Original Article

The Role of *FOXP3* Polymorphisms in Graves' Disease with or without Ophthalmopathy in a Turkish Population

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Abstract

Objectives: Forkhead box P3 (*FOXP3*) gene polymorphisms have been evaluated in many autoimmune diseases, including Graves' disease (GD), in different populations. However, those polymorphisms have not been analyzed in GD or Graves' ophthalmopathy (GO) in the Turkish population. In this study, we aimed to evaluate the frequency of *FOXP3* polymorphisms in GD with or without ophthalmopathy in a Turkish population.

Materials and Methods: The study included 100 patients with GO, 74 patients with GD without ophthalmopathy, and 100 age- and sex-matched healthy controls. In all study participants, rs3761547 (-3499 A/G), rs3761548 (-3279 C/A), and rs3761549 (-2383 C/T) single nucleotide polymorphisms (SNPs) were detected using the polymerase chain reaction-restriction fragment length polymorphism method. The chi-square test was used to evaluate genotype and allele frequencies. Odds ratios and 95% confidence intervals were calculated for genotype and allele risks.

Results: In the patient group (including GD with or without ophthalmopathy), the rs3761548 AC and AA genotype and rs3761549 CT genotype were significantly more frequent than in the control group (all $p<0.05$). No genotypic and allelic differences were observed for rs3761547 between the patient and control groups (all $p>0.05$). There was no statistically significant difference between the GO and GD without ophthalmopathy groups concerning the allele and genotype frequencies of all three *FOXP3* SNPs (all $p>0.05$).

Conclusion: The AC and AA genotypes of rs3761548 (-3279) and CT genotype of rs3761549 (-2383 C/T) were shown to be possible risk factors for GD development in the Turkish population. However, none of the three SNPs was shown to be associated with the development of GO in patients with GD.

Keywords: Forkhead box P3, Graves' disease, Graves' ophthalmopathy, single nucleotide polymorphisms

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Introduction

Graves' disease (GD) is an autoimmune disorder that causes diffuse enlargement of the thyroid gland and hyperthyroidism with elevated thyroid-specific autoantibody levels. GD is more common in women, and it usually occurs between the ages of 20 and 40 years.[1,2] Up to 50% of GD patients also develop Graves' ophthalmopathy (GO), which is another autoimmune disease that affects the orbital structures.[3] GO varies in clinical severity and is assessed according to the European Group on Graves' Ophthalmopathy (EUGOGO) classification.[3]

Thyroid-stimulating hormone receptor (TSHR) was shown to be the main autoantigen in GO, as in GD.[4] Both diseases have a complex pathogenesis involving interactions between genetic and environmental factors.[5] Among the genetic factors, *CTLA-4, TSHR, Tg, CD40,* and *PTPN22* polymorphisms and HLA class II gene variants were shown to be shared risk factors between GO and GD.[6] However, one polymorphism in *IL1A* was found to favor GO development in GD compared to GD patients without GO.[7] Additionally, another study in the Polish population showed that a *VDR* polymorphism may contribute to the development of GO.[8] From a clinical point of view, it is essential to identify patients with higher risk of developing GO in the course of GD, and we still need reliable genetic risk factors to act upon.

The immune system is primarily under the control of regulatory T-cells (Tregs).[9] Tregs were shown to be pivotal factors in the pathogenesis of human autoimmune diseases, including GD and GO.[10,11,12] The Forkhead box P3 (*FOXP3*) gene is located on the X chromosome, and its protein product FoxP3 is predominantly expressed in Tregs as a transcription factor. A deficiency of FoxP3 may impair the immunosuppressive effect of Tregs and lead to autoimmune diseases.[13,14] An association between *FOXP3* polymorphisms and the development of GD has been reported in different populations.[10,13,15,16,17,18,19,20,21,22] According to a recent meta-analysis of seven case-control studies, the *FOXP3* polymorphism rs3761548 was associated with GD susceptibility in Asians, and rs3761549 was associated in both Asians and Caucasians.[15] Despite several studies about the relationship between *FOXP3* single-nucleotide polymorphisms (SNPs) and GD susceptibility, none have been conducted in the Turkish population. Additionally, only two studies with small numbers of GO patients investigated the relationship between *FOXP3* SNPs and the risk of GO in GD patients.[13,17] Therefore, our study is the first to investigate three SNPs in the *FOXP3* gene (-2383 C/T, -3279 C/A, and -3499 A/G) in Turkish GD patients. In addition, we evaluated whether any of those SNPs favor GO development in a larger GD patient population.

