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Research Article

Investigation of the Association between rs2296283 and rs7337610 Polymorphisms of FLT-1 Gene with Behcet's Disease in the Population of Tehran-Iran

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Abstract

Background: Behcet's Disease (BD) is a rare autoimmune disease with undetermined etiology. In this study, the association of rs2296283 and rs7337610 polymorphisms of FLT-1 gene with the possibility for BD was investigated in Tehran population.

Methods: In this case-control study, 100 samples (50 patients with BD and 50 healthy individuals with no background of disease) were examined. PCR-RFLP procedure was performed to determine the rs2296283 and rs7337610 polymorphisms using the *HaeIII* and *MseI* enzymes, respectively. SNPStats software was used for the analysis of association study.

Results: The frequencies of CC, CT, and TT genotypes for rs2296283 polymorphism were 26, 35, and 39% in patients, and 32, 34, and 34% in healthy population, respectively ($p>0.05$). The frequency of TT, CT and CC genotypes for FLT-1 rs7337610 polymorphism in patients with BD was 18%, 31%, 51% and in healthy or control groups was 14%, 36% and 50%, respectively ($p>0.05$).

Conclusion: No significant relationship was observed between rs2296283 and rs7337610 polymorphisms and the BD. Study on a greater number of samples is proposed.

Keywords: Behçet's disease; FLT-1 gene; Polymorphism; rs2296283 and rs7337610

Introduction

Behcet's disease, also known as Behcet's syndrome, is a rare disease causes blood vessels inflammation, which leads in redness, pain, and swelling in the arms and legs. Large arteries inflammation, which can result in complications such as aneurysms and narrowing or blockage of blood vessels, can be observed throughout the body [1,2]. According to studies, BD is thought to be an autoimmune disease due to its positive response to immunosuppressive factors and autoantigens and T cells involvement [1].

This condition can cause many symptoms that may not be obvious at in early stages. Symptoms may include oral cavity sores, eye inflammation, rashes, skin lesions and genital sores [3,4]. The etiology of Behcet's disease is not clear yet. But it

is believed that an autoimmune process may be involved [5]. Furthermore, the genetic and environmental factors can also influence in the frequency of the disease [6]. Geographical living environment is another risk factor in the individuals, especially people from the Middle East and the Far East, including Turkey, Iran, Japan and China, are more likely to develop the disease [7]. Although Behcet's disease occurs in both men and women, it is usually more severe in men [8]. Iran is one of the most prevalent countries for BD (7641 cases in 2018) with oral aphthosis as the highest frequent symptom [9].

Several genes have been linked to the disease, but some researchers believe that viruses and bacteria may also cause Behcet's disease in people with certain genes to which they are sensitive [10,11]. Gene polymorphisms and environmental factors may be involved in the development of BD [12]. Several genes such as interleukin genes, IL-1A, IL-1B, IL- 1 receptor antagonist [13], IL-2 [14], tumor necrosis factor (TNF) [13], transporter associated with antigen processing (TAP) [15], Intercellular Adhesion Molecule-1 (ICAM-1) gene [16], endothelial nitric oxide synthase (eNOS) gene [17], glutathione S-transferase gene [18], N-acetyltransferase gene [19], and vascular endothelial growth factor (VEGF) gene polymorphisms have been related to BD susceptibility. Since VEGF expression is induced by these cytokines and also it is a potent stimulator for nitric oxide production by affecting endothelial cells [20], this study has been done with the purposes of evaluating the effects of FLT-1 (VEGFR) on patients with Behcet's syndrome. The FLT-1 gene encodes Vascular Endothelial Growth Factor Receptor (VEGFR) family [21]. This protein binds to VEGFR-A, VEGFR-B and plays an important role in angiogenesis and vasculogenesis [22]. The association of polymorphisms in FLT-1 gene with BD has been investigated in different studies [23,24]. In fact, the main aim of this study was to investigate the polymorphism of FLT-1 gene (VEGFR-1) in two loci rs2296283 and rs7337610 in two groups of BD and controls using PCR-RFLP in Tehran.

