthma (ARIA 2008) [16]. AR was defined as (1) persistent or discontinuous symptoms of anterior rhinorrhea, continuous sneezing, nasal obstruction, and itching; (2) nasal cavity local signs including discolored and edematous mucosa and watery drainage in the common or middle nasal meatus; and (3) positive serum antigen-specific IgE by the ImmunoCAP 100 system (Pharmacia, Uppsala, Sweden) and positive antigen SPT (Allergopharma, Reinbeck, Germany). The antigens mainly included dust mites, spring and autumn pollens, and mycetes. Subjects were considered sensitive to allergens if the measurement of IgE was equal to or above 0.35 kU/l. A positive SPT result was defined as a wheal greater than or equal to one half of the diameter of the histamine control and at least 3 mm larger than the diameter of the negative control [17]. Serologic and skin tests were performed by specialized technicians and nurses, respectively, whereas the AR diagnoses were made eventually by rhinologists.

2.2. Selection of polymorphisms in the human *FOXP3* gene

Eight polymorphisms were selected for genotyping in the patients and controls (Table 1). The SNPs were selected to extract the most genetic information based on CHB haplotype data using the HAPMAP database (<http://www.hapmap.org>) [18]. From this dataset, four SNPs (rs2280883, rs3761549, rs3761548, rs3761547) in *FOXP3* were selected using a pairwise tagging algorithm implemented in Haploview version 4.1 [19]. In addition, when we set the Hardy–Weinberg *p* value cutoff, minor allele frequency, and r^2 thresholds at 0.01, 0.05, and 0.8, respectively, the linkage disequilibrium (LD) pattern for *FOXP3* gene in our population exhibited strong LD in two groups of SNPs: rs2280883/rs3761548 ($r^2 = 1.00$) and rs3761549/rs3761547 ($r^2 = 1.00$), indicating that the SNPs in each group represent a common region (Fig. 1). Consequently, we chose rs3761548 and rs3761547 to represent the entire four loci or eventual genotyping. No exonic SNPs were included because no informative exonic SNPs are reported in the HAPMAP and other SNP databases. Six additional SNPs (rs3060515, rs2869211, rs2232364, rs2232365, rs2232366, rs2232368) from the previous literature were also included [20]. Additionally, direct-sequencing analysis of the coding region of the *FOXP3* gene was carried out for the detection of new SNPs.

2.3. SNP genotyping

DNA was isolated from peripheral blood leukocytes and collected in EDTA-treated tubes using the DNA Isolation Kit for Mammalian Blood (Roche, Indianapolis, IN). Isolated DNA from blood was stored at 4°C prior to use. Genotyping was performed by polymerase chain reaction (PCR) direct sequencing and details of the primers are given in Table 1. Among them, rs3060515 and rs2869211 were used as one common set of primers. The eight SNP candidates in the promoter and intron regions were chosen for

Table 1

Details of the primers used in the screening of SNPs in the promoter, coding, and intron regions of the *FOXP3* gene

Region	Locus	Primer
Promoter	rs3060515 (ATA-)	5'-GGCTGATCCTTTTCTGTCAG-3'
	rs2869211 (T/A)	5'-GGCATCAAGAGCTAAGAGAC-3'
	rs3761547 (A/G)	5'-GCACTCTGGCTCTCCATGCAT-3'
		5'-GGGGTCCAACGTGTGAGAAG-3'
	rs3761548 (A/C)	5'-GGGGTCCAACGTGTGAGAAG-3'
		5'-GTGGCCAGATGGACATCACC-3'
Coding	rs2232364 (C/T)	5'-AAGGAGCAACTGCTCCCCCA-3'
		5'-CTTCTTTAGGAAGTGTTCCTCG-3'
	rs2232365 (A/G)	5'-CCAGGCTTGAGGGCCAGAAA-3'
		5'-GGGTCTGGATCTCAGGGCAA-3'
	Exon1	5'-GTGGTGAGGGGAAGAAATCA-3'
		5'-CTGGGTACATCCCAGTGTAC-3'
	Exon2	5'-CAGCCAATGCCTGCTTTGAC-3'
		5'-GCACCTGTAGTCCAGGTAC-3'
	Exon3&4	5'-GTGGGCTTGCACTGCAGAGT-3'
		5'-AGTTTCAAGCCTCTGAGAC-3'
	Exon5&6	5'-TCAGGAGGGTGGGAAGTCA-3'
		5'-GGGATTGAACCCACAGTCT-3'
	Exon7&8	5'-CTCGTGGTCACCTGCATGGA-3'
		5'-GTCGCTCACCACAGATGAAG-3'
	Exon9	5'-GGCGACAGAGCAAGACTCAG-3'
		5'-AGAGCCTGTCAAGATTAGGA-3'
	Exon10	5'-ACGGGTGTTGACGGTGAGAT-3'
		5'-ATGAGGAGAACCCACTCTGAG-3'
	Exon11	5'-CCATGCTTACCCACATCT-3'
		5'-ACTTGAGGCCATCCAGTCA-3'
Intron	Exon12	5'-TGGGGTGTGTGGGTTGTGAC-3'
		5'-TGGGGTGTGTGGGTTGTGAC-3'
	rs2232366 (G/T)	5'-GGGAACCTTCCAGGGCCAGAT-3'
		5'-CAGCCCTGTCACTTCACTACT-3'
	rs2232368 (G/A)	5'-AGGGGTGTGAGAGGGAGACT-3'
		5'-TACCTGCTGCTCCAGAGACT-3'

