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FOXP3 and TGF-β Gene Polymorphisms in Allergic Rhinitis

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ABSTRACT

Background: Regulatory $CD4^+$T (Treg) cells are effective in maintaining immune tolerance. **Objective:** To investigate single nucleotide polymorphisms (SNPs) of Transforming Growth Factor β-1 (TGF-β1) and Forkhead Box Protein 3 (FOXP3) genes in Iranian patients with allergic rhinitis (AR). **Methods**: Variations at codons 10 and 25 of TGF-β1 and FOXP3 at positions -3279 A>C and -924 A>G were evaluated in AR patients and compared with controls. In a case-control study, 155 AR patients and 163 allergy-free controls were genotyped using polymerase chain reaction sequence-specific primer (PCR-SSP) technique. **Results:** The analysis of the frequency of these SNPs showed that the haplotype formed by FOXP3 -3279 A allele occurred significantly more frequently in patients than controls (odds ratio=1.44, 95% CI=1.312-2.66; p=0.001). **Conclusion:** Our results suggest that polymorphism in FOXP3 gene is associated with susceptibility to AR.

Keywords: Allergic Rhinitis, FOXP3, Gene Polymorphisms, TGF-β

INTRODUCTION

Allergic rhinitis (AR) is the most common chronic allergic inflammatory disease of the nasal mucosa, induced by IgE-mediated type I hypersensitivity. AR is consistent with over secretion of TH2 cytokines, regulatory T (Treg) cells defect and selective eosinophil accumulation following exposure to an allergen in genetically susceptible patients (1,2,3). AR is a global health problem, with a prevalence of 9-42% among the general population (4). Studies have considerably focused on the genetic basis of allergic diseases (5,6). FOXP3 and TGF-β proteins are specific markers for Treg cells development and function (7-9). Treg cells have an effective role in immune reaction and maintain peripheral tolerance against antigens, including auto immunogens and allergens by the production of anti-inflammatory cytokines such as IL-10 and TGF-β (10-12). T helper cytokines imbalance is remarkable in AR patients and may promote the production of the mucosal immunoglobulins E and A (IgE, IgA) and may result in the

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development of T herlper 2 cells in allergic individuals**.** Some studies have suggested that the polymorphisms of FOXP3 and TGF-β genes may functionally or quantitatively modify their secretion in autoimmune and allergic diseases, therefore leading to defects in the regulatory function of Treg cells. These defects reflect the role of these polymorphisms in several human immune-mediated diseases such as, psoriasis (13), systematic lupus erythematosus (14,15), autoimmune thyroid liver diseases (16,17), malignancies (18) and allergy (19-22). Therefore, evaluation of such polymorphisms may predict the susceptibility to certain diseases. In Iran, prevalence of AR is reported to be 10-15% and the tendency of increase in the prevalence has been observed (23-28). Hence, it is necessary to investigate the effect of FOXP3 and TGF-β polymorphisms on the susceptibility to allergic diseases in different populations. In this research, we evaluated the association between FOXP3 and TGF-β genes polymorphisms with susceptibility to AR and also studied the relationships between these variations with total serum IgE and IgA levels and the peripheral blood eosinophil counts.

MATERIALS AND METHODS

Study Population. In a case-control study, 155 AR patients (57 males and 98 females) were recruited from Allergy ward of Tooba clinic and BooAli university Hospital of Mazandaran province in Iran from April 2010 to March 2011. The 163 controls (61 males and 102 females) were also matched with controls according to age, gender and living habitat. The controls had no clinical features or family history of allergy and were recruited from the general population after undergoing a comprehensive medical screening. Patients were diagnosed based on symptoms such as: a typical history of sneezing, rhinorrhea, nasal obstruction, swollen turbinate, elevated total serum IgE level and eosinophil count more than 5% according to the criteria of AR and its Impact on Asthma (29). All subjects in this study were Iranians. The study was approved by the local ethics committee, and informed consent was obtained from all participants.

Samples Collection and Preparation. 5 ml of peripheral blood was taken under aseptic conditions and divided into 2 portions: 1.5 ml of whole blood was collected in sterile EDTA-containing tubes for DNA extraction and eosinophil counts, and the remainder was left for 30 to 60 minutes for spontaneous clotting at room temperature before being centrifuged at 3000 rpm for 10 minutes. Serum samples were kept at -20°C for determination of total IgE and IgA levels.