Materials and Methods

Sampling

This case-control study was performed on 100 samples in two groups: 50 samples from patients with Behcet's syndrome, which were confirmed by rheumatologists according to international standards [25] (Rheumatology Center of Iran, Tehran, Iran) and 50 samples from healthy individuals as a control average age ± standard deviation was 30.14±6.1 years. written informed consent was obtained from the patients. Five ml of peripheral blood were transferred to tubes containing EDTA and stored at -20 ˚C.

DNA Extraction and Polymerase Chain Reaction

DNA was extracted using modified salting-out method from peripheral blood samples [26]. The quantification of extracted DNA was performed using Spectronanophotometr at 260 nm and quality was measured by the 260/280 and 260/230 ratios. The integrity of genomic DNA was checked by agarose gel electrophoresis. PCR-RFLP method was used to determine the genotype of FTL-1 gene polymorphisms. Specific forward and reverse primers of FTL-1 gene were designed using Gene Runner program (Table 1). PCR reaction was performed in a final volume of 15 µL containing 15 ng of genomic DNA, 0.2 Unit/ µL each primer, 1.5 mM MgCL2, 0.2 Unit TaqDNA polymerase and 0.2 µM dNTP with the following amplification protocol: initial denaturation at 95˚C for 2 min, followed by 31 cycles of denaturation at 95˚C for 30 s, annealing at 57˚C for 30 s, and extension at 72˚C for 40 s. The program was completed with a final extension at 72˚C for 5 min. The PCR products were then analyzed by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide (EtBr) staining under UV.

Table 1: Primers of FTL-1 gene variants used in the presence study.

SNP ID	Nucleotide sequence 5′ ⇢ 3′	PCR Products
rs2296283	ATTGTCACTCTTGCTA-ACTTTCAG TTATTCAGTATGTG-CAGCTTCAAC	309 bp
rs7337610	ACTAGTTGTTTTCAGAG-CATTTGG TTGTTATAAGCTTGCGTTT-GAGG	372 bp

Restriction Fragment Length Polymorphism (RFLP)

The amplified products were digested using the different restriction enzymes and incubated for 16h at 37˚C. The amplified products of Flt-1 rs2296283 were digested with the enzyme *HaeIII*, whereas for Flt-1 (rs7337610), *MseI* restriction enzyme (Thermo Scientific) was used. Digestion was performed in a final volume of 10 µL containing 0.2 unit/µL of restriction enzyme, enzyme buffer 1x, 4 µL of PCR product and 4.8 µL of H2O. Fragments were separated on 12% acrylamide gel and stained with silver nitrate.

Statistical Analysis

SNPStats (online software) was used to test the association between the studied SNPs and Behcet's disease and also the Hardy-Weinberg equilibrium in controls. A P value of less than 0.05 was considered significant.

Results

In this study the genotype and allele frequency of rs2296283 and rs7337610 polymorphisms was investigated by PCR-RFLP and compared between a cohort of patients affected with Bechet's disease and normal control subjects. PCR products for FTL-1 gene in the rs2296283 locus was located at the molecular length of 309

bp. The amplified products of Flt-1 were digested with the restriction enzyme *HaeIII*. Results of polyacrylamide gel electrophoresis (12%) showed three genotypes TT, TC and CC. Homozygous CC (mutant C allele) with two bands of 171 and 92 bp. Homozygous TT (wild-type T allele) without cleavage (a band of 309 bp) and a heterozygous CT with three 309 bp, 171 bp and 92 bp bands was observed (Figure 1 and Table 2). The distribution of different genotypes (CC, CT, and TT) of FLT-1 rs2296283 polymorphism in patients and healthy subjects is presented in Table 3. The frequencies of CC, CT, and TT genotypes were 26, 35, and 39% in patients, and 32, 34, and 34% in healthy population, respectively.

Table 2: The effect of *HaeIII* and *MseI* restriction enzymes on FLT-1 gene polymorphisms.

