

Association study of mir-146a rs2910164 and rs57095329 polymorphisms with risk of Behcet's disease

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Abstract

Background & Aims: Behcet's disease (BD) is an autoimmune disease that usually occurs with oral, genital and ocular ulcers. The cause of this disease is unknown and environmental, genetic and immunological factors contribute to its development. In the present study, the relation between rs2910164 and rs57095329 polymorphisms of miR146a gene with the potential for BD was investigated in Iranian population.

Materials & Methods: In this case-control study, 100 patients with Behcet's disease and 100 Normal with no history of disease were studied as control. The polymorphism rs2910164 was determined by PCR-RFLP method using the *MnII* enzyme, and the polymorphism rs57095329 was determined by the ARMS PCR method for genotype. The data were analyzed using SPSS version 22.

Results: The prevalence of GG genotype in rs2910164 polymorphism showed no significant difference between patient and control groups ($p=0.156$). The Frequency of the genotype AA was also not significantly different in the rs57095329 polymorphism in both patient and control groups ($p=0.814$).

Conclusion: In this study, there was no significant relationship between rs2910164 and rs57095329 polymorphisms and the potential for BD. Research on a greater number of patients can be effective.

Keywords: Behçet's disease, miR146a, polymorphism, autoimmune disease.

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Introduction

Behçet's disease (BD) is a multi-systemic inflammatory disorder (1). The disease is characterized by four major symptoms including oral ulcers, genital ulcers, skin lesions and ocular lesions, and in some cases with minor signs of joint involvement, gastrointestinal tract ulcers, vascular and central nervous system symptoms. Although BD exists worldwide (2), it is more prevalent in northern China, Iran, Turkey and Turkish

German population (3). The mean age of onset is from the 2nd to 4th decade of life (4).

The cause of this disease is unknown (5); however, recent studies have shown that the disease may be triggered by environmental factors such as infection in patients with genetic predisposition (2). Genetic factors that predispose a person to BD are likely to play an important role in the development of this disease. Studies in BD patients showed that they have a strong genetic background and complex inheritance pattern (6).

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Several genes have been recognized to be associated with BD, the most common being in the HLA-B class (4). Genome-wide association studies (GWAS) have also found other potential susceptibility loci for BD, such as HLA-A*26, IL10, IL23R-IL12RB2, STAT4, GIMAP, CCR1, and KLRC4 (7). By using bioinformatics and molecular cloning methods, hundreds of miRNAs have been identified in plants, animals, and viruses. The miRNAs play an important role in various biological processes, such as development, proliferation and differentiation, carcinogenicity, metabolism, angiogenesis, and Inflammation (8). Dysregulation of miRNA expression and function is associated with a variety human disease including cancer, heart valve defect, neurodegeneration and autoimmunity (9), for example, miR146a plays an important role in autoimmune diseases like BD.

The miRNAs are short and non-protein coding RNAs that have a length of about 20-22 nucleotides and mediate post-transcriptional regulation by affecting mRNA stability and translational repression or activation (10-11). The miRNAs affect the functions of innate and adaptive immune responses. The miR146a plays an important role not only in these responses but also it plays an important role in autoimmune diseases. The miR146a has an effect on the expression of various genes such as IL-6¹, CD44, NCAM1², and NFAT5³, which are involved in the immune system, and thus affects immune responses (12).

The position of the miR146a gene is on human chromosome 5 and in the LOC285628 gene. The LOC285628 gene has two exons separated by the 16 kb sequence. The main sequence miR146a is located in the second exon (13). The miR146a gene polymorphisms, which interfere with maturation and function of miRNA, causes irregular expression of genes that are involved in

the immune system. The most important polymorphisms reported in this gene are rs2910164 single-nucleotide polymorphism G/C in the LOC285628 miR146a gene. This polymorphism is located on chromosome 5 and in the nucleotide position 160485411. G is an ancestral allele and C allele is mutated. The clinical significance of this polymorphism is unclear. Another polymorphism is rs57095329 and it is located on chromosome 5 and in the nucleotide position 160467840. A is an ancestral allele and G allele is mutated. The significance of clinical isn't clear. This polymorphism is a variation of single-nucleotid A/G in the LOC285628 gene.

