

Effect of *CTLA-4* and *TNF-α* Gene Polymorphisms on Inhibitor Development in a Turkish Cohort of Severe Hemophilia A Cases with Intron 22 Inversion Mutation: An Analytical Study

Intron 22 Mutasyon Taşıyıcısı Ağır Hemofili A Hastalarında *CTLA-4* ve *TNF-α* Gen Polimorfizmlerinin İnhibitör Gelişimi Üzerine Etkisi: Analitik Bir Araştırma

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ABSTRACT Objective: The development of neutralizing antibodies against infused factor VIII called inhibitor is the most challenging treatment complication in hemophilia A (HA) patients. Associated factors for inhibitor development are classified into 2 groups (genetics and non-genetics). Genetic factors other than mutation type of *F8* gene include family history, ethnical origin, human leucocyte antigen haplotype, and a number of polymorphisms in genes which play role in immune system. In this study, we aimed to analyze the association between 3 variants in 2 genes [c.-318C>T; rs5742909 and c.49A>G; rs231775 in *CTLA-4* and c.-308G>A; rs1800629 in tumor necrosis factor alpha (*TNF-α*)] and inhibitor development in a cohort of severe HA patients with intron 22 inversion (*inv22*) mutation. **Material and Methods:** The study included in 94 severe HA patients with *inv22*. Two groups were established according to the inhibitor status: inhibitor positive and inhibitor negative. We investigated 2 single nucleotide polymorphisms in *CTLA-4* (c.-318C>T; rs5742909 and c.49A>G; rs231775) and one in *TNF-α* (c.-308G>A; rs1800629) using Sanger sequencing in both groups. **Results:** In this study, no significant relationship between *CTLA-4* polymorphisms and inhibitor development was observed in severe HA patients with *inv22* mutation. However, the A allele for c.-308G>A variant in *TNF-α* was found to be associated with the increased risk for inhibitor development in those patients. **Conclusion:** In Turkish severe HA patients with *inv22* mutation, c.-318C>T and c.49A>G variants in *CTLA-4* gene are not associated with the inhibitor development, whereas c.-308G>A variant in *TNF-α*, the A allele is related to the risk of inhibitor development.

Keywords: Hemophilia; inhibitor; *CTLA-4*; *TNF-α*; intron 22 inversion

ÖZET Amaç: Hemofili A (HA) hastalarında faktör VIII'e karşı inhibitör adı verilen nötralizan antikorların gelişmesi en önemli tedavi komplikasyonudur. İnhibitör gelişimi ile ilişkili faktörler 2 gruba (genetik ve genetik olmayan) ayrılır. *F8* geni mutasyon tipi dışındaki genetik faktörler arasında aile öyküsü, etnik köken, insan lökosit antijen haplotipi ve immün sistemde rol oynayan genlerdeki birtakım polimorfizmler bulunur. Bu çalışmada, intron 22 inversiyon (*inv22*) mutasyonu taşıyan ağır HA hastalarından oluşan bir kohortta *CTLA-4* (c.-318C>T; rs5742909 ve c.49A>G; rs231775) ve tümör nekrozis faktör alfa (*TNF-α*) (c.-308G>A; rs1800629) gen polimorfizimleri ile inhibitör gelişimi arasındaki ilişkinin araştırılması amaçlanmıştır. **Gereç ve Yöntemler:** Çalışmaya, *inv22* mutasyonu taşıyan 94 ağır HA hastası dâhil edildi. Hastalar, inhibitör varlığına göre 2 gruba ayrıldı: İnhibitör pozitif ve inhibitör negatif. Her iki grupta da Sanger dizi analizi yöntemi kullanılarak *CTLA-4* geninde c.-318C>T; rs5742909 ve c.49A>G; rs231775 ve *TNF-α* geninde de c.-308G>A; rs1800629 polimorfizimleri araştırıldı. **Bulgular:** Bu çalışmada, *inv22* mutasyonu taşıyan ağır HA hastalarında *CTLA-4* polimorfizmleri ile inhibitör gelişimi arasında anlamlı bir ilişki saptanmamıştır. Bununla birlikte, *TNF-α* genindeki c.-308G>A varyantının A allelinin, bu hastalarda inhibitör gelişimi için artmış risk ile ilişkili olduğu bulunmuştur. **Sonuç:** *Inv22* mutasyonu taşıyan ağır HA hastalarında, *CTLA-4* genindeki c.-318C>T ve c.49A>G varyantları inhibitör gelişimi ile ilişkili değildir, oysa *TNF-α* genindeki c.-308G>A varyantı, A alleli, inhibitör gelişme riski ile ilgilidir.