SNP ID	Restriction Enzyme	Recognized Sequence	Genotypes	Length of fragments
Rs2296283	*HaeIII*	GG_^CC	CC	**92bp+171bp**
			TT	**309 bp**
			CT	**92bp+171bp+309bp**
Rs7337610	*MseI*	T^TAA	CC	**260bp+112bp**
			TT	**260bp+65bp+47bp**
			CT	**260bp+112bp+65bp+47bp**

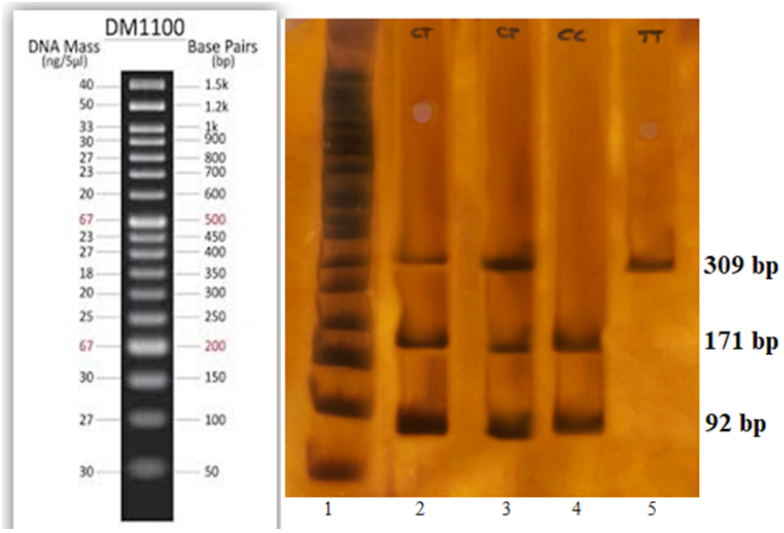

Figure 1: PCR-based restriction fragment length polymorphism analysis for FLT-1 gene at rs2296283 locus after enzymatic digestion using *HaeIII* restriction enzyme. Three genotypes TT, CT, CC were observed. Lane 1: 50 bp DNA marker; Lane 2 and 3: heterozygous CT; Lane 4: homozygous CC and Lane 5: homozygous TT in Behcet's patients.

The amplified PCR products of Flt-1 Rs7337610 polymorphism were digested using *MseI* restriction enzyme. Polyacrylamide gel electrophoresis revealed three genotypes, CC (mutant C allele) with two bands of 260 and 112 bp, TT (wild-type T allele) with 260, 65 and 47 bp and a heterozygous CT with four 260, 112, 65 and 47 bp bands was observed (Table 2 and Figure 2). The frequency of TT, CT and CC genotypes for FLT-1 Rs7337610 polymorphism in patients with Behcet's disease was 18%, 31%, 51% and in healthy or control groups was 14%, 36% and 50% respectively. The distribution of these genotypes is shown in Table 3. The characteristics of the studied population, which are described in Table 3, showed no significant differences for studied polymorphisms (rs2296283 and rs7337610) in the groups.

Table 3: Distribution of different genotypes for Rs2296283 and Rs7337610 polymorphisms in Behcet's disease and control.

SNP ID		Genotype	Patient	Control	OR	P-value
Rs2296283	Codominant	T/T C/T C/C	19(38.8%) 17(34.7%) 13(26.5%)	17(34%) 17(34%) 16(32%)	1.00 1.12(0.44-2.86) 1.38(0.52-3.67)	**0.81**
	Dominant	T/T C/T – C/C	19(38.8%) 30(61.2%)	17(34%) 33(66%)	1.00 1.23(0.54 – 2.79)	**0.62**
	Recessive	T/T - C/T C/C	36(73.5%) 13(26.5%)	34(68%) 16(32%)	1.00 1.30(0.55 – 3.11)	**0.55**
	Over dominant	T/T – C/C C/T	32(65.5%) 17(34.7%)	33(66%) 17(34%)	1.00 0.97(0.42 – 2.22)	**0.53**
Rs7337610	Codominant	C/C C/T T/T	25(51%) 15(30.6%) 9(18.4%)	25(50%) 18(36%) 7(14%)	1.00 1.20(0.50-2.90) 0.78(0.25-2.41)	**0.77**
	Dominant	C/C C/T – T/T	25(51%) 24(49%)	25(50%) 25(50%)	1.00 1.04(0.47 – 2.29)	**0.92**
	Recessive	C/C- C/T T/T	40(81.6%) 9(18.4%)	43(86%) 7(14%)	1.00 0.72(0.25 – 2.13)	**0.55**
	Over dominant	C/C – T/T C/T	34(69.4%) 15(30.6%)	32(64%) 18(36%)	1.00 1.27(0.55 – 2.95)	**0.57**

