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No Association between Foxp3 Promoter Region Polymorphisms (rs3761548 and rs2232365) and Crimean Congo Hemorrhagic Fever Disease

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Abstract

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne, viral disease that causes severe outbreaks in people throughout large geographical areas. The common feature of viral hemorrhagic fever (VHF) cases is the attack on the cells that initiate the antiviral response and the impaired immune response of the host. There is no study of the variation of the promoter region of *Foxp3* gene, which has been shown to be involved in inflammation in studies of autoimmune diseases, in relation to the risk or susceptibility of disease in CCHF patients. Therefore, the aim of our study was to investigate *Foxp3* rs3761548 and rs2232365 gene polymorphisms in patients with CCHF. Blood samples taken from 80 CCHF patients and 80 healthy subjects were used in the study. Genotyping was performed by PCR-RFLP followed by total DNA isolation. No statistically significant relationship between *Foxp3* gene polymorphisms rs2232365 and rs3761548 and CCHF was found.Although the pathophysiology of CCHF is still not fully understood, there are some mechanisms shown to be related to the immune system. Studies examining other variants in the *Foxp3* gene promoter region and of exon and intron mutations may yield beneficial results in terms of the association between gene and disease progression.

Key words: Crimean-Congo hemorrhagic fever, *Foxp3*, polymorphism

INTRODUCTION

Viral hemorrhagic fever (VHF) is a clinical syndrome characterized by fever and bleeding, caused by different viruses in humans [1]. CCHF, an acute VHF disease, has a high mortality rate in humans and comes from infected with the Crimean-Congo hemorrhagic fever virus (CCHFV). It is an Arbovirus belonging to the genus Nairovirus of the family of Bunyaviridae [2]. The virus spreads to the animals through the ticks and causes viremia without showing signs of disease. The transition from animals to humans is through body fluids and blood contact [3]. CCHF case was reported in 2002 for the first time in Turkey and since then, particularly in spring and summer times, CCHF outbreaks in Turkey's rural areas have been observed [4, 5]. In the host, the key targets of the CCHFV are mononuclear phagocytes, endothelial cells and hepatocytes; both natural and specific immunity are important for the host to protect against CCHFV. After entering the host, CCHFV meets the cells of the natural immune system, including monocytes and dendritic cells. The infection of mononuclear phagocytes and the depletion of lymphoid cells may cause immunological inactivation, protect the virus from phagocytosis and increase the systemic spread of the virus. However, the cells are infected producing a variety of cytokines, chemokines and inflammatory factors [6, 7].

The main function of regulatory T (Treg) cells is suppression of the immune response when it is not needed [8]. Tregs may perform by the immunosuppressive TGF-β and IL-10 cytokines secreted by the effector T cells or by contacting directly to the effector T cells [9].*Foxp3* gene encodes a transcription factor believed to be crucial for the development and function of Treg cells [10]. Polymorphisms in *Foxp3* gene may alter the gene's role functionally or quantitatively, therefore leads to functional CD4+CD25+Treg deficiency leading to some autoimmune/inflammatory diseases [11].

CCHFV infection can be fatal in serious cases and clinical course and outcome of the disease varies between individuals. There is no clear explanation about why some people's illness turns into serious while some patients only suffering from a mild infection. However, it is possible that mutations in the host genome may contribute to the development of symptomatic infection and the alteration of disease severity among individuals [12]. Polymorphisms in the promoter region of *FoxP3*may potentially affect the expression of the gene, by altering the specificity of the transcription factors binding to their binding sites and the transcription initiation kinetics [13]. There are five different polymorphic variants identified in the promoter region of *Foxp3*gene [14]. In this study, for the first time, two most common of the *Foxp3* promoter region polymorphisms (rs3761548 and rs2232365) were evaluated in terms of genotype and allele frequencies between patients with CCHF and healthy controls.

MATERIALS AND METHODS

Patients

The study group consisted of 80 patients who were diagnosed with CCHFV infection in the Infectious Diseases and Clinical Microbiology Service of the Cumhuriyet University Hospital, Sivas, Turkey. The control group was composed of 80 healthy volunteers who did not have any acute or chronic diseases. The study protocol was approved by the Ethics Committee of Cumhuriyet University School of Medicine. Individuals were informed and filled out the confirmation form before blood samples were collected from the patients and control groups.