examination in 193 AR individuals and 191 healthy controls. To screen the entire 12 coding region, we constructed nine sets of PCR primers according to the human genomic clone (Accession Number NT_079573) (Table 1) and PCR was employed among the DNA samples of the whole cohort of 157 AR patients and 118 controls, which were randomly selected from the study population. PCR was carried out in a 50-μl volume containing 100 ng of genomic DNA, 10 pmol of each primer, 2 units of HS-Taq DNA polymerase (TaKaRa, Dalian, China), and 20 nmol of each dNTP. The PCR profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 5 minutes.

2.4. Statistical analyses

The SPSS program version 13.0 was used to determine associations. χ^2 tests and Fisher's exact test were performed to determine whether an association existed for alleles between cases and controls. A corrected *p* value of <0.05 was considered statistically significant. We estimated odds ratios (OR) and 95% confidence intervals (95% CI) for the effect of *FOXP3* polymorphisms on AR risk. Bonferroni's multiple adjustments over the tested SNPs gave a significance threshold of 0.025 and 0.017 for whole-cohort analysis of heterozygotes and homozygotes and allergen-specific analysis, respectively. Gender was taken into account, with the males contributing a single allele to the analysis and females contributing two alleles. The AR cohort was then stratified by gender and tested against corresponding gender-specific control groups. The Breslow–Day test was performed to ensure heterogeneity between genders and the OR for the whole population was adjusted by Mantel–Haenszel methods. Furthermore, subanalysis restricted to the presence of different allergens, such as house dust mites, pollens, and mixed allergens was also performed to examine whether

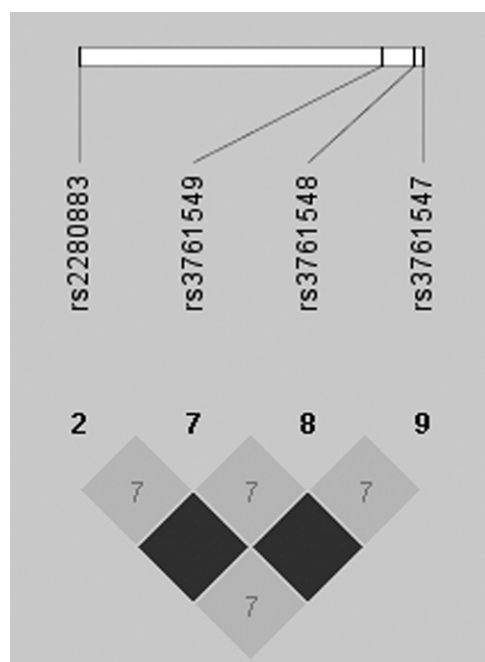


Fig. 1. Linkage disequilibrium (LD) plot for *FOXP3*. The LD plots were generated by Haploview 4.1. The white horizontal bar below the info track illustrates the location of SNPs on a physical scale. The shade of squares illustrates the strength of pairwise r^2 values on a black-and-white scale, where black indicates perfect LD ($r^2 = 1.00$) and white indicates perfect equilibrium ($r^2 = 0$). The r^2 LD value is also indicated within each square. Failed SNP and SNPs not in Hardy–Weinberg equilibrium or with low minor allele frequency are not illustrated.

the effect of associations within the population differed among the subgroups. The study power calculation for each analyzed parameter was calculated at the 5% significance level.