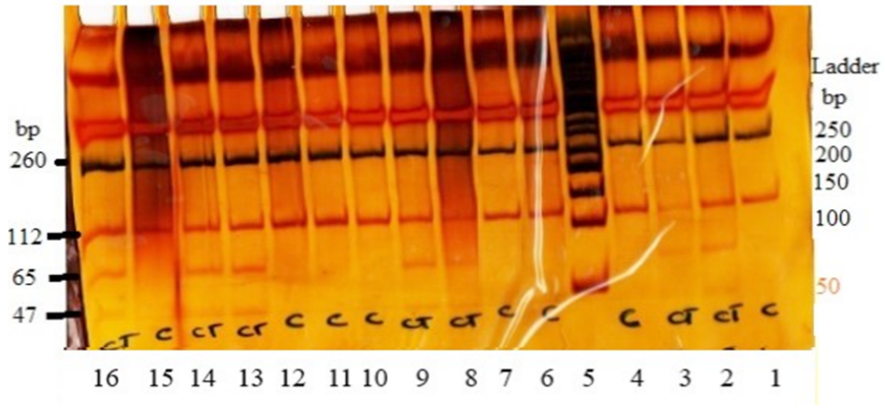

Figure 2: PCR-based restriction fragment length polymorphism analysis for the FTL-1 gene in the Rs7337610 locus after enzymatic digestion using *MseI* restriction enzyme. Lane 5: 50 bp Marker; Lanes 1, 4, 6, 7, 10, 11, 12 and 15: homozygotes CC; Lanes 2, 3, 8, 9, 13, 14 and 16: heterozygotes CT in Behcet's patients.

Discussion

BD is a systemic vasculitis identified by attacks of acute inflammation which can appear in almost any part of the body [27]. The most general indication is oral aphthosis, observed in more than 95% of patients [1]. VEGF and VEGFR (FLT-1; its receptor) play crucial roles in physiological and pathological angiogenesis, like cancer. Thus, VEGF-VEGFR complex is an important system for the treatment of neuronal degeneration [21]. Furthermore, $VEGF_{CSF}$ was markedly enhanced in patients with neuro-BD and MS [28]. Therefore, according to the several studies conducted on the relationship between different polymorphisms and BD, the objectives of this research are to determine whether FLT-1 gene polymorphism in two positions are correlated with BD. No previous research has

investigated the association between rs2296283 and rs7337610 polymorphisms with BD. However, Yuan et al. (2020) showed the frequency of allele T of rs2296283 was significantly lower in SLE patients (P=0.003, P=0.004) compared with healthy controls [29]. Furthermore, a study was performed to determine FLT-1 genetic variants (rs2296283, rs7337610 etc.) and their possible association with rheumatoid arthritis [22]. Slattery et al. (2013) has also studied the relationship between genetic variation in FLT1 (38 SNPs), etc. and colorectal cancer development and survival [30].

In the present study, two FLT-1 gene polymorphisms in BD in the Iranian population were studied for the first time. The results showed FTL-1 gene in the rs2296283 region increased in BD patients in TT homozygotes and declined in CC homozygotes. However, CT heterozygotes displayed no significant change. Data for FTL-1 gene polymorphism in rs7337610 locus revealed an enhance in TT homozygote and decrease in CT heterozygous, but no change was observed in CC homozygous. Results obtained from the comparison of two groups (Patient and Control), showed no significant difference between rs2296283 and rs7337610 polymorphisms with BD susceptibility ($P<0.05$). These results suggest that the variation in these regions is unlikely to play an important role in BD in our study population which can be due to: The relationship between the occurrences of polymorphisms in different populations. Due to the low prevalence of some alleles in the studied population, the probability of finding the homozygous mutant genotype also decreases. Especially if the sample size is small. Furthermore, Iran is a country with large population and contains different ethnic groups which can have their specific allele frequency which leads to achieve paradoxical results. Thus, if a large sample size can be examined, we can discuss the significance of these polymorphisms with BD patients in Iran more confidently.

The authors declare that they have no competing interests.

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