Considering the research done on the role of microRNA and the different results obtained regarding the effect of mirRNA146a on BD, in the present study, the relation between rs2910164 and rs57095329 polymorphisms of miR146a gene with the potential for BD was investigated in Iranian population.

Materials and Methods

In this study, 100 patients with BD in the age range of 16-62 that were identified by rheumatologists from all over Iran were studied. A total of 100 healthy individuals without any family history of BD from all over Iran in the same age range were studied as control sample. Having the knowledge and consent of the participants of the study, sterile syringes were used for drawing 5 ml of blood and blood were poured into tubes containing EDTA as anticoagulant suppliers. DNA extraction was performed using M & D kits. The rs2910164 polymorphism was investigated using PCR-RFLP method and genotyping the rs57095329 polymorphism was determined by ARMS-PCR. In Table 1, the sequence of specific primers for each reaction is presented. PCR reaction for rs2910164 in 15 µl containing 100 ng DNA, 1x PCR buffer, 0.2 mM

¹ Interleukin 6

² neural cell adhesion molecule 1

³ nuclear factor of activated T cells-5

dNTP, 5 pmol per primer, 0.2 mM MgCl₂, and 1 unit of Taq DNA Polymerase enzyme was conducted. Propagation was done in 30 cycles at 94 ° C for 50 seconds, 64.5 ° C for 50 seconds, 72 ° C for 50 seconds. Primary denaturation was carried out at a temperature of 94 ° C for 4 minutes and final elongation was done at 72 ° C for 10 minutes. PCR reaction for rs57095329 was carried out in 15 µl containing 100 ng DNA, 1x PCR

buffer, 0.23 mM dNTP, 5pmol per primer (Normal, Mutant, and Common), 1.3 mM MgCl₂, 1 unit of Taq DNA Polymerase enzyme. The PCR reaction was performed in 28 cycles at 95 ° C for 50 seconds, 67 ° C for 50 seconds, 72 ° C for 50 seconds. Initial denaturation was carried out at 95 ° C for 5 minutes and final elongation was done at 72 ° C for 10 minutes.

Table 1. Enzyme name, primer sequence and PCR product length

PCR product length	primer sequence	Enzyme	Polymorphism
273 bp	Forward: 5'- CCGATGTGTATCCTCAGCTTTG-3' Reverse:5'- CCCTGCTTAGCATAGAATTCAAG -3'	MnII	rs2910164
159 bp	TTGGAGCACGTGTCAGGAGCAG CGGGGCTGCGGAGAGTAAAGA CGGGGCTGCGGAGAGTAAAGG	-	rs57095329-R rs57095329-A rs57095329-G

The PCR product was digested on 12% polyacrylamide gel (PAGE) and analysed after staining with silver nitrate.

Statistical analysis of results:

SPSS version 22 was utilized to analyze the results of enzymatic digestion products and ARMS-PCR. The distribution of genotypes of each mutation, homozygote and heterozygote frequencies in two groups of patients and control were analyzed by chi-square test. P-value less than 0.05 was considered as a significant level. P-value and odds ratio were also calculated.

Results

In this case-control study, 100 patients with BD were included as case group and 100 normal individuals who had no history of BD in their families were included as control group. The length of the PCR product for polymorphism rs2910164 is 273 base pair (bp). In cases

with GG genotype, the PCR product in the vicinity of the MnII enzyme produces 22, 77 and 174 bp segments. CC genotype produces segments with 22, 32, 45 and 174 bp (Fig. 1). The rs2910164 polymorphism has two G and C alleles. The genotypic frequency observed in the studied groups in rs2910164 follows the Hardy-Weinberg Equilibrium. The frequency of GG genotypes in control and patient groups were 71% and 80%, GC genotype frequency in control and patient groups was 21% and 11%, respectively. The frequency of CC genotype in control and patient groups was 8% and 9%, respectively. The value of P-value is 0.156 and greater than 0.05, indicating no significant difference between these two polymorphisms in both control and patient groups. Also, the frequency of G allele in control and

patient groups was 81.5% and 85.5%, and the frequency of C allele in control and patient groups was 18.5% and 14.5%, respectively. The value of P-value is 0.281 and greater than 0.05. The risk of danger is OR = 0.747,

indicating no significant association between this polymorphism and C and G alleles in patient and control groups (Table 2).

