Assessment of the Relationship Between Ulcerative Colitis and Forkhead Box P3 Polymorphisms

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ABSTRACT

Background: *Ulcerative colitis (UC) is a chronic disease that does not have a definitive treatment and causes repetitive inflammation of the colon and impaired quality of life. The FOXP3 gene codes FOXP3 protein responsible for the development and function of regulatory T (Treg) cells. The rs2232365 A/G and the rs3761548 A/C polymorphisms of the FOXP3 gene were indicated to be associated with inflammation-related diseases such as UC. The effectiveness of Treg cells, which act as immune-suppressors in the control of inflammation, can be affected by these polymorphisms. The present study aimed to evaluate the association between these polymorphisms with UC.*

Methods: *The current study researched the FOXP3 gene polymorphisms in 146 patients with UC and in 292 healthy individuals by a real-time polymerase chain reaction (RT-PCR).*

Results: *The patients with rs2232365 G allele had a 1.44-fold higher UC risk than patients carrying other alleles (P = .013), and had significantly a 2.56-fold higher risk for the extent of UC (P = .001). Contrary, rs3761548 polymorphism did not reach statistically significant in any analysis.*

Conclusion: *This is the first study to reveal the relationship of the rs2232365 and the rs3761548 polymorphisms with UC in the Caucasian population. The rs2232365 has an important effect on the risk of UC. The current study suggests that these polymorphisms should be explored together with the FOXP3 expression and FOXP3⁺ Treg cell count in blood and colon tissue of UC patients to clarify the exact effect of FOXP3 polymorphisms on UC risk.*

Keywords: *FOXP3 variants, rs2232365, rs3761548, Ulcerative colitis*

INTRODUCTION

Ulcerative colitis (UC) is a chronic disease with an unknown etiology, resulting in inflammation of the colon, usually seen at the age of 30-40. Its frequency is approximately equal in terms of gender.[1] The defined feature of UC is repetitive mucosal inflammation which starts from the rectum and spreads to the proximal of the colon.[2] The fundamental reasons for ulcerative colitis are related to many factors such as genetics and environmental factors. Genetic factors have an important role in the persons with UC because the family of 8-14% of these patients has a history of inflammatory bowel disease.[3] Environmental factors affecting UC are known as diet and lifestyle, smoking, psychological status of individuals, food antigens, commensal bacteria, and viral infection such as cytomegalovirus.[3,4] There is no exact cure for UC; therefore, the annual financial burden of patients reaches approximately 13 billion dollars worldwide. The incidence and prevalence of UC are increasing day by day.[5] Whence, genetic studies have focused on this disease to reveal the underlying causes of this disease.

UC has been associated with about 240 genes in genome-wide association studies.[6] The highlights of these related genes, especially immunology-related ones, in UC susceptibility are *tyrosine kinase-2, human leukocyte antigen, interleukin-23 receptor, cytotoxic T-lymphocyte-associated protein 4, forkhead box P3* (*FOXP3*).[7,8] The *forkhead box P3* (*FOXP3*) gene expressed by regulatory T cells (Treg) is located on chromosome X (Xp11.23), and which codes a transcription factor FOXP3 protein responsible for the development and function of Treg cells.[7] Treg cells have a vital role in immune homeostasis, and provide immune tolerance against self- and non-self of antigens.[9] $CD4^+CD25^+$ $FOXP3^+$ Treg cells inhibit other T cells by binding interleukin-2 (IL-2), a T cell growth factor, with CD25 molecule in the inflamed colonic lamina propria of UC patients.[7,9] Moreover, the CTLA4 molecules

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of Treg cells contribute to this process through binding the CD-80 and CD86 molecules expressed on the surface of antigen-presenting cells.[10] Thereby, Treg cells prevent an aggressive T cell response to antigens, and provide immune homeostasis. FOXP3 expression' down-regulation or dysfunction is related to immunologic and inflammation-related diseases such as multiple sclerosis (MS),[11] rheumatoid arthritis (RA),[12] vitiligo,[13] and UC.[14]