3. Results

3.1. Population characteristics

Table 2 provides a summary of the demographic characteristics of the study population. Age and gender were well balanced between cases and controls. The cohort of 193 AR patients had a mean age 33 years and consisted of slightly more men (51.8%) than women (48.2%), whereas the 191 control individuals had a mean age of 37 years and consisted of slightly more women (51.3%) than men (48.7%). The doctors diagnosed the patients as AR by combining the skin test and serum-specific IgE data with the allergen-specific case history and physical examination. A total of 78.8% of the individuals demonstrated perennial nasal symptoms and the remainder reported seasonal reactions. When subjects were classified according to the allergen category, 110 (57%), 37 (19.2%), and 46 (23.8%) were allergic to house dust mites, pollens, and mixed allergens, respectively. We defined a mixed allergen allergy as individuals exhibiting two or more allergen-positive results from the skin test or serum examination.

3.2. Association analysis

The analysis of allele frequencies for the four tested SNPs (rs3060515, rs3761547, rs3761548, rs2232365) shown in Table 3 revealed significant associations ($p < 0.05$), with an AR outcome in the *FOXP3* gene. We did not detect any mutation in the remaining four selected SNP loci (rs2869211, rs2232364, rs2232366, rs2232368) in both cases and controls (data not shown). Table 3 presents the OR for risk allele and the corresponding OR for heterozygotes (OR_{Het}) and homozygotes (OR_{Hom}). No SNPs in the *FOXP3* gene indicated a significant association with AR. Regarding

the stratified analysis for heterozygotes and homozygotes of the four SNPs, the heterozygous allele in rs3761547 ($p = 0.037$, $OR_{Het} = 2.33$, 95% CI = 1.06–5.13) and rs3761548 ($p = 0.020$, $OR_{Het} = 3.12$, 95% CI = 1.21–8.04) appeared significant, whereas only rs3761548 remained significant following application of the Bonferroni correction for multiple testing for two tests ($p < 0.025$).

Likewise, stratification of the AR cohort and controls by gender failed to detect any evidence of an association between the genotyped SNP markers and AR and there were no differences between males and females (Table 4). The Breslow–Day test for the whole cohort indicated heterogeneity ($p > 0.05$) between genders. Table 4 includes the adjusted OR and 95% CI by Mantel–Haenszel methods, which contrast with the results in Table 3.

Subgroup analysis for the presence of different allergen allergies indicated a significant association for house dust mites (rs3060515, $p = 0.010$, $OR = 2.18$, 95% CI = 1.23–3.87; rs3761547, $p = 0.013$, $OR = 2.00$, 95% CI = 1.18–3.39) and pollens (rs3060515, $p = 0.035$, $OR = 0.15$, 95% CI = 0.02–1.16; Table 5), whereas only the former two loci in subjects with house dust mite AR attained the threshold required ($p < 0.017$) after application of Bonferroni's multiple adjustment for three tests.

In addition, we additionally sequenced all *FOXP3* exons in 157 AR cases (70 females and 87 males) and 118 control samples (59 females and 59 males). No SNPs or mutations among all 12 exons of *FOXP3* were reported in either of the AR subject groups compared with controls (data not shown).

4. Discussion

In this study, we aimed to evaluate the contribution of SNPs in the *FOXP3* gene region toward AR susceptibility in a Chinese population-based case–control association analysis. Whole-population and gender strata analyses revealed that no SNPs in *FOXP3* were identified as being significantly associated with AR. Regarding the stratified analysis for heterozygotes and homozygotes, the heterozygous allele in rs3761548 ($p = 0.020$, $OR_{Het} = 3.12$, 95% CI = 1.21–8.04, power = 63%) appeared significant. Subgroup analysis for the presence of different allergen allergies indicated a significant association for house dust mites (rs3060515, $p = 0.010$, $OR = 2.18$, 95% CI = 1.23–3.87, power = 71%; rs3761547, $p = 0.013$, $OR = 2.00$, 95% CI = 1.18–3.39, power = 68%). Additionally, no polymorphisms in the coding regions contributing to susceptibility to AR were noted. Our study provides the first evidence for the association of *FOXP3* polymorphism with AR in a Chinese population.