Materials and Methods

Participants

This prospective case-control study was conducted between January 2019 and January 2022 and included 174 patients with GD (74 without GO [non-GO] and 100 with GO) and 100 healthy controls. The diagnosis of GD was made in the Department of Endocrinology and Metabolic Diseases based on the guidelines of the American Association of Clinical Endocrinologists.[23] The diagnostic criteria included hyperthyroidism, elevated thyrotropin receptor antibody level, and typical thyroid ultrasound patterns. The presence of ophthalmopathy was evaluated by three ophthalmologists (K.S.C., B.T., and O.K.), and its severity was assessed according to the EUGOGO classification.[3] Patients who had GD for at least five years and did not have ophthalmopathy were included in the non-GO group. The healthy control group included age- and sex-matched individuals who had no history of GD, allergic diseases, or other autoimmune disease. Age at onset, disease duration, cigarette smoking status, and family history of GD were collected from hospital records.

We obtained written informed consent from all participants before collecting samples. The study protocol was approved by the Clinical Research Ethics Committee of Gazi University (decision no: 2018-824/1, date: 12.11.2018). This study also met the standards of the Declaration of Helsinki.

Sample Collection and Genotyping

Four milliliters of peripheral blood was collected from the patients and healthy controls in tubes with ethylenediamine tetraacetic acid for genotyping. Genomic DNA was isolated using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA). The genotypes of rs3761548, rs3761549, and rs3761547 in the *FOXP3* gene (gene ID: 50943, Xp11.23) were determined by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) assay.

For PCR, a total volume of 25 µL mixture was prepared using 50 ng genomic DNA template, 1×PCR buffer (Thermo Scientific™-Thermo Fisher Scientific Inc., USA) with 2 mM $MgCl_2$, 0.4 µmol of primers (IDT-Integrated DNA Technologies, Inc., USA), 0.20 mM dNTPs (Invitrogen-Thermo Fisher Scientific Inc., USA), and 1 U DNA polymerase (Thermo Scientific™-Thermo Fisher Scientific Inc., USA). In the standard PCR procedure, the first step is to denature the DNA at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 59 °C (rs3761547 and rs3761548) or 60 °C (rs3761549) for 30 seconds, and extension at 72 °C for 30 seconds. Ten µL of the PCR products were used for RFLP. The restriction enzyme Pst1 (New England Biolabs GmbH, Germany) was used for detection of rs3761547 (1 U at 37 °C for 16 h), BseN1 (BsrI) (New England Biolabs GmbH, Germany) was used for rs3761548 (1 U at 65 °C for 16 h), and PvuII (New England Biolabs GmbH, Germany) was used for rs3761549 (1 U at 37 °C for 16 h). Undigested and digested PCR products were analyzed by 3% agarose gel electrophoresis. The primers and restriction enzymes used for this assay and the product sizes before and after digestion were shown in Table 1.

Statistical Analysis

Statistical data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). We used Student's t-test for normally distributed variables and the chi-square test for categorical variables (sex, family history). The Hardy-Weinberg equilibrium (HWE) for SNP polymorphisms was calculated using the chi-square test. The chi-square test was also used to compare the genotype and allele frequency distributions of the polymorphisms in the patient and control groups. We used logistic regression analyses to determine odds ratios (ORs) with 95% confidence intervals (CIs) for specific genotypes and alleles. A p value <0.05 was considered statistically significant.

