



Association between polymorphisms in FOXP3 and EBI3 genes and the risk for development of allergic rhinitis in Chinese subjects

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ABSTRACT

Objective: To investigate whether polymorphisms in forkhead box protein 3 (FOXP3) and EBV-induced gene 3 (EBI3) genes are associated with allergic rhinitis (AR) in Chinese patients.

Methods: A population-based case-control association study design was used to assess the risk of AR conferred by single nucleotide polymorphisms (SNPs) in FOXP3 and EBI3 gene regions. DNA was extracted from 378 patients with AR and 330 healthy controls and analyzed for selected and tagged SNPs. Overall, 9 SNPs were selected and genotyped.

Results: In the single-locus analyses of AR risk, the allele frequencies of rs428253 in EBI3 gene were significantly different between the AR patients and control subjects ($P = 1.00E-04$); even after 10,000 permutations ($P < 0.05$). Logistic regression analyses, adjusted for age and gender, further showed a significant association between EBI3 rs428253 and protective effects against AR ($P = 0.015$, OR = 0.624 for CG/CC). The diplotype rs3761548-rs4824747 in FOXP3 gene with “AG” was associated with risk of AR ($P = 0.031$, OR = 1.755).

Conclusions: The findings of this study support the potential role of regulatory T cells and genetic variations in the regions around FOXP3 and EBI3 genes in modifying the risk for AR development in Chinese patients.

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1. Introduction

Allergic rhinitis (AR) is an inflammatory disease of the nasal mucosa induced by an immunoglobulin E (IgE)-mediated reaction in allergen-sensitized subjects. AR has increased in prevalence over the last decade, in particularly the industrialized nations, and currently affects up to 40% of the population worldwide [1]. Recent data from mainland China indicate that the prevalence of self-reported AR in major cities across China is high and ranges between 8.7% and 24.1%; with around 25% of all patients suffering from persistent symptoms [2].

Functional studies have demonstrated that regulatory T (T_{reg}) cells are an important sub-population of $CD4^+$ T cells that are essential for maintaining self tolerance and preventing autoimmunity, and for limiting chronic inflammatory diseases [3–5]. Some evidence suggests that T_{reg} cells play a critical modulatory role in

allergic and autoimmune diseases characterized by dysregulated peripheral tolerance [6,7]. Furthermore, it has been postulated that the development of an allergic response to common inhaled allergens occurs as a consequence of impairment in the numbers and/or function of allergen-specific T_{reg} cells [8,9]. Similarly, other studies have suggested that transcription factor forkhead box protein 3 (FOXP3; a member of the forkhead or winged helix family of transcription factors shown to be essential for the development and function of T_{reg} cells) [10,11] and EBV-induced gene 3 (EBI3; shown to be upregulated in $FOXP3^+$ T_{reg} cells and thought to be a downstream target of FOXP3) [12] may be involved in the aetiology of allergic diseases. As AR has multifactorial inheritance, it is possible that different combinations of genes and/or single nucleotide polymorphisms (SNPs) may change the inflammatory responses thus may increase the risk of phenotypic expression.

In view of these evidence, we hypothesized that genes involved in T_{reg} pathway regulation, such as FOXP3 and its downstream target gene, EBI3 as well as the surrounding genomic regions have any SNPs which may influence an individual's risk to develop AR. The aim of this study was therefore to examine whether polymorphisms in the FOXP3 and EBI3 gene are associated with an individual's susceptibility to develop AR in a Han Chinese cohort.

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2. Materials and methods

A population-based case-control association study design was used to assess the risk of AR conferred by SNPs in FOXP3 and EBI3 gene regions.

2.1. Study patients

Three hundred and seventy-eight patients suffering from AR were recruited from the outpatient clinic of Otolaryngology, Head and Neck Surgery Department at Beijing Tongren Hospital, between February 2010 to November 2010.