The *FOXP3* gene is localized in the small arm of the X-chromosome in the vicinity of the region showing linkage to many common autoimmune disorders, including type 1 diabetes, juvenile idiopathic arthritis, and rheumatoid arthritis, and is identified as an autoimmune locus [21,22]. Bassuny *et al.* [20] reported an association between a microsatellite marker within intron 0 of *FOXP3* and type 1 diabetes in a Japanese population. Subsequently, a detailed and comprehensive haplotype survey of five *FOXP3* polymorphisms including the (GT)_n promoter microsatellite was carried out in a large type 1 diabetes cohort [23]. However, this

Table 2
Demographic characteristic of the study population

Characteristic	AR cases (n = 193)	Controls (n = 191)
Age, mean (range) (years)	33 ± 14 (6–67)	37 ± 14 (7–77)
Sex, M/F, No. (%)	100 (51.8)/93 (48.2)	93 (48.7)/98 (51.3)
Perennial/seasonal, No. (%)	152 (78.8)/41 (21.2)	
Allergen category, No. (%)		
House dust mites	110 (57.0)	—
Pollens	37 (19.2)	—
Mix	46 (23.8)	—

Table 3
Allergic rhinitis whole-association analysis

SNPs	Controls (<i>n</i>)				Patients (<i>n</i>)				OR (95% CI)	<i>p</i> value	OR _{Het} (95% CI)	<i>p</i> value	OR _{Hom} (95% CI)	<i>p</i> value
	Hom	Hom	Het	Total	Hom	Hom	Het	Total						
	Dom	Res			Dom	Res								
rs3060515	159	28	1	188	157	31	4	192	1.22 (0.71–2.10)	0.495	4.05 (0.45–36.65)	0.371	1.12 (0.64–1.96)	0.777
rs3761547	151	30	10	191	136	30	21	187	1.42 (0.88–2.28)	0.186	2.33 (1.06–5.13)	0.037	1.11 (0.64–1.94)	0.777
rs3761548	146	29	6	181	148	23	19	190	1.18 (0.72–1.96)	0.525	3.12 (1.21–8.04)	0.020	0.78 (0.43–1.42)	0.454
rs2232365	106	63	16	185	113	53	25	191	0.93 (0.62–1.40)	0.754	1.47 (0.74–2.90)	0.308	0.79 (0.50–1.24)	0.358

Hom Dom = homozygous dominant; Hom Res = homozygous recessive; Het = heterozygous; OR_{Het} = odds ratios for heterozygotes; OR_{Hom} = odds ratios for homozygotes; CI = confidence interval.

study did not replicate the previous findings and indicated no evidence of an association between *FOXP3* and type 1 diabetes. Similarly, Sanchez *et al.* [24] were unable to detect an association with the GT microsatellite and several autoimmune diseases, although intriguing allele frequency differences were observed between the Spanish and Japanese control subjects. The role of *FOXP3* in other autoimmune diseases has also recently been investigated, again with discrepant results [25–27]. Although the studies focused on SNPs or microsatellites and are not completely consistent, no evidence exists of *FOXP3* increasing the risk of developing a common autoimmune disorder within different populations.

Allergic sensitization is defined as the production of IgE against environmental antigens such as house dust mites, grass pollen, and animal proteins and can lead to diseases including asthma, rhinitis, and atopic dermatitis [28]. Activation of Th2 cells is thought to have a major role in allergic sensitization, although it is unknown why atopic individuals generate a Th2-type T-cell response, whereas nonatopic individuals do not. One possibility is that regulatory mechanisms prevent IgE responses to allergens in nonatopic individuals. Potential mechanisms include the suppression of Th2 responses by regulatory T lymphocytes, although treatment with allergen-derived T-cell peptides results in allergen-dependent recruitment to the skin of Th1, rather than regulatory T cells, to cutaneous late-phase reaction sites [29]. Recent studies have indicated that mucosal induction of tolerance against dietary antigens is associated with the development of CD4⁺CD25⁺ T cells in both animals and humans [30,31]. Another study has indicated that following antigen inhalation, CD4⁺CD25⁺ T cells play a key immunomodulatory role [32]. Recently, Lee *et al.* demonstrated that the numbers of CD4⁺CD25⁺ T_{reg} cells and mRNA expression of functional molecules of T_{reg} are related to airway allergy and disease severity [33]. It was also indicated that asthmatic patients have decreased *FOXP3* protein expression within their CD4⁺CD25⁺ T_{reg} [34]. Likewise, decreased *FOXP3* gene expression was recently reported in nasal secretions from patients with AR [35]. To establish clinical and pathological relevance of the reported association, we can hypothesize that the SNPs in the *FOXP3* locus would reduce the gene expression in CD4⁺CD25⁺ T_{reg} cells. In the present study, we did not report a significant association of SNPs in *FOXP3* with AR when whole-population and gender strata analyses were performed. Subgroup analysis for the presence of different allergen