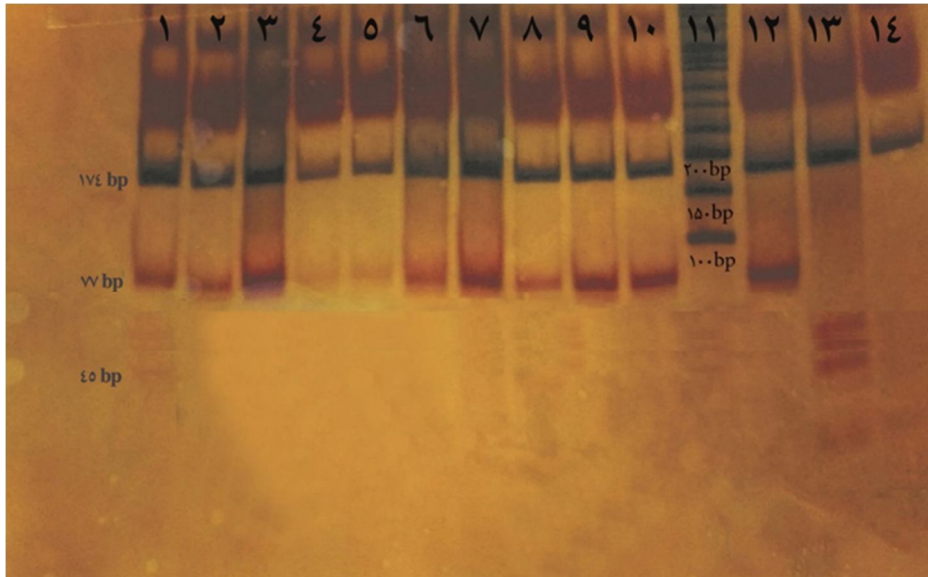


Fig 1. Acrylamide gel represents fragments obtained from rs2910164 digestion by MnlI enzyme. Wells 1 and 2: GC, Wells 3 to 7: GG, Wells 8: GC, Wells 9 and 10: GG, Wells 11: Size marker 50 bp, Wells 12: GG, Wells 13 and 14: CC.

Table 2. Genotype frequency, allelic frequency and significant level of the rs2910164 polymorphism in patient and control groups

Genotype	Patient group N=100 (%)	Control group N=100 (%)	P-value
GG	80 (80%)	71 (71%)	0.156
GC	11(11%)	21 (21%)	
CC	9 (9%)	8 (8%)	
Allele	Patient group N=100 (%)	Control group N=100 (%)	P-value
G	171 (85.5%)	163 (81.5%)	0.281
C	29 (14.5%)	37 (18.5%)	

The length of the PCR product for polymorphism rs57095329 is 159 base pair (bp). The PCR product of this polymorphism produces three genotypes: AA, AG and GG (Fig. 2). The rs57095329 polymorphism has two alleles (A and G). In rs57095329, the frequency of AA genotype in control and patient groups was 87% and 84%, respectively. The frequency of AG genotype in

control and patients groups was 11% and 14%, and the frequency of GG genotype in control and patient groups was 2%. The P-value was 0.814 and greater than 0.05, which shows no significant difference in this polymorphism in both control and patient groups. The frequency of allele A in control and patient groups was 92.5% and 91%, and the frequency of G allele in control

and patient groups was 7.5% and 9%, respectively. The P-Value was 0.586 and greater than 0.05 and also risk of danger is $OR = 0.819$, indicating no significant association between A and G alleles and this polymorphism in patient and control groups (Table 3).

It should be noted that the allelic and genotypic frequencies in this group also followed the Hardy-Weinberg equilibrium.