Results

Characteristics of the Participants

One hundred GD patients with GO, 74 GD patients without GO, and 100 control participants were enrolled in this study. There was no significant difference in age and sex distribution among the groups. The mean age was 36.1±12 years for controls and 37±12.6 years for the study group (p=0.33). The respective distributions of males and females were 24.3% and 75.7% in the non-GO group, 26.0% and 74.0% in the GO group, and 32.0% and 68.0% in the control group (p=0.48) (Table 2).

FOXP3 SNP Allele and Genotype Frequencies

The distribution of allelic and genotypic frequencies of the three analyzed *FOXP3* polymorphisms in the patient group (non-GO and GO) and the control group, along with their associations with the risk of GD, are summarized in Table 3.

Table 1. Primers for *FOXP3*

Position	Enzyme	Forward primer (5'→3') Reverse primer (3'→5')	Amplicon size	RFLP pattern
-2383 C/T, rs3761549	Bsrl1	gcctggcactctcagagcttcaa cgacaccacggaggaagagaaga	487 bp	CC-329bp, 15 bp AC-487bp, 329bp, 158bp AA-48bp
-3279 C/A, rs3761548	Pst1	cctctccgtgctcagtgtag gcctcagccttcgccaata	261 bp	CC-184bp, 127bp, 77bp CT-261bp, 184bp, 127bp, 77bp TT-261bp, 127bp
-3499 A/G, rs3761547	Pvu11	gcaatcctcctctcgcacac tgcagggcttcaagttgacag	158 bp	AA-158bp AG-158bp, 123bp, 35bp GG-123 bp, 35 bp

FOXP3: Forkhead box P3, RFLP: Restriction fragment length polymorphism

In controls, all SNPs were in HWE (rs3761547, p=0.871; rs3761548, p=0.126; and rs3761549, p=0.068). For the patient group, rs3761547 (p=0.992) and rs3761548 (p=0.143) were in HWE, whereas rs3761549 was not in HWE (p=0.036).

For rs3761547, the frequencies of the AA, AG, and GG genotypes respectively were 147 (84.5%), 26 (15%), and 1 (0.5%) in GD patients and 90 (90%), 10 (10%), and 0 (0%) in the control group. The statistical analysis showed that the distribution of alleles and genotypes of the *FOXP3* rs3761547 polymorphism were not significantly different in GD patients and controls (p=0.23 and p=0.17, respectively) (Table 3).

For rs3761548, the frequencies of the CC, CA, and AA genotypes were 50 (28.7%), 74 (42.5%), and 50 (28.7%) in the patient group (non-GO and GO), compared to 66 (66%), 26 (26%), and 8 (8%) in the control group, respectively. The distribution of genotypes differed significantly between study patients and healthy controls (p<0.0001). When the most common genotype in the control group, CC, was used as a reference, it was found that the CA genotype was associated with a higher risk of GD (OR: 3.8; 95% CI: 2.1-6.7). Moreover, the frequency of the A allele was significantly higher in patients than controls (50% vs. 21%, p<0.0001), showing that carriers of the A allele have a significant risk for developing GD (OR: 3.8; 95% CI: 2.5-5.6) (Table 3).

For rs3761549, the respective frequencies of the CC, CT, and TT genotypes were 131 (75.3%), 35 (20.1%), and 8 (4.6%) in the patient group (non-GO and GO) versus 88 (88%), 10 (10%), and 2 (2%) in the control group. The genotypic distribution differed significantly between GD patients and healthy controls (p=0.02). Using the most common genotype in the control group (CC) as a reference, the CT genotype was found to be associated with a higher risk of GD (OR: 2.3; 95% CI: 1.1-4.9). Additionally, the frequency of the T allele was significantly higher in patients than controls (14.7% vs. 7%, p=0.007), showing that carriers of the T allele have a significant risk for developing GD (OR: 2.3; 95% CI: 1.2-4.2) (Table 3).