All patients had a history of AR for at least 1 year and fulfilled all of the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines [13] criteria for AR, including (i) presence of persistent or discontinuous symptoms of anterior rhinorrhoea, continuous sneezing, nasal obstruction and itching, (ii) demonstration of a pale and edematous nasal mucosa, nasal discharge and swollen inferior turbinates by nasal endoscopy, and (iii) positive skin prick test (SPT) to a panel of common allergens as shown below (Allergopharma, Reinbeck, Germany) and/or positive serum antigen-specific IgE, measured by the ImmunoCAP 100 system (Pharmacia, Uppsala, Sweden). A diagnosis of AR was further confirmed by the presence of symptoms induced by exposure to an allergen shown to produce a strong positive skin test response.

The tested antigens included house dust mite (HDM) (Der f and Der p); seasonal grass pollens (Gaint Ragweed; Mugwort; Lamb's quarters; Humulus; Chenopodium album and so on); animal hair (especially dog and cat); molds (indoor and outdoor mustiness or floricultural environment) and cockroach. 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 [14]. Subjects were also considered to be sensitized to allergens when the serum IgE was ≥ 0.35 kU/L.

AR patients with (i) comorbid asthma, eczema, or any other allergic disease; (ii) hypertension, diabetes or other chronic diseases; or (iii) tumor in the nasal cavity or any other inflammatory nasal disease were excluded. The diagnosis of asthma was confirmed by a chest physician according to Global Initiative for Asthma (GINA) guidelines [15].

A total of 330 healthy control volunteers; with no history of allergic or any nasal disease and not demonstrating any abnormal clinical features in the nasal cavity; were recruited from an ethnically similar local population to determine background population allele frequencies. Moreover all the controls were examined for serum total IgE.

All subjects were of Han Chinese ethnic origin from the Beijing region, China, and provided written informed consent prior to entry in the study. The study protocol was approved by the Ethics Committee of Beijing Tongren Hospital and performed in accordance with the guidelines of the World Medical Association's Declaration of Helsinki.

2.2. Selection of polymorphisms in the FOXP3 and EBI3 genes

The International Haplotype Mapping (HapMap) (www.hapmap.org) SNP databases were used to select tagging SNPs (tSNPs) in the FOXP3 and EBI3 gene region, and the screened region was extended 10 kilobases upstream of the annotated transcription start site and downstream at the end of the last exon in each gene. The tSNPs were selected to extract most genetic information in the region using the CHB genotyping data from the HapMap database (HapMap data rel 27 Phase II+III, Feb2009) [16]. Genotyping data was obtained for 14 tSNPs for FOXP3 and 12 tSNPs for EBI3 in this

dataset and loaded in the Haploview software version 4.2 (<http://www.broad.mit.edu/haploview/haploview-downloads>) [17]. Further selection of the eventual tSNPs to be investigated was then made using a pairwise tagging algorithm [17]; setting the Hardy-Weinberg p value, minor allele frequency (MAF), and r^2 thresholds at 0.01, 0.05 and 0.8, respectively. The linkage disequilibrium (LD) patterns of the selected genes in the CHB population exhibited strong LD in several groups of tSNPs (r^2 greater than or equal to 0.8), indicating that most common SNPs can be captured by a subset of tagging SNPs [18]. Consequently, we selected 4 SNPs for FOXP3 (rs2294018, rs3060515, rs2232365, rs3761548) and 4 SNPs for EBI3 (including rs428253, rs6613, rs353698, rs2302164) to represent the entire 26 loci for genotyping. Additionally, to ensure all the positive SNP loci presented in our previous study, i.e., rs3761548, rs3060515 and rs3761547 [19], are included, we added rs4824747 for investigation as well. Here we choose rs4824747 to replace rs3761547 (rs4824747–rs3761547 $r^2 = 1.00$) because of the Sequenom platform genotyping requirements.

2.3. Genotyping

DNA was isolated from peripheral blood leukocytes, using the DNA Isolation Kit for Mammalian Blood (Roche, Indianapolis, USA), and stored at 4 °C prior to further investigation within 2 days. The genotyping of the selected SNP was performed using iPLEX chemistry on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Sequenom, San Diego, California) according to the manufacturer's instructions [20]. The polymerase chain reaction (PCR) and extension primers were designed using the MassARRAY Assay Design 4.0 software and the details of the primers were showed in Supporting information, Table 1.