Anahtar Kelimeler: Hemofili; inhibitör; *CTLA-4*; *TNF-α*; intron 22 inversiyon

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Hemophilia A (HA) is an X-linked inherited bleeding disorder caused by a deficiency in the coagulation factor VIII (FVIII) results from disease causing mutations in the *F8* gene. The prevalence of HA is about one in 5,000 male birth. According to the level of FVIII, phenotype is classified into 3 groups. While in severe HA FVIII level is below <1%, it is >5% in mild HA. In severe hemophilia patients, intron 22 inversion (inv22) mutation caused by intrachromosomal rearrangement constitutes almost 40% of cases. FVIII protein replacement is used for the treatment of HA, but this therapy can sometimes be noneffective due to the development of neutralizing antibodies (inhibitors) against the administered FVIII protein. Inhibitor development represents the most challenging complication of HA treatment. This complication develops in approximately 25% of severe HA patients.¹⁻³

The underlying mechanism of inhibitor development is not completely clear. As a classical multifactorial polygenic trait, factors related to inhibitor development are categorized into 2 groups (genetics and non-genetics). The major genetic risk factor is the mutation type causing HA. Other genetic factors are an inhibitor history in the family, human leucocyte antigen haplotype, ethnic origin, and polymorphisms of some genes related to immune system including CTLA-4 (cytotoxic T-lymphocyte antigen-4), tumor necrosis factor alpha (TNF- α), interleukins, and transforming growth factor beta 1 etc.⁴⁻⁷

In order to investigate the effects on the development of inhibitor in severe HA patients for the first time in Türkiye, we genotyped 2 polymorphic sites in CTLA-4 and one site in TNF- α in a selected cohort.

MATERIAL AND METHODS

STUDY GROUP

Our study cohort consisted of 94 severe HA patients who were diagnosed according to the guidelines published by World Federation of Hemophilia.⁸ All the patients enrolled in the study had inv22 mutation in *F8* gene and they were classified into 2 groups. According to the definitions established by International Society on Thrombosis and Hemostasis, patients

were noted to express inhibitors which were documented on 2 different occasions, above 0.6 Bethesda units (BU) per mL using the Nijmegen modification of Bethesda test.⁹ Clinical data were collected including patient information such as gender, age, age at first administration of FVIII replacement. The patients defining gross hemorrhage such as CNS hemorrhage or uncontrolled bleeding during or after a surgery were excluded.

Genotyping of CTLA-4 and TNF- α

Genomic DNA was obtained from 2 mL peripheral blood using a Gentra Puregene Blood Kit (QIAGEN), in accordance with the manufacturer's instructions.

We investigated two single nucleotide polymorphisms (SNPs) in CTLA-4 (c.-318C>T; rs5742909 and c.49A>G; rs231775) and one in TNF- α (c.-308G>A; rs1800629). The genomic region including these SNPs were amplified by polymerase chain reaction (The primers are given in the Table 1). The amplicons were sequenced on capillary 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment was performed using the CLC Genomics Workbench.

STATISTICAL ANALYSIS

To analyze the data, statistical package IBM SPSS version 25 (IBM Corp., Armonk, NY, USA) was used. The chi-squared test was used to compare differences including genotypes, and alleles between inhibitor-positive patients (IPP) and inhibitor-negative patients (INP) and $p < 0.05$ was considered as statistically significant.

The study was approved by the Ethical Committee of the Ege University Medical Faculty (date: October 02, 2018, no: 18-10/11) and samples from the patients were obtained in accordance with the Helsinki Declarations.

TABLE 1: Primers list used in this study.

SNP	Forward primer	Reverse primer
CTLA4:c.-318C>T	AAATGAATTGGACTGGATGGT	TTACGAGAAAGGAAGCCCGTG
CTLA4:c.49A>G	AACACATTTCAAAGCTTCAGGA	AACAAATGAAACCCAGGTAGGA
TNF:c.-308G>A	CTGAAGCCCTCCAGTT	AAAGTTGGGGACACACAAGC

SNP: Single nucleotide polymorphism.

TABLE 2: The association between inhibitor development and 3 SNPs (genotype and allele).

SNP	Genotype	Inhibitor negative n (%)	Inhibitor positive n (%)	p-value	
CTLA-4 (49A>G)	AA	32 (46.4)	13 (52)	0.311	
	AG	31 (44.9)	12 (48)		
	GG	6 (8.7)	-		
	A	95 (68.8)	38 (76)		0.340
	G	43 (31.2)	12 (24)		
CTLA-4 (-318C>T)	CC	57 (82.6)	20 (80)	0.758	
	CT	11 (15.9)	5 (20)		
	TT	1 (1.5)	-		
	C	125	45 (90)		0.905
	T	13 (9.4)	5 (10)		
TNF (-308G>A)	GG	69 (100)	20 (80)	0.000	
	GA	-	5 (20)		
	AA	-	-		
	G	138 (100)	45 (90)		0.000
	A	-	5 (10)		

SNP: Single nucleotide polymorphism.

Written informed consent for genetic testing was obtained from all subjects or their parents/guardians.

RESULTS

Following the exclusion of the cases with the history of gross hemorrhage at the diagnosis, in the study unrelated 94 severe HA patients were included. All the patients enrolled had inv22 mutation in *F8* gene. Twenty five (26.5%) of them were inhibitor-positive.

CTLA-4 Genotype Distribution

In *CTLA-4* gene, for both variants (c.-318C>T and c.49A>G), no significant differences were found between 2 groups according to the allele frequency or genotype (Table 2).