Comparison of genotypic and allelic distributions of each of the three *FOXP3* SNPs between the GO and non-GO groups showed no statistically significant difference (Table 4). Furthermore, the association between the *FOXP3* genotypes (-2383 C/T and -3279 C/A) and demographic variables was also carefully analyzed. We found no association between the investigated *FOXP3* polymorphisms and patients' age, family history, sex, or smoking status (Table 5).

Discussion

FoxP3 is predominantly expressed in CD34+ CD25 Tregs and plays a critical role in maintaining the suppressive function of Tregs.[13,14] Genetic variations in the *FOXP3* gene play a role in the pathogenesis of GD by weakening the suppressive functions of Tregs and enhancing autoimmune responses.[15,16,20] Although the relationship between *FOXP3* SNPs and GD has been demonstrated in several studies in different populations, few studies to date have compared the frequency of *FOXP3* SNPs between GD patients with and without orbitopathy.[13,17] This is the first study that investigated the relationship between three

Table 2. Demographic characteristics of groups

Variables	Non-GO n=74	GO n=100	Control n=100	p value
Age at onset of disease (mean ± SD)	35.5±12.1	37.8±12.4	-	0.66
Sex, n female/male	56/18	74/26	68/32	0.48
Duration of disease (years), mean ± SD	9.1±3.0	6.7±5.2	-	0.15
Family history (positive/negative)	38/36	58/42	-	0.42

GO: Graves' ophthalmopathy, SD: Standard deviation

Table 3. Genotypic and allelic distribution of the study and control groups				
Genotype	**Control group n=100**	**Study group (non-GO + GO) n=174**	**p value**	**OR (95% CI)**
rs3761548 (-3279 C/A)				
CC	66 (66%)	50 (28.7%)	-	1.0[a]
AC	26 (26%)	74 (42.5%)	**<0.0001**	3.8 (2.1-6.7)
AA	8 (8%)	50 (28.7%)	**<0.0001**	8.25(3.6-18.9)
Allele				
C	158 (79%)	174 (50%)	-	1.0[a]
A	42 (21%)	174 (50%)	**<0.0001**	3.8 (2.5-5.6)
rs3761549 (-2383 C/T)				
CC	88 (88%)	131 (75.3%)	-	1.0[a]
CT	10 (10%)	35 (20.1%)	**0.02**	2.3 (1.1-4.9)
TT	2 (2%)	8 (4.6%)	0.3	8.25 (0.5-12.9)
Allele				
C	186 (93%)	297 (85.3%)	-	1.0[a]
T	14 (7%)	51 (14.7%)	**0.007**	2.3 (1.2-4.2)
rs3761547 (-3499 A/G)				
AA	90 (90%)	147 (84.5%)	-	1.0[a]
AG	10 (10%)	26 (15%)	0.23	1.58 (0.73-3.43)
GG	0	1 (0.5%)	-	-
Allele				
A	190 (95%)	320 (92%)	-	1.0[a]
G	10 (5%)	28 (8%)	0.17	1.67 (0.73-3.43)

[a]The first listed allele/genotype is considered the reference value, bold values indicate statistical significance. Frequencies of genotypes and alleles were compared using chi-square test
GO: Graves' ophthalmopathy, OR: Odds ratio, n: Number, CI: Confidence interval

common polymorphisms in the *FOXP3* gene (rs3761549 [-2383 C/T], rs3761548 [-3279 G/T], and rs3761547 [-3499 T/C]) and GD in a Turkish population. The results showed that the AC and AA genotypes of -3279 and the CT genotype of -2383 are possible risk factors for GD. However, the development of GO in GD patients could not be associated with the investigated *FOXP3* polymorphisms in our study population.