PCR was carried out in standard 384-well plates, using 5 μ L of a reaction mix comprising 10 ng of genomic DNA, 0.5 units of Taq polymerase (HotStarTaq, Qiagen), 500 μ mol of each deoxynucleotide triphosphate, and 100 nmol of each primer per well. PCR thermal cycling was carried out in an ABI-9700 instrument (GeneAmp PCR system 9700, ABI, California) for 15 min at 94 °C, followed by 45 cycles of 20 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C. At the end of cycling, 2 μ L of containing 0.3 units of Shrimp Alkaline Phosphatase was added to each well, and the reaction incubated at 37 °C for 20 min. The reaction was inactivated for 5 min at 85 °C.

After adjusting the concentrations of extension primers to equilibrate signal-to-noise ratios, the post-PCR primer extension reaction of the iPLEX assay was carried out in a final volume of 9 μ L extension reaction mixture containing 0.2 μ L of termination mix, 0.04 μ L of DNA polymerase (Sequenom, Inc.), and 625 to 1.250 nmol/L extension primers. A 200-short-cycle program was used for the iPLEX reaction; involving an initial denaturation by incubation for 30 s at 94 °C, followed by incubation for 5 s at 94 °C, five cycles of 5 s at 52 °C, and 5 s at 80 °C. Forty additional annealing and extension cycles were looped back to 5 s at 94 °C, five cycles of 5 s at 52 °C and 5 s at 80 °C, and the final extension was carried out at 72 °C for 3 min. The final reaction mixture was cooled to 4 °C and each sample was desalted using 6 mg of clean resin and a dimple plate. The final product was transferred to a 384-well Spectro-CHIP (Sequenom, Inc.), and analyzed in a Compact Mass Spectrometer, using the MassARRAY Typer 4.0 Software. The PCR assay was arrayed with two no-template controls and four duplicated samples in each 384-well format as quality controls. All genotyping results were generated and checked by an investigator blinded to the clinical status of the subject from whom the sample was derived.

2.4. Statistical analyses

Data were initially processed for suitability for further statistical evaluation using the Haploview version 4.1 software.

Hardy-Weinberg equilibrium (HWE) of each SNP was assessed in controls only and a threshold $P < 0.001$ was regarded to indicate deviation from HWE. In addition, we assessed the MAF, non-missing genotype percentage and other criteria in the AR cases as well as controls to filter the data. Among them, minor allele frequency (MAF) and non-missing genotype percentage thresholds were set at <0.001 and $<75\%$, respectively.

Differences in frequencies of the alleles and genotypes between the AR subjects and control subjects were evaluated using the chi-square test and HWE was tested by the chi-square test for goodness of fit according to the web-based program: <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>; and a P -value of 0.05 was considered significant. Akaike's information criteria (AIC) were used to select the most parsimonious genetic model for each SNP. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression analysis, adjusted for age and gender. Stratification analyses were also performed by variables of interest, such as gender. These analyses were conducted using the STATA statistical package (version 11.0; Stata Corp LP, College Station, TX, USA).

The pairwise linkage disequilibrium (LD) among the SNPs was examined using Lewontin's standardized coefficient D' and LD coefficient r^2 [21], and haplotype blocks were defined by the method of Gabriel et al. [22] in Haploview 4.2 with default settings (the CI for a strong LD was minimal for upper 0.98 and low 0.7 and maximal for a strong recombination of 0.9, and a fraction of strong LD in informative comparisons was at least 0.95). In addition, PHASE 2.1 Bayesian algorithm [23] was used to estimate the haplotype frequencies. The HAPLO.STATS package developed by Schaid et al. [24] (www.mayo.edu/hsr/Sfunc.html) in the software language R was used for the haplotype analysis. This method, based on the generalized linear model framework, allows adjustment for possible confounding variables and provides both global and haplotype-specific tests. Haplotypes with a frequency of less than 0.03 were pooled into a combined group and empirical P -values, based on 100,000 simulations, were computed for the global and individual haplotype score tests. Diplotype (haplotype dosage, an

estimate of the number of copies of the haplotype) was the most probable haplotype pair for each individual. Unconditional logistic regression analyses, adjusted for age and gender, were conducted to estimate ORs and 95% CIs for participants carrying one to two copies versus zero copy of each common haplotype for the dichotomized diplotypes.

