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# Relationship Between Elevated FVIII Level and 3' UTR Variations of F8 Gene in Turkish Patients with Venous Thrombosis

# Türk Venöz Trombozlu Hastalarda Yüksek FVIII Düzeyi ile F8 Geninin 3' UTR Varyasyonları Arasındaki İlişki

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ABSTRACT

Objective: Venous Thrombosis (VT) is an important medical disorder caused by genetic and environmental factors. This study investigated the sequence variants in the 3' untranslated region (UTR) of Factor 8 gene in 30 patients with high FVIII plasma levels and 30 healthy individuals with normal FVIII plasma levels.
Materials and Methods: The plasma levels of FVIII protein were measured in blood samples using the Static Timing Analyze Kit. The 3' UTR region of F8 gene was amplified by PCR using 6 primers pairs. Single strand conformation analysis (SSCA) and DNA sequencing analysis were carried out for determination of the sequence variants.
Results: While the FVIII protein level was 200 ± 64 IU / dl in patients, it was detected as 120 ± 32 IU / dl in controls. In the 3 'UTR of the F8 gene were observed different SSCA patterns of some individuals and nucleotide change rs1050705 of the F8 gene. Therefore, we did not find a significant relationship between sequence variants in 3' UTR of F8 gene and elevated FVIII levels.
Conclusions: VT does not seem to be associated with the sequence variant in 3' UTR of the F8 gene but small-scale studies will draw attention to studies on the 3' UTR regions of the genes to elucidate complex disorders such as venous thrombosis.



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ÖZET

Amaç: Venöz Tromboz (VT), genetik ve çevresel faktörlerin neden olduğu önemli bir tıbbi hastalıktır. Bu çalışmada, yüksek FVIII plazma seviyelerine sahip 30 hastada ve normal FVIII plazma seviyelerine sahip 30 sağlıklı bireyde Faktör 8 geninin 3' transle edilmeyen bölgesindeki (UTR) dizi varyantları araştırıldı.
Materyal ve Metot: Statik Zamanlama Analiz Kiti kullanılarak kan örneklerinde FVIII proteininin plazma seviyeleri ölçüldü. F8 geninin 3' UTR bölgesi, 6 primer çifti kullanılarak PCR ile çoğaltıldı. Dizi varyantlarının belirlenmesi için tek zincirli konformasyon analizi (SSCA) ve DNA dizi analizi yapıldı.
Bulgular: FVIII protein düzeyi hastalarda 200 ± 64 IU/dl iken kontrollerde 120 ± 32 IU/dl olarak tespit edildi. F8 geninin 3' UTR'sinde bazı bireylerin farklı SSCA paternleri ve F8 geninin rs1050705 nükleotid değişikliği gözlendi. Bu nedenle, F8 geninin 3' UTR'sindeki dizi varyantları ile yüksek FVIII seviyeleri arasında anlamlı bir ilişki bulamadık.
Sonuç: VT, F8 geninin 3' UTR'sindeki dizi varyantı ile ilişkili görünmemektedir, ancak küçük ölçekli çalışmalar, venöz tromboz gibi karmaşık bozuklukları aydınlatmak için genlerin 3' UTR bölgelerine yönelik çalışmalara dikkat çekecektir.



## 1. Introduction

Thrombosis is pathologic coagulation of blood in vessels. Venous Thrombosis (VT) is a major problem worldwide, and it causes more than 0.5 million deaths in a year in the European Union (1). Thrombosis is a complex multifactorial disease caused by the interactions between genetic and environmental factors. Most of the investigations on the level of factors involved in this disease are physiological studies. In addition, many studies have shown that the high factor VIII (FVIII) plasma level is hereditary (2,3). These studies indicated that a high plasma level of FVIII is a higher threat for venous thrombosis than for arterial thrombosis (4,5,6). Plasma levels of FVIII are higher than or equal to 150 IU/dl in 16% of all venous thrombosis patients, whereas FVIII plasma levels are higher than 123 IU/dl in 4% of all arterial thrombosis patients (5). The risk for VT depends on the level of FVIII. In other words, elevated FVIII plasma levels may increase