Measurement of IgE and IgA. Total IgE level was measured by ELISA using Accu-Bind IgE Quantitative Kits (Lake Forest, California, USA) and Serum total IgA was measured using a Nephelometric method (MININEPHTM Human IgA KIT, UK) according to the manufacturer's instructions. Eosinophil counts were determined based on the eosinophil percent per total white blood cells with giemsa staining in direct smears.

DNA Extraction and Genotyping. Genomic DNA was extracted from Whole blood using a DNA extraction kit (Roche, Germany) according to the manufacturer's instruction. Genotyping was conducted by using the polymerase chain reaction with sequence-specific primers (PCR-SSP). The Primer sequences (13) for genotyping are shown in Table 1. Human EGFR (30) and GAPDH (31) genes were used as an internal control. PCR amplification was performed according to an established protocol. Briefly, the procedure was performed in a total volume of 25 µl solution containing 50 ng of ge-

nomic DNA, 200 μM of each dNTP (mixture of dATP, dTTP, dCTP, dGTP), 0.2 μM of each primer, 1.5 mM of MgCl2, 10 mM of Tris hydrochloride (pH 8.3), and 1 U of Taq DNA polymerase (Fermentas, Italy). Cycling conditions included an initial denaturation step of 94°C (4 minutes), followed by 35 cycles at 94°C (30 sec.), 61°C (30 sec.) and 72°C (40 sec.), and a final extension step of 5 minutes at 72°C. PCR products were separated on a 1.5% agarose gel electrophoresis and visualized by 0.5 μg/ml ethidium bromide staining under an ultraviolet illuminator. 10% of the subjects were randomly genotyped in duplicate for each polymorphism, and the results were 100% concordant.

Table 1. Primers used in the genotyping SNPs in the FOXP3 and TGF-β genes.

Gene	Position	Method	Primer sequences	PCR product
FOXP3	-3279 A>C (rs3761548)	PCR-SSP	Forward: 5′-CTGGCTCTCTCCCCAACTGA-3' Forward: 5′-TGGCTCTCTCCCCAACTGC-3' Common Reverse: 5′-CAGAGCCCATCATCAGACTCTCTA-3'	334 bp C: 333 bp
	-924 A>G (rs2232365)		Forward: 5'-CCCAGCTCAAGAGACCCCA-3' Reverse: 5'-GGGCTAGTGAGGAGGCTATTGTAAC-3' Forward: 5'-CCAGCTCAAGAGACCCCG-3' Reverse: 5'-GCTATTGTAACAGTCCTGGCAAGTG-3'	A: 442 bp G: 427 bp
TGF-β	+869 T>C (rs1982073)		Forward(C): 5′-GCAGCGGTAGCAGCAGCG-3′ Forward(T): 5′-AGCAGCGGTAGCAGCAGCA-3′ Common reverse: 5′-TCCGTGGGATACTGAGACAC-3′	241 bp
	+915 G>C (rs1800471)		Forward(G): 5′-GTGCTGACGCCTGGCCG -3′ Forward(C): 5′-GTGCTGACGCCTGGCCC -3′ Common reverse: 5′- GGCTCCGGTTCTGCACTC-3	233 bp

Statistical Analysis. Chi-square analysis was used to test for the deviation of genotype distribution and for the comparison of differences in genotype combinations among groups. The risk associated with individual alleles or genotypes was calculated as the odds ratio with 95% confidence intervals (CI) using SPSS 18 software. One-way analysis of variance (ANOVA) was applied for comparison of the total serum IgE and IgA levels and the peripheral blood eosinophil counts in different genotypes. P value of less than 0.05 was considered statistically significant.

RESULTS

Determination of Immunoglobulins and the Eosinophil Counts. A total of 318 unrelated subjects (118 males, 200 females) living in north of Iran were included in this study. Summary of the demographic characteristics of study population is illustrated in Table 2.

Table 2. Demographic Characteristics of AR Patients and Controls.