The statistical power for the present study was calculated using G*Power 2 software (<http://www.psych.uni-duesseldorf.de/aap/projects/gpower/>).

3. Results

3.1. Population characteristics

The demographic characteristics of the study population are shown in Table 1. Both the AR and control groups were well matched with respect to age ($P = 0.059$), although the AR group consisted more males (60.1%) than females (39.9%). In contrast with this, there were approximately equal numbers of males and females in the control group (53.0% vs 47.0% m/f) ($P = 0.213$). The mean total serum IgE measurements for AR and control groups were 325.4 ± 549.1 and 69.1 ± 120.1 IU/ml respectively ($P = 0.000$); confirming the AR/non-AR status of both groups. The statistical power of AR vs. control was 96.47%.

3.2. Individual SNP association analysis

The initial quality tests for the SNPs in the EBI3 and FOXP3 genes selected for genotyping demonstrated that one SNP (rs6613) in EBI3 region and one SNP (rs3060515) in FOXP3 gene was not suitable for study because of deviation from HWE ($P < 0.001$) and MAF value <0.001 respectively (data not shown). Thus the data for these two loci were therefore excluded from further analyses. Here HWE was calculated only for the SNPs on chromosome 19. Overall, a total of 7 SNPs, including 3 from chromosome 19 and 4 from chromosome X were chosen as shown in Table 2. The 3 SNPs from chromosome 19 were selected from

Table 1
Demographic characteristics of the study population.

Demographic index	AR ($n = 378$)	Controls ($n = 330$)	P
Age mean (range) (years)	27.1 ± 14.8 (5–71)	36.2 ± 15.2 (6–78)	0.059
Sex, male/female, no. (%)	227 (60.1)/151 (39.9)	175 (53.0)/155 (47.0)	0.213
Total IgE mean (range), kU/l	325.4 ± 549.1 (6.9–5000)	69.1 ± 120.1 (2–906)	0.000

AR = Allergic rhinitis subjects.

Table 2
SNPs genotyped for EBI3 and FOXP3 genes.

Gene: locus and OMIM no. ^a	No.	SNP_ID	Chromosome pPosition	Intermarker distances (bp)	Genic location	Base change	MAF ^d			P^g	P value for HWE ^h test
							NCBI ^e	Case ^f	Control		
EBI3: 19p13.3 OMIM: 605816	1	rs428253	4180913 ^b	11035	Intron 1	G/C	0.178	0.160	0.223	1.00E-04	0.3311
Intergenic: 19p13.3	1	rs353698	4191948 ^b	6057	–	A/G	0.267	0.266	0.293	0.2181	0.5425
CCDC94: 19p13.3	1	rs2302164	4198005 ^b	–	5' near gene	C/G	0.189	0.170	0.162	0.9732	0.7303
CCDC22: Xp11.23	1	rs2294018	48985575 ^c	17255	Intron 3	A/G	0.384	0.339	0.437	0.2721	–
FOXP3: Xp11.23 OMIM: 300292	1	rs2232365	49002830 ^c	2355	Intron 2	A/G	0.430	0.395	0.367	0.6293	–
	2	rs3761548	49005185 ^c	10782	Intron 1	A/C	0.198	0.206	0.172	0.4492	–
PPP1R3F: Xp11.23	1	rs4824747	49015967 ^c	–	Intron 1	G/T	0.233	0.190	0.202	0.0879	–

^a OMIM, Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/Omim>).

^b SNP position in chromosome 19 in the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>).