For polymorphism -3279, an association between genotypes AA and AC and autoimmune diseases such as systemic lupus erythematosus and vitiligo has been shown in the literature.[10,12] The association with GD varies in the literature according to ethnicity. Although there are genotypic differences, the -3279 polymorphism has been reported to be a risk factor for GD in the Asian population.[10,13,17,19,21,22] It has been noted that the AC genotype of -3279 in the Kashmiri population, the AA and AC genotypes of -3279 in the Chinese Han population, and the AA genotype of -3279 in the female Southwest Chinese Han population pose a risk for GD.[13,19,22] In addition, a high frequency of the A allele was reported in patients with high thyroid-stimulating hormone (TSH) levels or low TSHR levels.[21] Like many studies, the A allele was observed more frequently in the GD group in the current study.[13,17,21] There are no studies in the Caucasian population reporting risk factors for -3279 polymorphisms.[18,20] Similar to the Kashmiri and Polish populations, the genotype distribution did not significantly differ between males and females in our study population (Table 5).[17,20] However, in the Asian population, the genotype distribution was reported to be significantly different between males and females.[13,22]

Polymorphism -2383 has been reported to increase GD susceptibility similarly to polymorphism -3279.[13,17,18,20] Bossowski et al.[20] reported that among Caucasians, the CT genotype of the -2383 polymorphism was more common in healthy females. Shehjar et al.[17] reported that the TT genotype of the -2383 polymorphism was a risk factor for developing GD in the Kashmiri population, but there were no sex differences in allelic or genotypic frequency distribution. In another study conducted in the Chinese Han population, carriers of the TT genotype of -2383 had a higher free triiodothyronine level than those with the CC/CT genotypes, but there was no significant difference between GD and control groups regarding genotype frequencies.[13] In our study, we found an association between the development of GD and the CT genotype of -2383, and the T allele was significantly more frequent in the study group. However, the genotype distribution did not differ significantly between males and females in our study population (Table 5). The differences in allelic and genotypic associations with GD in studies may be explained by ethnic differences.

Table 4. Genotypic and allelic distribution of non-GO and GO patients				
Genotype	**Non-GO**	**GO**	**p value**	**OR (95% CI)**
	n=74	**n=100**		
-3279 C/A				
CC	18 (24.3%)	32 (32%)	0.26	1.38 (0.7, 2.72)
AC	34 (45.9%)	40 (40%)	0.43	0.8 (0.44, 1.47)
AA	22 (29.7%)	28 (28%)	0.80	0.92 (0.47, 1.78)
Allele			0.38	1.21 (0.79, 1.85)
A	78 (47.3%)	96 (48%)		
C	70 (52.7%)	104 (52%)		
-2383 C/T				
CC	58 (78.4%)	73 (73%)	0.41	0.75 (0.37, 1.51)
CT	10 (13.5%)	25 (25%)	0.61	2.13 (0.95, 4.77)
TT	6 (8.1%)	2 (2%)	0.06	0.23 (0.05, 1.18)
Allele			0.71	0.89 (0.49, 1.63)
C	116 (84%)	171 (85.5%)		
T	22 (16%)	29 (14.5%)		
-3499 A/G				
AA	62 (83.8%)	85 (85%)	0.82	1.1 (0.48, 2.51)
AG	12 (16.2%)	14 (14%)	0.70	0.16 (0.06, 0.43)
GG	0	1 (1%)	-	-
Allele			0.75	1.13 (0.52, 2.46)
A	156 (82.9%)	184 (92%)		
G	12 (7.1%)	16 (8%)		

Frequencies of genotypes and alleles were compared using chi-square test
GO: Graves' ophthalmopathy, OR: Odds ratio, n: Number, CI: Confidence interval