The polymorphisms that existed in the promoter region of the *FOXP3* gene (especially the rs2232365 A/G and the rs3761548 A/C) may influence *FOXP3* gene expression by interaction with *cis*-acting elements and transcription factors (GATA-3, E47, c-Myb). Therefore the Treg cells'' activation and function can abrogate.[15,16] Especially, these polymorphisms have associated with different diseases such as multiple sclerosis,[11] vitiligo,[13] psoriasis,[17] allergic rhinitis,[18] hepatocellular carcinoma,[19] and UC.[14] But, there is no work regarding the influence of these polymorphisms on UC in the Caucasian population until now.

For the first time, this study explored the association between UC and these polymorphisms using 146 patients with UC and in 292 healthy persons by RT-PCR in a Turkish population that is a Caucasian population.

MATERIALS AND METHODS

This work was ratified by the Çukurova University Ethics Committee in Turkey. The individuals in this study were recruited at Balcalı Hospital of Çukurova University from November 2013 to October 2019. The participants in this study signed an informed consent form respecting the usage of their blood samples. This study was realized according to the Helsinki declaration endorsed at the World Medical Association gathering in Edinburgh. One hundred 46 cases with UC were diagnosed using endoscopic, radiological, clinical, and pathological findings based on Lennard-Jones criteria[20] were registered as the UC patient group. The activity of UC was determined with colonoscopy examination according to the Truelove and Witts Activity Index.[21] To provide the statistical power, the severity of UC disease was categorized into subgroups as mild/moderate and severe. Likewise, the extent of UC was categorized as distal colitis (proctitis/left sided) and extensive colitis (extensive/pancolitis). The participants have immunological diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and other immune-related diseases excluded from this work. Simultaneously, 292 healthy persons were included in the current study as the control group. These variants of all study subjects were established by the RT-PCR method. Biochemical parameters, endoscopy, and histopathological findings of all participants were used for statistical analysis. The participants' blood and serum specimens were frozen at −80°C until analysis.

Main Points

- *Ulcerative colitis (UC) is a chronic disease with an unknown etiology, resulting in inflammation of the colon; therefore, it is necessary to investigate host genetic factors in detail to understand the molecular pathogenesis of ulcerative colitis.*
- *This is the first study to reveal the relationship of FOXP3 polymorphisms with UC in a Turkish population which is a Caucasian population.*
- *FOX gene rs2232365 polymorphism has an important effect on the risk of UC.*

The Isolation of Genomic DNA, and the rs2232365 and rs3761548 Variants' Analysis

According to the kit's prospectus, all study subjects' genomic DNA was extracted from their blood samples by PCR Template Preparation Kit (Roche, Germany). The alleles of these variants were identified by RT-PCR. RT-PCR protocol, in brief, 5 μL (about 100 ng) extracted genomic DNA was mixed with 10 μL of Roche Probe Master Mix, 4 μL of distilled water, and 1 μL of SimpleProbe reactive in a total volume of 20 μL. Afterward, the RT-PCR process were implemented as first denaturation at 96°C (10 min), and whereupon throughout 43 cycles, denaturation at 96°C (10 s), annealing at 60°C (12 s), and extension at 72°C (15 s), and ultimate melting conditions were at 96°C (30 s) and 40°C (3 min). The variants have a melting peak at a specific temperature as follows: the A allele of rs2232365 at 66.04°C and the G allele of rs2232365 at 60.04°C; the A allele of rs3761548 at 66.43°C and the C allele of rs3761548 at 60.28°C). To ensure quality control, the genotyping was done in the absence of disease situation information of the individuals. A 20% of random specimens of participants were determined twice by diverse experts, and reproducibility was 100 %.