^c SNP position in chromosome X in the NCBI dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>).

^d MAF, minor allele frequency.

^e MAF for Chinese in the NCBI dbSNPs database.

^f Allergic rhinitis subjects.

^g P value for difference in allele distributions between allergic rhinitis and control subjects.

^h HWE, Hardy-Weinberg equilibrium, only for SNPs in chromosome 19.

Table 3
Genotype frequencies of 7 tag SNPs among AR and control subjects, and their associations with AR risk.

Gene	SNP ID	Genotype	AR subject		Control subject		P (2 df) ^a	Logistic regression	
			No.	Frequency	No.	Frequency		OR (95%CI)	p ^b
EBI3	rs428253	CC	221	73.18%	152	59.38%	4.38E-05	1.00 (referent)	
		CG	81	26.82%	94	36.72%		0.624 (0.426–0.914)	0.015
		GG	0	0.00%	10	3.91%		NA ^c	NA ^c
Intergenic	rs353698	AA	170	51.83%	152	50.67%	0.037	1.00 (referent)	
		AG	144	43.90%	120	40.00%		1.038 (0.773–1.393)	0.803
		GG	14	4.27%	28	9.33%		0.606 (0.351–1.045)	0.071
CCDC94	rs2302164	GG	263	72.45%	220	70.51%	0.223	1.00 (referent)	
		GC	82	22.59%	83	26.60%		1.334 (0.604–2.942)	0.476
		CC	18	4.96%	9	2.88%		1.048 (0.767–1.433)	0.768
CCDC22	rs2294018	GG	221	60.88%	179	57.37%	0.652	1.00 (referent)	
		GA	60	16.53%	56	17.95%		0.705 (0.462–1.078)	0.107
		AA	82	22.59%	77	24.68%		1.180 (0.855–1.630)	0.315
FOXP3	rs2232365	AA	198	55.00%	170	55.19%	0.532	1.00 (referent)	
		AG	69	19.17%	50	16.23%		1.003 (0.654–1.539)	0.989
		GG	93	25.83%	88	28.57%		1.319 (0.966–1.803)	0.082
FOXP3	rs3761548	CC	268	74.03%	242	77.07%	0.524	1.00 (referent)	
		CA	52	14.36%	36	11.46%		1.065 (0.672–1.691)	0.786
		AA	42	11.60%	36	11.46%		1.443 (0.954–2.183)	0.083
PPP1R3F	rs4824747	GG	283	77.53%	229	72.70%	0.345	1.00 (referent)	
		GT	43	11.78%	45	14.29%		0.801 (0.516–1.242)	0.321
		TT	39	10.68%	41	13.02%		1.126 (0.753–1.685)	0.562

AR = Allergic rhinitis.
^a Global P values [2 degrees of freedom (df)]: genotype frequencies in AR and control subjects were compared using a χ^2 test with 2 df.
^b P values from unconditional logistic regression analyses, adjusted for age and gender.
^c NA, not available because of the rarity of genotype.

EBI3 and coiled-coil domain containing 94 (CCDC94) genes, within an intergenic region spanning 17092 bp; whereas the 4 SNPs from chromosome X were selected from CCDC22, FOXP3, protein phosphatase 1, regulatory subunit 3F (PPP1R3F) genes, within an intergenic region spanning 30392 bp. All genotype distributions in the controls were consistent with those expected from the HWE (all $P > 0.05$) (see Table 2).

The genotype distributions of 7 selected tSNPs in AR and control subjects are also summarized in Table 2. In the single-locus analyses of AR risk, the allele frequencies of rs428253 were significantly different between the AR and control subjects ($P = 1.00E-04$), even after 10,000 permutations ($P < 0.05$).

Age and gender adjusted logistic regression analyses further revealed that in the codominant-effect model, as assessed by the AIC, a significant protective effect against AR was associated with EBI3 rs428253 ($P = 0.015$, OR = 0.624, 95% CI = 0.426–0.914 for CG/CC), compared with wild-type carriers (Table 3). Stratified analysis showed that neither age nor gender were significantly associated with increased risk of AR for any variant (data was not shown).