allergies, however, reported a significant association for different allergen allergies, indicating a possibly different sensitized mechanism of different allergens. Stratified analysis for heterozygotes and homozygotes also demonstrated a significant result. However, the potential relationship between the *FOXP3* gene and developing house dust mite AR requires further study.

Interestingly, although we have gleaned genetic information encompassing all sequences across the entire *FOXP3* exon in both patient and control groups, we did not detect any SNP changes in such regions. To date, the findings are valid only for the Chinese Han population. Although HAPMAP-CHB data indicate that no common polymorphisms exist among exons of the *FOXP3* gene, we should take this information for reference only, rather than as certain results. Consequently, two interpretations are possible with regard to the present results. First, no polymorphisms in the *FOXP3* coding regions exhibited an association with AR in the Chinese population. Second, *FOXP3* might be related to our AR phenotype, but we did not find mutations in the selected samples because of the allele frequency. Further study in a larger study population is needed.

By contrast, we were able to replicate eight SNPs in noncoding areas and reported significant associations. The simple paradigm of promoter-driven exon-based gene expression is losing ground gradually. We now understand that gene expression is far more complex. Gene expression is controlled by proximal and distant enhancers and silencers, epigenetic modulation of the gene locus, secondary regulation by microRNA, gene splicing, SNPs, and other factors. In this sense, the association between the etiology of AR and *FOXP3* is more likely related to the mRNA changes in the gene, consistent with previous reports [33,35]. Further AR case–control association studies should be performed to investigate the changes in mRNA level of the *FOXP3* gene in Chinese Han population-based study samples.

Taken together, the present findings provide evidence for an association between the *FOXP3* gene and AR. Future studies will be aimed at further elucidating the mechanisms underlying allergen sensitization. Larger cohorts of patients with these conditions in a variety of ethnic populations are still necessary to increase our knowledge base for the *FOXP3* gene and might shed light on studies of the etiology of AR. Such advances might also enable more appropriate exploitation of *FOXP3* for the prevention, diagnosis, treatment, and monitoring of AR and other allergic diseases.

Table 4
Male cohort and female cohort association analysis and heterogeneity test

Locus	Males		Females		Breslow–Day test p value	Mantel–Haenszel OR (95% CI)
	OR (95% CI)	p value	OR (95% CI)	p value		
rs3060515	1.18 (0.51–2.76)	0.830	1.30 (0.64–2.63)	0.480	0.871	1.25 (0.73–2.15)
rs3761547	1.78 (0.87–3.64)	0.154	1.21 (0.63–2.30)	0.623	0.426	1.44 (0.89–2.32)
rs3761548	1.16 (0.53–2.50)	0.845	0.86 (0.46–1.61)	0.748	0.559	0.97 (0.59–1.58)
rs2232365	0.99 (0.55–1.78)	1.000	0.87 (0.49–1.56)	0.662	0.762	0.93 (0.62–1.40)

Table 5
Allergen-specific association analyses of SNPs in *FOXP3* and AR

Locus	House dust mites		Pollens		Mixed allergens	
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
rs3060515	2.18 (1.23–3.87)	0.010	0.15 (0.02–1.16)	0.035	0.52 (0.17–1.57)	0.344
rs3761547	2.00 (1.18–3.39)	0.013	0.76 (0.29–1.94)	0.656	0.84 (0.36–1.95)	0.836
rs3761548	0.98 (0.54–1.80)	1.000	1.34 (0.58–3.10)	0.503	1.47 (0.69–3.13)	0.314
rs2232365	0.89 (0.55–1.44)	0.713	0.92 (0.45–1.88)	0.857	1.13 (0.59–2.16)	0.742

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