the risk of venous thrombosis through induction of acquired APC resistance and/or increased thrombin levels (5). The FVIII level in plasma is regulated in a complex way. Most of the FVIII protein circulates in blood plasma in the form of a complex with von Willebrand factor (VWF) (7,8) that the level of which is accepted to be related to endothelial stimulation (9,10) and the factors like blood group (11,12). The molecular principle of high levels of FVIII protein is partially known, it is accepted that it consists of genetic and acquired factors. There are few studies on the polymorphisms in the F8 gene and plasma levels of FVIII (4,5,13,14).

MicroRNAs (miRNAs) are a class of non-coding, single-stranded, short RNAs (about 21-23 nucleotides in length). miRNAs are significant regulatory molecules in plants and animals. miRNAs regulate gene expression by mRNA cleavage, translational repression, and mRNA decay initiated by miRNA-guided rapid deadenylation (15). In other words, miRNAs are actively involved in gene regulation in the cell, in the timing of development, cell differentiation (16), apoptosis (17), organ development (18) and metabolism (19). The existing evidence have shown that a dysregulated miRNA expression is associated with the onset and progression of immunological disorders, such as multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis (20). miRNAs are complementary to one or more mRNAs and post-transcriptionally down-regulate gene expression by binding target 3′ UTR mRNA sequences in mammalians. Small non-coding RNAs provide a new perspective to explain the change in functionality of the 3 'UTR variant (21). This study aimed to define the possible relationship between high FVIII levels and unknown nucleotide variations in 3' UTR of the F8 gene in Turkish patients with venous thrombosis.

## 2. Materials and Methods

Thirty patients with documented VT and 30 healthy individuals were included in this study. Individuals in the control group had not a cardiovascular disease and had a familial history of VT. All tests were performed by the relevant clinics with the consent of the patients, and since our study consists of a retrospective evaluation of these test results, ethics committee approval was not required. The written informed consent form was obtained from the study participants in accordance with the Declaration of Helsinki. All participants were recruited from the Department of Hematology, Istanbul Medical Faculty, Istanbul University and from the Turkish population.

### 2.1. Genomic DNA Isolation and Static Timing Analysis

Five ml of peripheral blood sample was collected in EDTA vacutainer tube from patients and healthy individuals. Genomic DNA was isolated from peripheral blood leukocytes using the PureLink™ Genomic DNA Mini Kit (Invitrogen, USA). In addition, blood plasma samples were used to measure FVIII levels using the Static Timing Analysis-Immunodeficiency FVIII Kit. (Stago, USA).

### 2.2. Polymerase Chain Reaction

PCR technique was used to amplification of 3' UTR. In the PCR were used specific oligonucleotides primer pairs (Table 1). PCR amplification was performed in 25 µL reaction mixture including 150 ng/mL of DNA, 1X PCR-buffer (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), 1.5 mM MgCl (Fermentas) for all PCR fragments, 200 µM dNTPs (Fermentas) 10 pmols of primers, and 1 U of Taq DNA polymerase (Fermentas). The amplification program was carried out at 95 °C for 2 min followed by 30 cycles of 45 sec at 94 °C, 45 sec at 53 °C (fragment 3), 45 sec at 55 °C (fragment 1) or at 57 °C (fragment 2, 4, 5 and 6), 45 sec at 72 °C, and a final extension at 72 °C for 5 min in Veriti Thermal Cycler (Applied Biosystems, Invitrogen). PCR products were displayed by electrophoresis on 2% agarose gel stained with ethidium bromide and the product size was checked using a 100 bp DNA ladder.