3.3. LD analysis and haplotype block structure

Fig. 1 shows plots of the pairwise LD (r^2 and D') values for the tag SNPs and LD structures in the selected region of chromosome X (Fig 1-A). The LD plot indicated that two identical blocks with high LD; block 1 encompassing the 3' region of CCDC22 and the 5' region of FOXP3 and block 2 encompassing the 3' region of FOXP3 and the 5' region of PPP1R3F (Fig 1-B); were associated with AR.

3.4. Haplotype analysis

Table 4 summarizes the associations between frequencies of the haplotypes in blocks rs2294018–rs2232365 and rs3761548–rs4824747 and risk of AR. None of the haplotypes investigated was found to be associated with an increased risk in the studied cohort. Assessment of associations between the frequencies of diplo-

types in the two blocks and risk of AR, however, indicated that diplotype rs3761548–rs4824747 with “AG” was significantly associated with AR risk (for one-copy, $P = 0.031$, OR = 1.755 [95% CI = 1.054–2.92]) (Table 5).

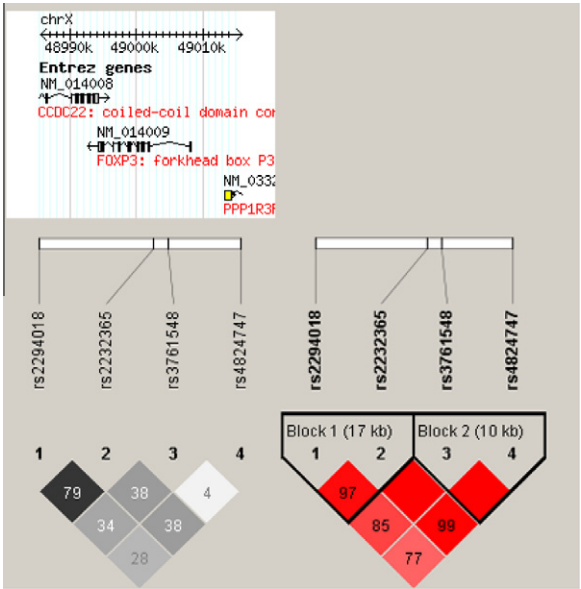


Fig. 1. Graphical representation of the SNP locations and LD structure of each gene. The figure was composed of chromosome scale (the top line with even division), the transcription string (the thick bars represent exon (yellow) or UTR (blue), the thin lines represent intron), SNP scale (the hollow bar with scales representing SNPs location), and graphic of LD (black-and-white) or block definition (flamulated). (A) The measure of LD (D') among all possible pairs of SNPs is shown graphically according to the shade of color, where white represents very low D' and dark represents very high D' . (B) The numbers in squares are D' values ($D' \times 100$). The measure of LD (r^2) among all possible pairs of SNPs is shown graphically according to the shade of color (B), where white represents very low r^2 and scarlet represents very high r^2 . The numbers in squares are r^2 values ($r^2 \times 100$).

Table 4

Associations between common haplotypes in chromosome X and AR risk.

Block	AR subjects		Control subjects		P^a	P_{sim}^b	Hap. Score ^c	Logistic regression		Global score test ^f
	No.	Frequency	No.	Frequency				OR (95% CI)	p^d	
Block1: rs2294018–rs2232365										
GA	481	63.62%	406	61.52%	0.4377	0.8531	0.18554	1.00 (referent)		Global-stat = 1.77767, df = 3, p -val = 0.61981, P_{sim}^b = 0.63474
AG	236	31.22%	225	34.09%	0.2816	0.6589	−0.44186	0.939 (0.741–1.191)	0.604	
GG	36	4.76%	25	3.79%	0.4144	0.36894	0.90586	1.368 (0.778–2.404)	0.276	
AA	3	0.40%	4	0.61%	NA ^e	NA ^e	NA ^e	0.417 (0.087–1.994)	0.273	
Block2: rs3761548–rs4824747										
CG	499	66.01%	425	64.39%	0.4647	0.91966	0.09949	1.00 (referent)		Global-stat = 2.94736, df = 2, p -val = 0.22908, P_{sim}^b = 0.23035
CT	121	16.01%	127	19.24%	0.0915	0.16871	−1.3808	0.814 (0.606–1.092)	0.170	
AG	136	17.99%	108	16.36%	0.4353	0.19943	1.28527	1.217 (0.900–1.646)	0.202	

AR = Allergic rhinitis.