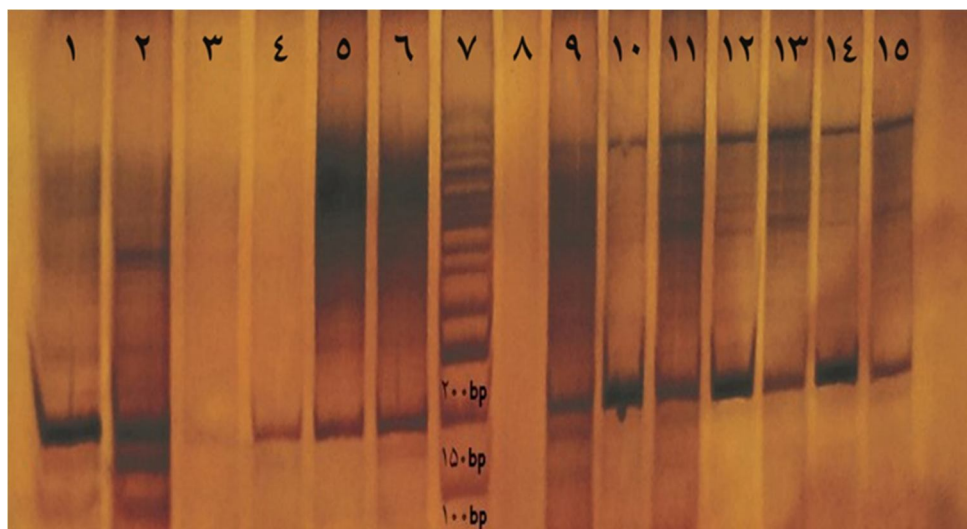


Fig 2. Acrylamide gel Represents ARMS-PCR products Position of rs57095329. Wells 1 and 2: AA, Wells 3 and 4: GG, Wells 5 and 6: AG, Wells 7: Size marker 50 bp, Wells 8 and 9: GG, Wells 10 and 11, Wells 12 and 13, Wells 14 and 15: AG

Table 3. Genotypic and allelic frequency and significant level of rs57095329 polymorphism in patient and control group

Genotype	Patient group N=100 (%)	Control group N=100 (%)	P-value
AA	84 (84%)	87 (87%)	0.814
AG	14(14%)	11 (11%)	
GG	2 (2%)	2 (2%)	
Allele	Patient group N=100 (%)	Control group N=100 (%)	P-value
A	182 (91%)	185 (92.5%)	0.586
G	18 (9%)	15 (7.5%)	

Discussion and Conclusion

BD is a rare systemic vasculitis associated with mucosal, ocular, vascular, articular, neurological, gastrointestinal, urinary and pulmonary lesions. The exact cause of the disease is unknown, but both the

cellular and the humoral immunity and the environmental factors may play a role in it (14).

Self-antigens become problematic and cause immune responses against a person's own cells and tissues by several immune constituents such as autoreactive antibodies and T cells. This abnormal

response to one's own tissue antigens causes several presentations that are categorized as a distinct type of disease of phenotypes, based on the tissues and the antigens being targeted (15). As stated, miRNAs affect the functions of inherent and actuated immune responses. Meanwhile, miR146a not only plays an important role in these responses but also has a key role in autoimmune diseases (12). Dysregulated miRNA expression can cause serious complications in the immune system, and also the increasing evidence has shown that miRNAs play a critical role in the development of autoimmunity disease (15). Different studies have linked several SNPs to BD. SNPs can interfere with miRNA maturation and function, causing dysregulated expression of immune-related genes (16). Considering the role of miR146a in autoimmune diseases such as BD and the effect of different polymorphisms on this gene; in this study, for the first time the relationship between miR146a gene and BD in Iran has been addressed. In the present study, based on the frequency of genotypes and alleles (Table 2 and 3) of polymorphisms rs2910164 and rs57095329 in both patient and control groups (P-value greater than 0.05) no significant differences were observed between the polymorphisms rs2910164 and rs57095329 in both patient and control groups.

Various studies have been conducted on the relationship between polymorphisms rs2910164 and rs57095329 with other autoimmune diseases.

Jimenez Morales et al. (2012) examined the relationship between rs2910164 G / C polymorphism and systemic lupus erythematosus, asthma, and juvenile rheumatoid arthritis (JRA) in pediatric Mexican patients. The study group consisted of 979 pediatric patients (asthma: 402, systemic lupus erythematosus: 367 and JRA: 210) and 531 control subjects without inflammatory or autoimmune diseases. Genotyping was performed using 5' exonuclease technique. The Genotype distribution of the rs2910164 polymorphism

was in Hardy Weinberg equilibrium in each group. There was no significant difference in distribution of this polymorphism between cases and controls. However, stratification by gender showed a significant difference between asthmatic and control females, in which the C allele was significantly associated with the protection of asthma. Evidence suggests that rs2910164 may play an important role in childhood-onset asthma but it does not apply to systemic lupus erythematosus and JRA. Related studies may be more likely to contribute to the role of a single nucleotide polymorphism in immune-mediated diseases (17).