Genotyping

DNA isolation was performed using the phenol-chloroform method[15] from 10 ml blood samples taken from each individual. The *Foxp3* promoter region was amplified by polymerase chain reaction (PCR) to include the region -924. Forward and reverse primer sequences were 5'-TGGAGGGCTTTCAAGGTGAGGA-3' and 5′-GGGGAGTTGGATTGGGTGCA-3' respectively. The method for PCR included an initial denaturing at 94ºC for 4 min, followed by 30 cycles at 94ºC for 1 min, 58ºC for 30 s, 72ºC for 30 s with a final extension at 72ºC for 10 min. The resulting

374 bp amplicons were incubated with *BsMI*(New England Biolabs) restriction endonuclease enzyme at 37ºC for 1 hour and then separated on 3% agorose gel. For the AG genotype 374+188+186 bp fragments, for the GG genotype 188+186 bp fragments and for AA genotype 374 bp fragment were obtained following restriction digestions and agarose gel electrophoresis separation.

For *Foxp3* -3279 SNP, forward and reverse primer sequences were 5'CTGACATGCCTCCATCAT-3' and 5'-TGCAGGGCTTCAAGTTGACAG-3' respectively. The method for PCR included an initial denaturing at 94ºC for 4 min, followed by 30 cycles at 94ºC for 1 min, 55ºC for 30 s, 72ºC for 30 s with a final extension at 72ºC for 10 min. The resulting 361 bp amplicons were incubated with *Pst*1 (Nzytech) restriction endonuclease enzyme at 55ºC for 1 hour and then separated on 3% agarose gel. For the CC genotype 138+223 bp fragments, for the CA genotype 361+138+223 bp fragments and for the AA genotype 361 bp fragment were obtained following restriction digestion and agarose gel electrophoresis separation.

Statistical Analysis

The data were analyzed using the SPSS 22.0 (SPSS, Chicago, IL, USA) program. Chi square and Fisher's exact tests were used to compare the data. OR and 95% confidence intervals for obtained results were given. $p<0.05$ was considered as significant in all tests.

RESULT AND DISCUSSION

In our study, blood samples taken from 80 CCHF patients (40 males and 40 females) and from 80 healthy volunteers (36 males and 44 females) without any disease were used. The mean age of the patients and control groups is 48,69±17.47 and 43,35±15.79, respectively. Another demographic data of the study group have been evaluated.

Single nucleotide polymorphisms in the promotor region of *Foxp3* gene -924A/G (rs2232365) and -3279A/C (rs3761548) were analyzed in the patient and control groups. We did not find significant difference between the two groups for these polymorphisms.

When the patient and control group were analyzed in terms of rs3761548 polymorphism genotype distributions, wild type AA genotype appeared to be close to each other in patient (47.62%) and control (55%) group. AC heterozygote and CC homozygote polymorphic genotypes were determined as 9.52% and 42.86% to 15% and 30% respectively in patient and control groups. When these data were statistically evaluated no significant difference was observed between two groups (see Table 1).

Table 1: Distribution of *Foxp3* gene promoter region polymorphism rs3761548 genotype and allele frequency in CCHF patient and control groups

	Control (n=80)	Case (n=80)	*p* value	OR (%95CI)
Genotype				
AA	44 (%55)	38 (%47.62)		
AC	12 (%15)	8 (%9.52)	0.79	0.74 (0.074-5.98)
CC	24 (%30)	34 (%42.86)	0.48	1.62 (0.42-6.62)
Genotype				
AA	44 (%55)	38 (%47.62)		
CC+AC	36 (%45)	42 (%52.38)	0.34	1.35 (0.722-2.526)
Allele				
A	100 (%62.5)	84 (%52.5)		
C	60 (%37.5)	76 (%47.5)	0.07	1.5 (0.964-2.359)

When the patient and control groups were examined for the other promoter region polymorphism rs2232365, there was no statistically significant difference in genotype distribution between two groups for this polymorphism. As well as the genotype distribution, wild type AA genotype appears to have similar values in the patient (42.86%) and control (55%) groups. AG heterozygous and GG homozygous polymorphic genotypes were determined as 23.81% and 33.33% to 25% and 20% respectively in patient and control groups. When allelic frequencies were compared, it was determined that the polymorphic allele was significantly higher in the patients than in control subjects (p=0.017). The genotype and allelic distributions for the *Foxp3* rs2232365 variants are shown in Table 2.