Table 5. Analysis of the association between *FOXP3* genotypes and demographic variables in the patient group (GO and non-GO)						
Characteristics	***FOXP3* -2383 C/T**			***FOXP3* -3279 C/A**		
	CC (n=131)	**CT+TT (n=43)**	**p**	**CC (n=50)**	**AC+AA (n=124)**	**p**
Age at onset			0.11			0.49
≤40 years	76 (58%)	31 (72.1%)		33 (66%)	17 (34%)	
>40 years	55 (42%)	12 (27.9%)		74 (59.7%)	50 (40.3%)	
Sex			0.42			0.57
Female	100 (76.3%)	30 (69.8%)		39 (30%)	91 (70%)	
Male	31 (23.7%)	13 (30.2%)		11(25%)	33 (75%)	
Family history			0.48			0.87
Positive	70 (53.4%)	26 (60.5%)		27 (28.1%)	69 (71.9%)	
Negative	61 (46.6%)	17 (39.5%)		23 (29.5)	70.5 (55%)	
Smoking			0.48			>0.99
Yes	73 (77.7%)	21 (22.3%)		26 (52%)	63 (48%)	
No	58 (72.5%)	22 (27.5%)		24 (50.8%)	61 (49.2%)	

Genotype frequencies were compared using chi-square test
GO: Graves' ophthalmopathy, *FOXP3*: Forkhead box P3

For polymorphism -3449, we found no statistically significant difference between groups, consistent with the literature.[10,13,17,20,21,22] Only one study in the literature reported that free triiodothyronine and thyroxine levels and the -3499 A/G polymorphism were associated with GD.[20] It can be surmised that -3499 is not associated with altered *FOXP3* expression and does not affect Tregs functions.

In our study, none of the *FOXP3* genotypes or alleles was found to be associated with GO despite the higher number of GO patients in our study population. Similarly, Zheng et al.[13] and Shehjar et al.[17] could not show any associations between *FOXP3* SNPs and ophthalmopathy in GD in Asian populations. In the literature, Aydın et al.[24] found an association between the endothelin receptor type A (*EDNRA*) C+70G gene and the development of ophthalmopathy in GD patients. Another study reported that new SNPs in *CD74* (AG genotype of rs2569103) increased the risk of developing GO by affecting adipocyte proliferation and differentiation.[25] There are still many unanswered questions about the risk of ophthalmopathy in GD. For a better understanding of ophthalmopathy development, both genetic and non-genetic factors should be evaluated.

Study Limitations

Our study has several limitations. First, the number of participants in each group was limited, which might have reduced the power of the research and prevented us from showing significant differences between subgroups, such as gender and the presence of orbitopathy. Second, the study included only a small proportion of the GD patients in Türkiye. Therefore, whether our results could be generalized to the Turkish population is unclear. Third, we could not do a haplotype analysis due to the limited size of the study population. Fourth, our study was not longitudinal, and we could not control the other GO-related factors in study groups that might confound the risk of GO development in GD patients.

Conclusion

This study is the first to explore the association between *FOXP3* polymorphisms and GD with and without GO in a Turkish sample. We showed that the AC and AA genotypes of -3279 and the CT genotype of -2383 may be risk factors for GD development in our study population. However, we could not find any association between *FOXP3* SNPs and GO development in GD. More extensive population studies or meta-analyses of available data may reveal the impact of *FOXP3* polymorphisms on the risk of GO development in patients with GD.

Ethics

Ethics Committee Approval: The study protocol was approved by the Clinical Research Ethics Committee of Gazi University (decision no: 2018-824/1, date: 12.11.2018).

Informed Consent: Obtained.

Authorship Contributions

Surgical and Medical Practices: K.S.C., B.T., O.K., Concept: F.Y.T., K.S.C., S.G.E., O.K., Design: F.Y.T., K.S.C., S.G.E., O.K., Data Collection or Processing: F.Y.T., K.S.C., S.G.E., Analysis or Interpretation: F.Y.T., K.S.C., S.G.E., Literature Search: F.Y.T., K.S.C., Writing: F.Y.T., K.S.C., S.G.E.

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