**Table 1.** The oligonucleotide primer pairs for PCR

| Primer name | Primer sequence |
|---|---|
| Fragment 1-F | F-5’ ACC GTT ACT GAC TCG CTA CC |
| Fragment 1-R | R-5’ GAA GGA GTA ATC TGG GAG CA |
| Fragment 2-F | F-5’ GAG GGT GCA TCC AAT TTA AC |
| Fragment 2-R | R-5’ TCT CCA TTT TGC AGA TTG TC |
| Fragment 3-F | F-5’ TGG AAC AAA GCA TGT TTC AG |
| Fragment 3-R | R-5’ AGC TTT CAA CAA TTG CAT CC |
| Fragment 4-F | F-5’ AAA GCC ATT TGG TCT TAA TTC T |
| Fragment 4-R | R-5’ TTT CTG TTT TCA CCA GTC CA |
| Fragment 5-F | F-5’ CCG TGA CTG AAA ACT AGA GTC C |
| Fragment 5-R | R-5’ GGG TCA AGC AGG ATT ATT AGA |
| Fragment 6-F | F-5’ GGC AAA TGG AAA ACA GGA GAT CC |
| Fragment 6-R | R-5’ CTC AAA GGC ATT TGT TTG TAT GTG |

### 2.3. Single-Strand Conformation Analyses (SSCA) and DNA Sequencing Analysis

The unknown nucleotide variations were determined by single-strand conformation analyses (SSCA) (22). Two different conditions for SSCA were used; it is 4% stacking polyacrylamide gel and 6% separating polyacrylamide gel. These polyacrylamide

gels (non-denaturing) was prepared from 30% acry:bisacry (29:1) in a 10xTBE buffer containing 10% APS and TEMED. Polyacrylamide gel was made and poured in 200x200x1 mm glass plates; It was polymerized for a minimum of 1 hour. Five µl of the PCR product was mixed with 15 µl of SSCA gel loading buffer (10 mM NaOH, 0.05% xylene cyanol, and 0.05% bromophenol blue in 95% formamide) and was denatured at 95°C for 5 min. The mixture of PCR samples was instantly cooled on ice for 5 minutes and loaded into a polyacrylamide gel well. Samples were electrophoresed using a Protean® II XL Cell (Bio-Rad) vertical electrophoresis cell. The central cooling core of Protean® II XL Cell (Bio-Rad) connects to a tap water line. Gels were pre-run for 30 min at 150 V and a run for 4.5-5 h at 400 V. DNA fragments after electrophoresis was visualized by silver staining technique. DNA samples belonging to different SSCA products detected by Direct DNA Sequencing Analysis System were analyzed. Direct DNA Sequencing Analysis System was built with Applied Biosystems 8 capillary 3500 devices. DNA sequence analysis results were analyzed with programs such as Sequencing Analysis Software and Sequencing-SeqScape Software v2.7. DNA sequence analysis results were compared in the NCBI Basic Local Alignment Search Tool and differences were detected.

## 3. Results

This study included 30 patients (19 male, 11 female; mean age: 23±17) and 30 healthy individuals (16 male, 14 female; mean age: 50±11 years). The healthy control group was selected from individuals over the age of 40 to eliminate the advanced age risk factor for venous thrombosis.

Plasma FVIII levels of patients with VT and controls were determined as 200±64 IU/dl and 120±32 IU/dl, respectively. FVIII levels were found to be significantly higher in the VT patient group compared to the healthy ones. PCR was successfully performed with 6 specially designed primers. We examined single nucleotide variants in 3' UTR of the F8 gene in 30 patients with a high level of FVIII and 30 controls using SSCA.

The different patterns were observed of 3' UTR of the F8 gene in some individuals. A nucleotide exchange F8 rs1050705-G was detected in two men and one woman three thrombosis patients with high levels of FVIII. To date, no potential binding miRNA to the site of the F8 rs1050705 variant has been detected.