	Controls (No=163)	AR Patients (No=155)	P value *
Age (y) (mean ± SD)	30.1 ± 10.8	30.2 ± 10.33	NS*
Gender (Male/Fmale)	61/102	57/98	NS*
Eosinophil (%), (mean ± SD)	1.7 ± 0.87	5.2 ± 3.65	0.001**
Serum IgE (IU/ml) (mean ± SD)	74.1 ± 48.3	286.6 ± 166.8	0.001**
Serum IgA(g/l) (mean ± SD)	1.93 ± 0.63	2.11 ± 0.79	0.03**

Abbreviation: NS = not significant, SD= standard deviation
* Values were determined by two-sided x^2 test
**Statistically significant differences between groups were tested using Student's t-test

Age and gender were well balanced between the case and the control groups. There was a significant increase in total IgE levels and eosinophil count in the AR groups compared with the control group (p=0.001). Serum total IgA in AR patients was significantly more than control group. However, the level of IgA in both groups was normal.

FOXP3 and TGF-β Genotypes. The frequencies of genotypes and alleles in all cases and controls are shown in Table 3. Because of FOXP3 is located on the X-chromosome, data analyses were divided into female and male groups. The frequency of the FOXP3-3279 AA genotype and an allele in the patient group increased significantly in comparison with the controls ($p<0.05$). In contrast, no significant differences of allele and genotype frequencies of FOXP3 -924 A>G SNP were observed between AR patients and the control subjects in both female and male groups. In addition, there was no statistically significant differences between TGF-β gene of the case and the control groups ($p>0.05$). FOXP3 -3279 A>C SNP was associated with disease activity indexes (Table 4).

Statistically, there were significant increases in IgE levels and the eosinophil counts in FOXP3 -3279 AA group compared with the homozygous CC in both female and male groups. However, there was no significant difference in the serum IgA levels in the genotypes ($P>0.05$). Also, there was no relationship between the disease activity indexes and FOXP3 -924 A>G or TGF-β SNPs (Table 4).

Table 3. Genotype and allele frequencies of FOXP3 and TGF-β SNPs in cases and controls and their association with a risk for AR.

Position	Gender (No)	Genotype/ Allele	Controls No (%)	AR patient No (%)	Odd Ratio [a] (95% CI)	P [b]
FOXP3	Female	AA	5 (4.9)	18 (18.4)	1:00 (Reference) [c]	
-3279 A>C		AC	58 (56.9)	57 (58.2)	0.16 (0.05-0.5)	0.003
		CC	39 (38.2)	23 (23.5)	0.60 (0.319-1.13)	
	Male	A	21 (34.4)	30 (52.6)	1:00 (Reference)	0.035
		C	40 (65.6)	27 (47.4)	0.47 (0.22-0.99)	
	Total	A	89 (33.6)	123 (48.6)	1:00 (Reference)	0.001
		C	176 (66.4)	130 (51.4)	0.53 (0.37-0.76)	
FOXP3	Female	AA	76 (74.5)	59 (60.2)	1:00 (Reference)	
-924 A>G		AG	22 (21.6)	32 (32.7)	2.25 (0.63-5.06)	0.094
		GG	4 (3.9)	7 (7.1)	1.2 (0.314-4.6)	
	Male	A	48 (78.7)	46 (80.7)	1:00 (Reference)	0.786
		G	13 (21.3)	11 (19.3)	0.88 (0.36-2.17)	
	Total	A	222 (83.8)	196 (77.5)	1:00 (Reference)	0.075
		G	43 (16.2)	57 (22.5)	1.71 (1.11-2.64)	
TGF-β	Total	TT	62(38.0)	58 (37.4)	1:00 (Reference) [c]	
+869 T>C		TC	89 (54.6)	80 (51.6)	1.51 (0.66-3.44)	0.52
		CC	12 (7.4)	17 (11.0)	1.57 (0.71-3.50)	0.62
		T	213 (65.3)	196 (63.2)	1:00 (Reference)	
		C	113 (34.7)	114 (36.8)	1.64 (1.16-2.33)	
TGF-β	Total	GG	143 (87.7)	126 (81.3)	1:00 (Reference)	
+915 G>C		GC	18 (11.0)	25 (16.1)	2.27 (0.41-12.60)	0.26
		CC	2 (1.2)	4 (2.6)	1.44 (0.23-8.73)	0.09
		G	304 (93.3)	277 (89.4)	1:00 (Reference)	
		C	22 (6.7)	33 (10.6)	1.65 (0.94-2.89)	

Logistic regression analyses were used for calculating odds ratios with 95% confidence interval
[b] Was determined by x^2 test (for genotype) or Fisher exact test (for alleles) from a 2×2 and 2×3 contingency table
[c] The first allele or genotype is considered as reference

Table 4. Analyses of serum total IgE and IgA levels and peripheral blood eosinophil counts between the genotypes of FOXP3 and TGF-β genes SNPs.