Statistical Analysis

The sufficient sample size and 80% statistic power were calculated with Quanto software using minor allele frequencies of these polymorphisms in HapMap, the prevalence of the disease in the population, and the effect of minor allele on phenotype. All analysis was got by using the IBM SPSS software (USA). Comparisons in demographical variables between both groups were done the

Independent samples *t*-test or Mann–Whitney *U* test for continual variable, and chi-square test for categorical variables. The haplotype frequency and linkage disequilibrium (LD) analyzes were got using the website: https://www.snpstats.net/analyzer.php. Logistic regression analysis with adjustment for age, gender, and smoking was got to determine UC risk factors in inheritance models between both groups. To provide statistical power, the total allele's frequencies were used in subgroups of severity and extent of UC. All results are exhibited as odds ratios with 95% CI and analyzes were two-sided. *P* values < .05 were accepted statistically significant.

RESULTS

The characteristics of the cases with UC and controls were represented in Table 1. The male rate in the case and control groups was 58.9 and 51.4%, respectively. Differences between the variables of UC patients and those of the controls were significant as expected, including hematocrit (HCT), white blood cells (WBC), sedimentation, and C-reactive protein (Table 1).

The rs2232365 and rs3761548 Polymorphisms' Allele and Genotype Distributions

The total allele distributions of rs2232365 polymorphism were 46.3 and 53.7% for A and G, respectively. Furthermore, rs3761548 polymorphism's total allele frequencies were 41.3 and 58.7% for A and C, respectively. A significant association was determined between both groups in terms of allele distribution of rs2232365 polymorphism ($P = .013$), but not found for rs3761548 (Table 2). Additionally, when both groups were analyzed according to gender, it did not find a significant association between these variants' allele and genotype frequencies (Table 3). Further, there was statistically an association between the extent of UC and rs2232365 polymorphism, but the rs3761548 was not significant (Table 5). The severity of UC was not related to both polymorphisms (Table 5).

Table 1. Distribution of Selected Characteristics in Case and Control Groups

Variable	Cases (UC Patients) (%), *n* (%),	Controls (Healthy Persons) (%), *n* (%),	*P*
Age†	49 (24-81)	47 (17-85)	.57[a]
Sex, male (%)	86 (58.9)	150 (51.4)	.14[b]
Smoking, (%)	44 (30.1)	80 (27.4)	.55[b]
Extent of UC, n (%)			
Proctitis/left sided	82 (56.2)	-	
Extensive	30 (20.5)	-	
Pancolitis	34 (23.3)	-	
Severity of UC, n (%)			
Mild type	82 (56.2)	-	
Moderate type	50 (34.2)	-	
Severe type	14 (9.6)	-	
Treatment options (%)			
5-ASA (aminosalicylic acid)	96 (65.3)	-	
5-ASA + AZA (azathioprine)	6 (4.2)	-	
5-ASA + steroid	20 (13.9)	-	
5-ASA + AZA + steroid	20 (13.9)	-	
5-ASA + AZA + anti-TNF	4 (2.8)	-	
HCT (μL)‡	37.90 ± 6.41	40.83 ± 5.28	.001[c]
WBC (10^3/μL)†	7.69 (4.31-20.20)	6.70 (5.90-9.70)	.002[a]
Sedimentation (mm/h)†	19 (2-104)	12 (5-25)	.001[a]
C-reactive protein (mg/dL)†	0.64 (0.1-72.1)	0.33 (0.1-1.3)	.001[a]

[a]*P* values were calculated by Mann–Whitney test. [b]*P* values were calculated by chi-square test. [c]*P* values were calculated by Student's *t*-test. †Data were shown as median (min–max). ‡Data were shown as mean ± SD.

Table 2. The Association Between FOXP3 Polymorphisms and the Risk of UC

	Cases, *n* ases, his	Control, *n* ontrol, is	*P*	OR (95% CI)
For rs2232365				
Allele frequency, n (%)				
A	118 (40.4)	288 (49.3)		1.00 (Reference)
G	174 (59.6)	296 (50.7)	0.013[a]	1.44 (1.08-1.91)
For rs3761548				
Allele frequency, n (%)				
C	160 (54.8)	354 (60.6)		1.00 (Reference)
A	132 (45.2)	230 (39.4)	0.10[a]	1.21 (0.96-1.69)

[a]*P* values were calculated by logistic regression analysis after adjusted for age, sex, and smoking.