## 4. Discussion

The thrombosis was dependent on complicated interactions like deficiencies in protein C, in anti-thrombin, and in protein S, APC resistance, high FVIII level, and prothrombin (FII) 20210G>A allele (5, 23). A high plasma level of FVIII is a significant risk factor for VT. The molecular principle of high FVIII protein level, consisting of genetic and environmental factors, is only partially known. If it is supposed a cutoff value that results in 2% prevalence in the healthy population, it is determined that the prevalence of unexplainable high FVIII levels in patients with thrombosis varies between 11% and 18% (24). For this reason, a high FVIII level can be suggested that is not only an obviously increased relative risk but also a considerable attributable risk. High FVIII level may be an important risk factor as factor V Leiden (FV Leiden) and F2 gene 3' UTR 20210G>A mutation are the most common defects of the inherited thrombophilia (24).

Several studies were focused on the possible relationship between F2 gene mutation, FV Leiden, protein C, and venous thrombosis in the Turkish population (13, 26). In our previous study, the frequencies of SNPs in F8 gene exons were determined in 20 venous thrombosis patients who have elevated levels of FVIII, and the statistical analysis showed no significant difference. (13). Bittar et al. (26) determined one variation in the 3' UTR region of F8 gene that presents in only one in 75 venous thromboembolism patients. They suggested that genetic variation in F8 3' UTR region is not associated with elevated FVIII levels (26). Some other studies have not identified any polymorphism in the 3' terminus and the promoter regions of the F8 gene in 62 thrombosis patients with high FVIII level (27).

MicroRNAs are created from endogenous hairpin-shaped transcripts and play an important role in the regulation of many genes as the posttranscriptional (28). They can bind to a partly complementary site in 3′ UTR of mRNAs and usually block protein translation or stimulate the degradation of their target mRNAs (28). Specific SNPs, which are also called miR-SNPs can locate predicted miRNA target sites within 3′ UTR of mRNAs. Such variations have the potential to affect the efficiency of miRNA binding on its target region, create a new binding site or destroy binding sites (29). In a subgroup of patients with postmenopausal breast cancer who responded poorly to letrozole chemotherapy, a polymorphism study was performed on the 3' UTR region of the aromatase gene. It was found that there was a relationship between rs4646 polymorphism and letrozole chemotherapy poor response in this patient group (30). In patients with spontaneous venous thromboembolism, 1444C> T

polymorphism was detected in the 3' UTR region of the active protein C gene. But, there was no significant relationship between this polymorphism and venous thromboembolism (31). In this study, we provide the first systematic analysis of the 3' UTR of F8 gene region in patients with VT and elevated FVIII levels. We did observe different DNA patterns and nucleotide exchange of 3' UTR of F8 gene in patients subjects. The nucleotide exchange in the 3' UTR region of F8 gene is responsible for the formation of different DNA patterns. However, we cannot claim that these nucleotide changes in the F8 gene 3' UTR of patients with venous thrombosis are associated with the modified of post-transcriptional regulation via miRNAs.

A nucleotide exchange F8 rs1050705-G was detected in three thrombosis patients. Vossen et al. determined F8 rs1050705 variations were in control women with a similar minor allele frequency for F8 rs1050705-G for control women (27,5%) and men (26,7%). Vossen et al. F8 rs1050705 was related to an increased risk of venous thrombosis in men (32).

It has been demonstrated that nucleotide changes in miRNA binding target regions have an effect on the risk of diseases like Parkinson's disease, colorectal cancer, and childhood asthma (33). Thus, nucleotide variations in possible miRNA binding sites may have an effect on the expression of the target protein by altering the FVIII protein plasma levels and increasing the risk of venous thrombosis.

## 5. Conclusion

We examined 3' UTR of F8 gene region in a small population of patients individuals and did observe the different DNA patterns and nucleotide exchange in participants. Our findings show that there is no significant difference in DNA nucleotide changes of the 3 'UTR of the F8 gene in patients with venous thrombosis and healthy subjects. Also, in our previous study, no pattern differences have been observed in the promoter region and exons in F8 gene between venous thrombosis patients and the healthy control group (13). These findings may provide data for further studies on risk factors and thrombosis, focusing on the 3 'UTR regions of genes to elucidate complex disorders such as venous thrombosis.

**Conflict of Interest:** The authors report no conflicts of interest and are responsible for the content and writing of the paper.

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