Table 2: Genotype and allele distribution of *Foxp3* gene promoter region rs2232365 variant for the patient and control groups.

	Control (n=80)	Cases (n=80)	*p* value	OR (%95CI)
Genotype				
AA	44 (%55)	34 (%42.86)		
AG	20 (%25)	19 (%23.81)	0.8	1.21 (0.25-5.94)
GG	16 (%20)	27 (%33.33)	0.35	2.086 (0.45-10.58)
Genotype				
AA	44 (%55)	34 (%42.86)		
AA+AG	36 (%45)	46 (%57.14)	0.12	1.648 (0.881-3.102)
Allele				
A	108 (%67.5)	87 (%54.37)		
G	52 (%32.5)	73 (%45.63)	**0.017**	1.74(1.105-2.75)

CCHF disease, which is the result of CCHFV infection, constitutes an important public health problem in our country. Therefore, it is important to conduct studies to determine the isolation, biology, virulence factors and genetic diversity of CCHFV strains in our country in order to find an effective vaccine and develop strategies and tools for the diagnosis and treatment the disease. In this study for the first time two of *Foxp3* gene promoter region variations (rs3761548 and rs2232365) were examined in patients with CCHF. While there was no significant difference between the patient and control groups in terms of both polymorphisms, the polymorphic allele of rs2232365 polymorphism was found to be higher in patients than in healthy controls.

Recent studies have shown that *Foxp3* polymorphisms increase susceptibility to some autoimmune diseases. In CCHF disease, after the virus is introduced into the host one of the earliest targets is the immune cells. The impairment of the immune response is due to the impairment of the functions of the cells that initiate the response to the effect [16]. Any defect in Treg cells leads to the development of immunological diseases [17]. In a study of mice with reduced Treg cells, these mice have not been able to develop an appropriate immune response against HSV-2 virus and it has been shown that infection progresses through the nervous tissue to the central nervous system [18]. In other studies, in the cases of CCHF resulting with death, of interleukins; IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α levels were found to be higher in patients who died that patients who survived [2, 19]. From these studies, Treg cells are thought to be necessary not only in stopping the immune response but also in improving the appropriate immune response to infection [8]. Polymorphisms determined in the promoter region of *Foxp3* gene have been shown to cause deficiency of Treg cells [20]. In this study, it is thought that there is an association between disease and *Foxp3* in terms of the relation between *Foxp3* and Treg cells, the role of Treg cells in immunological system and the effect of immunity in the pathogenesis of CCHF and two of the promoter region variations have

been studied for this purpose. In a polymorphism study with Toll-like receptors that are active in immune system, Engin et al. found that Toll-like receptor 8 and 9 polymorphisms are more important in patients that controls [12]. Kaya and colleagues found that in a comprehensive study of some cytokines and TNF-α, IL-6 and TNF-α at a high concentration were strongly associated with mortality in CCHF [21]. In another study, contrary to these findings, researchers not found effect of IL-6 and TNF-α polymorphisms on CCHF prognosis and death [22]. On the other hand, both studies have shown that CCHFV alters the homeostatic mechanisms of the host organism in two different ways. The first effect is on direct homeostasis and platelet-related endothelial cell whereas the second effect is an indirect effect on the endothelium via immunological and inflammatory pathways [2, 23]. When determining the purpose of our study, these contradictory results and effect mechanisms of CCHFV have been taken into account and a gene known to be affected in the immune system and not previously studied in CCHF has been selected. In the case-control study, there was no significant association between the variations and the disease. However, to better understand the role of autoimmunity in disease pathogenesis, it may be useful to examine in *Foxp3* gene; other variations in the promoter region and exonic or intronic polymorphisms with more detailed parameters in larger study groups.

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