The Relationship Between FOXP3 Variants and UC Risk

To explore whether the increased risk of UC by these variants, we implemented logistic regression analysis with adjustment for age, sex, and smoking between both groups (Table 2). The G allele of rs2232365 polymorphism was statistically significant in terms of 1.44-fold an increased UC risk ($P = .0131.44$ (1.08-1.91)) (Table 2). In addition, although it increased the risk of UC, there was no significant relationship between these polymorphisms and male and female groups (For rs2232365 G risk variant $P = .055$, OR = 1.72 (0.99-2.92)), for rs3761548 A risk variant $P = .36$, OR = 1.30 (0.74-2.28) in males; $P = .45$, OR = 1.18 (0.77-1.82); $P = .17$, OR = 1.35 (0.88-2.07) in females, respectively) (Table 3). Likewise, although the risk genotypes of these polymorphisms increased the risk of UC, they could not reach a statistically significant value in females (Table 3). Further, the patients carrying rs2232365 G allele had a 2.56-fold greater risk of the extent of UC than patients having other alleles, and it was significant ($P = .001$, OR = 2.56 (1.57-4.17)). As for rs3761548, although A allele increased 1.58-fold the risk of UC extent, it was not significant ($P = .054$) (Table 5).

The rs2232365 and rs3761548 Polymorphisms' Haplotype Analysis

LD was strong for these variants in females ($D' = 0.99$, $r^2 = 0.74$). The males have only one allele of this gene because of *FOXP3* gene is located on the chromosome X. Therefore, LD value with 100% probability is 1. The haplotype analysis showed that the haplotypes of these variants were not related to UC risk in females (Table 4).

DISCUSSION

We investigated for the first time the effect of the *FOXP3* gene polymorphisms (rs2232365 and rs3761548) on the risk of UC predisposition using a case–control group in a Turkish population, which is a Caucasian population. There are only 2 studies so far, but one is a preliminary study of the other, and it is written in Chinese; the main study reported that these polymorphisms of the *FOXP3* gene are associated with the risk of UC predisposition in Chinese patients.[14,22] Xia et al.[14] also reported that associations of upregulation of FOXP3 expression and the risk alleles of these polymorphisms with UC patients when comparison with healthy controls. Moreover, reported the expression of *FOXP3* gene decreased in UC patients carrying risk alleles of rs2232365 and rs3761548 compared with UC patients no having risk alleles of these polymorphisms. Additionally, they indicated that the severe UC patients carry more frequent risk alleles compared with mild and moderate UC patients.[14] There is no study other than this study about these polymorphisms. Therefore, these polymorphisms of the *FOXP3* gene were selected as the aspirant polymorphisms because of their vital role in immune-related UC disease.

In the current study, total allele distributions of these variants in the control group were 49.3% and 60.6% for rs2232365 A and rs3761548 C variants. The allele distribution of rs2232365 polymorphism was statistically significant between UC patients and controls, but rs3761548 was not significant. In the current study, the individuals with G allele of rs2232365 polymorphism had a 1.44-fold higher UC risk than those who had not got G alleles ($P = .013$, OR = 1.44 (1.08-1.91)). As for rs3761548, this polymorphism's A allele increased a 1.21-fold UC risk when compared with control persons, but not significant ($P = .21$). The study reported from China that these variants' total allele frequencies were 63.6 and 78.6% for rs2232365 A and rs3761548 C, respectively.[14] They have reported that a significant relationship between the allele frequencies of these variants and UC risk in females and males, separately. In a female group of that study, these polymorphisms' risk alleles, rs2232365 G and rs3761548 A, increased a 1.38- and a 1.51-fold UC risk as compared to control group ($P = .012$ and $P = .004$, respectively).[14] Likewise, Xia et al. reported that in males with UC, these polymorphisms' risk variants were very meaningful to increase UC risk compared with healthy males ($P = .014$, OR = 1.59 and $P = .031$, OR = 1.58, respectively).[14] Conversely, when we analyzed an association between these polymorphisms with UC risk according to