TNF- α Genotype Distribution

In *TNF- α* gene, for the c.49A>G, the A allele frequency was found to be 0% in INP and 10% in IPP. Of INP, all were identified as homozygous for the G allele (GG), no patient were homozygous for the A allele (AA), or heterozygous (GA), compared with 20 (80%), 0 (0%) and 5 (20%) of IPP, respectively. There was significant difference between 2 groups (Table 2).

DISCUSSION

The most challenging complication during the treatment of HA is the development of neutralizing antibodies for the FVIII protein. To foresee which HA patients are at risk for inhibitor development and to identify susceptibility factors that lead to inhibitor formation would allow the use of suitable therapies. Here, in this research, we focused on severe HA patients specifically having inv22 mutation and aimed to investigate relationship between 3 different polymorphic variants in 2 immune system related genes (CTLA-4 and TNF- α) and inhibitor development.

The inhibitor development for the FVIII protein in HA patients is a T helper (Th) cell-dependent process where B-lymphocytes and antigen presenting cells (APCs) play role. APCs present molecules of the FVIII protein administered to the T cell receptor. The second signal at the same time is produced by the interaction between CD28 on Th cells and CD80/86 molecules on APCs. CTLA-4 is a receptor that competes with CD28 for the interaction with CD80/86 molecules. This mechanism leads to the lower activity of T cell. If this CTLA-4 interaction is blocked, the proliferation of T cell and the activity of B cell is increased.^{4,10}

The c.-318C>T variant in *CTLA-4* gene was reported to be associated with the higher activity of the promoter region, a negative effect on the interaction between APCs and Th cell, so it might be related to a lower risk of inhibitor development.^{11,12} Astermark et al. in 2007 reported a significant protective association between inhibitor development and c.-318C>T variant in *CTLA-4* in 123 siblings (63 inhibitor patients) with severe HA.¹³ Marchione et al. in 2017 confirmed this finding in a large cohort of severe HA with inv22 and other type of mutations, separately.¹⁴ In contrast to Astermark's and Marchione's studies, Pinto et al. in 2012 reported no association between inhibitor development and c.-318C>T in *CTLA-4* in a group of Indian severe HA cases.¹⁵ Similar to the Pinto's research, Agostini et al. showed no significant relationship in Brazilian patients with severe HA.¹⁶ In our study, similar to the study of Marchione et al., we focused on the patients with intron 22 mutation.¹⁴ In our cohort, there was no significant protective relationship between c.-318C>T in *CTLA-4* and inhibitor development.

The c.49A>G variant in *CTLA-4* gene results in incomplete glycosylation in the endoplasmic reticulum and it decreases surface/total ratio of the protein. Its function might be affected by this.^{11,17} The inhibitory effect of the *CTLA-4* protein on T cells is less affected in patients with the G allele. Astermark et al., in their cohort, showed no significant relationship between inhibitor development and the c.49A>G variant in *CTLA-4* gene.¹³ In contrast to Astermark et al., Marchione et al. in their Argentinian cohort with inv22 mutation showed that the G allele is associated with significantly higher risk for the development of inhibitor.¹⁴ In this study, we found no significant association.

In addition to the immune related genes interacting with APCs-Th cell signal, cytokines less or more directly play role in the antibody-mediated immunity. Therefore, SNPs in some cytokine genes may also be involved in inhibitor formation. TNF- α , as an important cytokine, has a potent immunomodulatory and proinflammatory effect and SNPs in this gene have been reported to be related with autoimmune disease.¹⁸ Astermark et al. reported the association between the -308A/A genotype and inhibitors in a HA cohort. The cases they analyzed include 124 se-

vere HA patients and 75 of them were the patients with inversion mutation.¹⁹ Pavlova et al. analyzed c.-308G>A polymorphism in TNF- α in HA patients to evaluate its effect on inhibitor development. The authors showed that individuals with homozygous for the allele A (AA) presented a higher risk of inhibitor development in severe HA.²⁰ In another study conducted by Zhang et al. (2011), this SNP was analyzed in 140 Chinese Han patients with HA and the authors confirmed the same result in their cohort.²¹ But studies showed that the A allele is not associated with the increased risk. Here, in this study, in our cohort, while all inhibitor-negative HA patients with inv22 mutation were found to have the G allele homozygously, 80% of inhibitor-positive cases had GG genotype. In our inhibitor-positive group, there were no AA genotype as well, however, 20% of inhibitor-positive group was found to have heterozygous for the A allele. According to this result, we showed that the A allele is related with the increased risk for the development of inhibitor in severe HA patients with inv22 mutation.

Our study is the first study analyzing the effect of *CTLA-4* and TNF- α polymorphisms on the development of inhibitor in a Turkish cohort of severe HA patients with inv22 mutation. A small number of patients in this study is the main weak point. It resulted from the choice of only the patients with inv22 mutation. The aim of the choice of this cohort was to provide perfectly homogeneous group.

CONCLUSION

In Turkish severe HA patients with inv22 mutation, c.-318C>T and c.49A>G variants in *CTLA-4* gene are not associated with the inhibitor development, however, for the c.-308G>A variant in TNF- α , the A allele is related to the risk of inhibitor development.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or mem-

bers of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

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