Position	Gender	Genotype (No)	IgE		IgA		Eosinophil	
			mean ± SD	P*	mean ± SD	P*	mean ± SD	P*
FOXP3 -3279A>C	Female	AA (23)	386.7±183.4	0.001	2.25±0.76	0.101	4.87±4.01	0.003
		AC (115)	148.1±136.6		1.92±0.68		3.27±2.98	
		CC (39)	158.9±144.7		1.98±0.57		2.53±1.70	
	Male	A (51)	216.7±181.8	0.014	2.19±0.85	0.203	4.62±3.93	0.016
		C (67)	144.3±132.8		2.00±0.76		3.02±3.18	
FOXP3 -924A>G	Female	AA (135)	176.8±169.6	0.399	1.93±0.64	0.303	3.25±2.90	0.888
		AG (54)	171.1±143.4		2.05±0.70		3.26±2.80	
		GG (11)	243.0±169.3		2.18±0.68		2.81±1.94	
	Male	A (94)	169.2±160.7	0.396	2.08±0.77	0.696	3.87±3.87	0.366
		G (24)	200.3±154.4		1.96±0.92		3.12±2.19	
TGF-β +869T>C	Total	TT (120)	154.98±150.6	0.073	1.9±0.7	0.12	3.3±2.9	0.36
		TC (169)	185.78±160.2		2.0±0.7		3.4±3.2	
		CC (29)	224.31±198.2		2.2±0.7		4.2±3.8	
TGF-β +915G>C	Total	GG (269)	176.6±163.4	0.92	2.0±0.7	0.81	3.4±3.1	0.59
		GC (43)	185.1±151.7		2.0±0.6		3.2±3.0	
		CC (6)	164.5±158.2		1.8±0.7		5.3±2.1	

* Values were statically analyzed by ANOVA.

DISCUSSION

Treg cells have an important role in immune response and the maintenance of peripheral tolerance against foreign antigens by secretion of anti-inflammatory cytokines such as IL-10 and TGF-β in allergic patients (10-12). Our results suggested that the FOXP3-3279 A alleles were associated with an increased risk of AR in an Iranian population. Therefore, it might suggest that -3279 A allele is considered as a risk allele. In addition, the total serum IgE and peripheral blood eosinophil count in FOXP3 -3279AA genotype were significantly more than FOXP3 -3279CC and AC genotypes. These results showed that Treg functions differently or inadequatly in individuals carrying this SNP. We speculated that subjects having allele A may be more susceptible to regulation than patients with allele C. However, the frequency of the FOXP3-924 A>G SNP between females and males was not significant. Since, FOXP3 is located on the X-chromosome (females have two X-chromosomes while males have only one X-chromosome), this study showed different genes polymorphism frequencies for the males and females (Table 3 and 4). A case-control study by Foder et al. Demonstrated that females homozygous for rs3761548 foxp3 polymorphism are protected against AR (32).

Present data is consistent with previous studies which showed FOXP3 rs3761548 is associated with AR (19,20). Myeong Lee et al. (33) reported that patients with AR had a significantly lower FOXP3 gene expression than the controls. Furthermore, our results is in agreement with that of Shen et al. (34) who demonstrated that FOXP3 rs3761548 AA genotype, leads to a defective transcription of Foxp3 gene in psoriatic patients.
Foxp3, a gene marker produced not only from the $CD4^+$ T cells, but also from $CD8^+$Tcells, is correlated with the levels of anti-proinflammatory cytokines such as TGF-β and IL-10. Increases or decreases in the expression of TGF-β have been linked to numerous diseases. Some studies showed that genetic polymorphisms at position +869 T>C (Leu-to–Pro) and +915 G>C (Arginine- to- Proline). In TGF-β gene may change TGF-β functionally or quantitatively (35,36). Gentile et al. suggested that TGF-β genotypes contribute in the pathogenesis of AR and asthma (37). In addition, Kim et al. showed that TGF-β polymorphisms may contribute to the development of rhino sinusitis (38). In contrast, our study showed that polymorphisms in these regions cannot be associated with susceptibility to AR.
In conclusion, FOXP3 polymorphisms appear to contribute to the risk of AR. Also, this study has provided the first genetic data on the FOXP3 gene in our population and a basis for searching immune-mediated disease-related FOXP3 haplotype.

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