Table 3. Alleles and Genotypes Frequency Distribution of Foxp3 Polymorphisms According to Sex As Well As the Association with the Risk of UC

rs2232365	Allele/Genotype	Cases, *n* (%)	Controls, *n* %	*P*	OR (95% CI)
Male, n (%)		86 (58.9)	150 (51.4)		
	A	34 (39.5)	79 (52.7)	-	1.00 (Reference)
	G	52 (60.5)	71 (47.3)	.055[b]	1.72 (0.99-2.92)
Female, n (%)		60 (41.1)	142 (48.6)		
	A	50 (41.7)	130 (45.8)	-	1.00 (Reference)
	G	70 (58.3)	154 (54.2)	.45[b]	1.18 (0.77-1.82)
	AA	6 (10)	24 (16.9)	.45[a]	1.00 (Reference)
	AG	38 (63.3)	82 (57.8)	.22[b]	1.85 (0.69-4.92)
	GG	16 (26.7)	36 (25.4)	.28[b]	1.81 (0.62-5.29)
Dominant	AA/AG + GG	6/54	24/118	.19[b]	1.84 (0.71-4.77)
Recessive	AA + AG/GG	44/16	106/36	.80[b]	1.09 (0.55-2.18)
Overdominant	AA + GG/AG	22/38	60/82	.49[b]	1.25 (0.67-2.33)
rs3761548					
Male, n (%)		86 (58.9)	150 (51.4)		
	C	52 (60.5)	100 (66.7)	-	1.00 (Reference)
	A	34 (39.5)	50 (33.3)	.36[b]	1.30 (0.74-2.28)
Female		60 (41.1)	142 (48.6)		
	C	56 (46.7)	154 (54.2)	-	1.00 (Reference)
	A	64 (53.3)	130 (45.8)	.17[b]	1.35 (0.88-2.07)
	CC	10 (16.7)	36 (25.4)	.31[a]	1.00 (Reference)
	CA	36 (60)	82 (57.8)	.28[b]	1.57 (0.79-3.54)
	AA	14 (23.3)	24 (16.8)	.15[b]	2.06 (0.78-5.45)
Dominant	CC/CA + AA	10/50	36/106	.19[b]	1.68 (0.76-3.69)
Recessive	CC + CA/AA	46/14	118/24	.32[b]	1.47 (0.70-3.09)
Over dominant	CC + AA/CA	24/36	60/82	.78[b]	1.09 (0.59-2.03)

[a]*P* values were calculated by the chi-square test. [b]*P* values were calculated by logistic regression analysis. *P* values were adjusted for age and smoking.

gender groups, we did not find any statistically significant for both polymorphisms, albeit increased UC risk.

This work also analyzed the association of these polymorphisms with the severity and extent of UC. While rs2232365 polymorphism significantly increased the risk of UC extent, rs3761548 variant was not significant. However, there were not found any statistically significant the effect of both polymorphisms on the severity of UC disease. In contrast to us, Xia et al. [14] reported that the

Table 4. Frequency Distribution of Haplotypes of Foxp3 gene rs2232365 and rs3761548 Polymorphisms in Female of Both Groups

	Frequency			
Haplotypes FOXP3 rs2232365 A/G and rs3761548 C/A	Cases	Controls	OR (95%CI)	*P*
GA	0.533	0.458	1.00 (Reference)	-
AC	0.418	0.457	0.74 (0.45-1.22)	.22
GC	0.050	0.084	0.53 (1.20-1.40)	.20
Global haplotype association P-value: .28				

Table 5. Relationship Between FOXP3 Polymorphisms and the Clinical Features of UC Patients

FOXP3	Severity of UC		Extent of UC	
rs2232365, n (%)	Mild/Moderate, n ld/M	Severe, n ver	Distal colitis, n sta	Extensive colitis, n xten
A	108 (40.9)	10 (35.7)	84 (50)	36 (28.1)
G	156 (59.1)	18 (64.3)[a]	84 (50)	92 (71.9)[b]
rs3761548				
C	142 (53.8)	18 (64.3)	98 (59.8)	62 (48.4)
A	122 (46.2)	10 (35.7)[c]	66 (40.2)	66 (51.6)[d]