^a P value for difference in haplotype frequency between AR and control subjects.^b Generated by permutation test with 100,000 times simulation.^c A positive (or negative) score for a particular haplotype would have suggested that the haplotype was associated with increased (or decreased) risk of AR.^d P values from unconditional logistic regression analyses.^e NA, not available because of the rarity of genotype.^f df, degrees of freedom.**Table 5**

Diplotype analysis of common haplotypes in chromosome X with AR risk.

Block	0-Copy		1-Copy logistic regression			2-Copy logistic regression			<i>P</i> (2df) ^b
	AR/C	OR (95%CI)	AR/C	P ^a	OR (95%CI)	AR/C	P ^a	OR (95%CI)	
<i>Block1: rs2294018-rs2232365</i>									
GA	95/91	1.000 (referent)	85/72	0.210	1.357 (0.842–2.186)	198/167	0.685	1.081 (0.744–1.570)	0.762
AG	352/312	1.000 (referent)	16/11	0.550	1.289 (0.560–2.967)	10/7	0.437	1.526 (0.526–4.430)	0.736
GG	222/182	1.000 (referent)	76/71	0.718	1.082 (0.707–1.654)	80/77	0.517	0.879 (0.596–1.298)	0.626
<i>Block2: rs3761548-rs4824747</i>									
CG	93/90	1.000 (referent)	71/55	0.085	1.579 (0.940–2.653)	214/185	0.727	1.068 (0.737–1.548)	0.625
CT	296/244	1.000 (referent)	43/45	0.417	0.813 (0.492–1.342)	39/41	0.246	0.745 (0.453–1.224)	0.395
AG	284/258	1.000 (referent)	52/36	0.031	1.755 (1.054–2.923)	42/36	0.604	1.148 (0.686–1.909)	0.504

AR = Allergic rhinitis subjects; C = control subjects.

^a P values from unconditional logistic regression analyses.^b Global P values [2 degrees of freedom (df)]: diplotype frequencies in cases and controls were compared using a χ^2 test with 2 df.

4. Discussion

In this study, we aimed to evaluate the contribution of SNPs in the FOXP3 and EBI3 genes towards AR susceptibility in Han Chinese subjects by employing a population-based case-control association analysis. In the single-locus analyses of AR risk, the allele frequencies of rs428253 were significantly different between the AR and control subjects ($P = 1.00E-04$). Logistic regression analyses, adjusted for age and gender, further revealed that significant protective effects against AR were associated with EBI3 rs428253 ($P = 0.015$, OR = 0.624 for CG/CC). While AR risk was not found to be associated with any specific haplotype in either block rs2294018–rs2232365 or block rs3761548–rs4824747, the diplotype rs3761548–rs4824747 with “AG” was found to be associated with significant increased risk of AR ($P = 0.031$, OR = 1.755) in this population. To our knowledge this is the first study to demonstrate the contribution of the polymorphisms in both FOXP3 and EBI3 genes involved in the T_{reg} pathway to the susceptibility of an individual to developing AR in the present population.

As T_{reg} cells are thought to play a pivotal role in maintaining the balance within the T-cell populations and their effects, it has been suggested that modifications in T_{reg} -cell function/signaling may lead to deviated immune responses [25]. Indeed, Lee and

colleagues [26] have provided evidence that the levels of CD4+CD25+ T_{reg} cells in pediatric patients with AR and bronchial asthma are related to airway allergy and disease severity. Moreover, it has been suggested that the FOXP3 gene, localized in the short arm of the X-chromosome, is particularly important as it appears to specifically control regulatory T-cell function [27]; with deletions or change in function in the human FOXP3 gene resulting in the development of severe immune dysregulation with polyendocrinopathic and enteropathic X-linked syndrome [28]. Similarly, other studies have demonstrated that asthmatic and AR patients have decreased FOXP3 protein expression within their CD4+CD25+ T_{reg} [29,30].

The findings of our study for SNPs in the FOXP3 gene are in accordance with other studies. We have previously reported that rs3761548 in FOXP3 gene was significantly associated with AR in a relatively small cohort of Chinese subjects [19]. Similarly, in another cross-sectional population-based study Suttner and colleagues [31] investigated the influence of related SNPs in FOXP3 gene on atopic diseases in a sample of 3099 German children and demonstrated that specific genetic variations within and in regions around FOXP3 may modify the risk for hay fever. More recently, Fodor and colleagues [32] performed a case-control study to investigate the role of polymorphisms in FOXP3 gene in the Hungarian population. The authors demonstrated that females homozygous

for the FOXP3 rs3761548 allele (A/A) were protected against AR, whereas females who were either wild types (C/C) or heterozygote carriers (C/A) of this allele were more susceptible to AR. In the present study, although we could not replicate the the association between FOXP3 polymorphisms (rs3761548, rs3060515 and rs3761547) and AR presented in our previous study [19], we have demonstrated the diplotype rs3761548-rs4824747 with “AG” may increase the risk of developing AR in Han Chinese subjects.

EBI3, first identified in B lymphocytes based on its induction following infection with EB virus, is a member of the IL-12 heterodimeric cytokine family [33], which can associate with either p28 to form the heterodimeric cytokine IL-27, or with the p35 subunit of IL-12 to form the EBI3/p35 heterodimer (also known as IL-35) [12,34,35]. Collison and colleagues [12,34,35] have demonstrated that in mice IL-35 was expressed constitutively by CD4⁺CD25⁺Foxp3⁺T_{reg} cells, and was likely to contribute to their suppressive activity. Moreover, T_{reg}-cell restriction of this cytokine occurred because EBI3 was a downstream target of FOXP3, a transcription factor that is required for T_{reg}-cell development and function [12]. Although a recent study has reported an association between deficiency in CD4⁺CD25⁺T_{reg} cell suppressive activity and decreased expression of molecular mediators of EBI3 and FOXP3 in a murine AR model [36], Tong and colleagues [37] have demonstrated that the development and suppressive function of T_{reg} to inhibit effector T cell proliferation was not affected by EBI3-deficiency. Similarly, a study in humans has shown that EBI3 transcripts were detected only in activated T_{eff} cells, but not in resting or activated T_{reg} cells [38], suggesting that human T_{reg} cells do not express detectable amounts of IL-35. In the present study we explored the potential molecular mechanism/s underlying the role of EBI3 in AR. We detected that the allele frequencies of rs428253 located in the intron of EBI3 gene were significantly different between the AR cases and the controls and that EBI3 rs428253 (CG/CC) was associated with a significant protective effect against the risk of AR.

In summary, our study has confirmed the findings of others on the association between the FOXP3 gene in subjects with AR. Furthermore, our study has for the first time provided evidence also for an association between the EBI3 gene and development of AR in Chinese Han subjects. Although the genetic contribution of FOXP3 and EBI3 was not identified with strong statistical significance (i.e. lower *P* values), the current data imply that potential genetic variations in the region of and around FOXP3 and EBI3 genes may modify the risk for AR. However, further functional analyses are needed to determine whether the associations of SNPs in FOXP3 LD block observed with AR may be influenced by polymorphisms in genes close to FOXP3 and/or locus regulatory elements in the area. Similarly, the precise roles of EBI3 and FOXP3 in T_{reg} cell development and function need to be elucidated in larger cohorts of subjects with AR from different ethnic and cultural backgrounds to provide a better understanding of the mechanisms underlying the aetiology of AR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.humimm.2012.07.319>.

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