P values were calculated by logistic regression analysis after adjusted for age, sex, and smoking. [a]*P* = .59, OR = 1.25 (0.55-2.80); [b]*P* = .001, OR = 2.56 (1.57-4.17); [c]*P* = .29, OR = 0.65 (0.29-1.45); [d]*P* = .054, OR = 1.58 (0.99-2.52).

severe UC patients have the majority carried risk alleles and/or genotypes of these polymorphisms than patients with mild and moderate UC in females and males, and it is statistically significant. As for the extent of UC, their results are not in line with our findings for rs2232365 polymorphism. Xia et al.[14] reported there is no statistical significance for both polymorphisms. Additionally, when we also performed haplotype analysis, we did not find any statistically significant between UC risk and haplotypes of these polymorphisms. Similarly, Xia et al.[14] did not find any significant results related to UC risk in haplotype analysis of these polymorphisms.

There are studies investigating the relationship between these polymorphisms and some diseases. The results of these studies are also controversial. Some studies found these polymorphisms significant in relation to the disease they investigated, while others found no association. Some of them reported in the different diseases, including psoriasis,[23] Crohn's disease,[24] Wilms' tumor,[25] and breast cancer[26] were not statistically significant for allele distributions of these polymorphisms, and they are in line with results of the current study. Contrarily, a few studies, vitiligo,[13] idiopathic recurrent pregnancy loss[27] and renal diseases,[28] reported a statistically significant association with these polymorphisms. The discrepancy among these works' results may be due to inadequate sample size, the errors in statistical analysis, ethnicity, gene-gene, and gene-environment interactions. In addition, the males are hemizygous for the *FOXP3* gene, which is located at the X chromosome; therefore, though males have only a single allele, females have got 2 alleles. This may be one of the reasons for the discrepancy between the results.

Some studies investigated *FOXP3* gene expression in UC disease, and they have reported that the FOXP3 expression, mRNA and protein, were upregulated in the inflamed colonic mucosa of UC patients to prevent intestinal inflammation.[14,29] Especially, the rs2232365 and rs3761548 polymorphisms influence FOXP3 expression because of located within the DNA-binding sites of GATA-3 and Sp-1 transcription factors in the promoter region of *FOXP3* gene.[13,25] Moreover, it was reported that UC patients carrying risk alleles of rs2232365 and rs3761548 variants have lower FOXP3 expression, therefore lower FOXP3$^+$ Treg cell frequencies, in colonic tissue, especially in the patients with severe UC.[14,30] Therefore, in the case of low FOXP3 expression, regulatory T cells cannot inhibit excessive immune response, resulting in excessive inflammation in colon tissue.

As with almost any study, the current study has several limitations: (a) the patients with UC and controls included in the current study were from Adana and surrounding provinces. The present work does not represent all patients with UC and individuals with health in the Caucasian population. (b) Since the number of samples decreases in the relationship analysis with these polymorphisms in terms of gender, the statistical power decreases, so it is imperative to work with a larger sample number in the Caucasian population. (c) This study only investigated the relationship of *FOXP3* polymorphisms with UC. Therefore, FOXP3 polymorphisms with FOXP3 expression and FOXP3+ Treg cell count have not been established.

In conclusion, the present work demonstrates the relationship between UC disease and the FOXP3 gene polymorphisms in the Turkish population, a Caucasian population. The results of this study demonstrated that the *FOXP3* gene rs2232365 G allele has an important effect on the UC risk, but not for rs3761548 polymorphism. The current study suggests that these polymorphisms should be explored together with the FOXP3 expression and FOXP3$^+$ Treg cell count in blood and colon tissue of UC patients to clarify the exact effect of *FOXP3* polymorphisms on UC risk.

Ethics Committee Approval: The study was approved by the Committee for Ethics of Medical Experiment on Human Subjects, Çukurova University Faculty of Medicine in Adana in Turkey.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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