Weigang Zhang et al. (2014) examined the association of rs2910164 in miR146a and the risk of psoriasis in the Chinese Han population. The study group included 521 Han Chinese patients with psoriasis and 582 healthy controls. Polymorphism rs2910164 was genotyped by RFLP-PCR technique. The increased risk of psoriasis was associated with GG and miR146a CG genotypes. In addition, G allele was associated with a decrease in miR146a levels in psoriasis lesions. These findings suggested that the G allele in miR146a may decrease the suppression effect on the proliferation of keratinocytes probably through the decreased inhibition of the target gene, EGFR, which may account for the increased risk of psoriasis in this study population (18).

You Li et al. (2015) examined the relationship between the miR146a polymorphisms (rs2910164, rs57095329) and multiple sclerosis in the Chinese Han population. A group of 525 patients and 568 healthy subjects were genotyped to detect two polymorphisms by SNaPshot. There was no significant difference in the distribution of two polymorphisms (rs2910164 and rs57095329) between patients and controls ($P > 0.05$). However, stratification by gender revealed a statistically significant difference in the frequency of the genotype rs2910164 between MS patients and control females ($P = 0.009$). The stratification analysis by subgroup suggested that the miR146a rs2910164 C allele poses a

higher risk of the developing PRMS ($P = 0.018$). Furthermore, the C rs2910164 allele was significantly associated with increased expression of miR146a in patients with PRMS ($P = 0.025$). Also, patients with the rs2910164 C allele released more TNF- α and IFN- γ , but not IL-1 β , compared with individuals carrying the homozygous GG genotype ($P < 0.05$) (19).

Qingyan Zhou et al. (2014) studied the association between the miR146a and Ets-1 gene polymorphisms with BD and VKH⁶ in the Chinese Han population. The study group included 809 patients with BD, 613 patients with VKH and 1132 normal controls. Polymorphisms rs2910164 miR146a, rs57095329 and rs6864584, rs10893872 Ets-1 and rs1128334 were by genotype PCR-RFLP technique. The miR-146a expression was examined in peripheral blood mononuclear cells (PBMCs) by real-time PCR. The produced cytokines were also measured by ELISA method. A significant decrease was observed in the frequency of homozygous rs2910164 CC genotype and C allele in patients with BD compared to control ($P_c^a = 1.24 \times 10^{-5}$, OR 0.61, $P_c^a = 1.33 \times 10^{-4}$, OR 0.75). The expression of miR146a in GG cases was 2.45-fold and 1.99-fold higher, respectively, than CC and GC cases. There was no relationship between other single-nucleotide polymorphisms and BD and its main clinical features. No association was found between the five SNPs tested with clinical manifestations in VKH. Production of IL-17, TNF α and IL-1 β in CC rs2910164 cases was significantly lower than that in GG cases. No effect was observed in the production of IL-6 and MCP-1, and expression of IL-8 was higher in CC cases (20).

Tuba Oner et al. (2015) studied the association of BD with the NFKB1 rs28362491, pre-miRNA-146a rs2910164 and pre-miRNA-499 rs3746444 polymorphisms, as well as the analysis of their single and combined effects on their susceptibility in a Turkish

population. The polymorphisms were evaluated using PCR-RFLP technique in 100 BD patients and 145 healthy subjects. The frequency of rs28362491 genotype was significantly higher in BD patients. Also, miRNA-499 rs3746444 homozygous (TT) genotypes showed higher risk in BD patients. In addition, the frequency of T allele of rs3746444 was a risk factor for BD. Furthermore, there was a significant difference between the two groups concerning the miRNA-146a rs2910164 polymorphism and it was found that homozygous CC and C allele of rs2910164 polymorphism were protective factors against BD. There was no significant difference between the multiple comparisons of rs28362491-rs2910164 and rs28362491-rs3746444 in the patients and the control groups (21).

Considering the studies done in different societies and comparing them with the present study, the cause of no significant differences in SNPs of rs2910164 and rs57095329 in the samples studied could be due to factors such as:

1. Studies conducted in different regions of the world revealed contradictions regarding the level of significance of these polymorphisms with BD. This difference in the results of studies in different countries can be due to the association of the occurrence of the polymorphisms in different populations.

2. The low prevalence of some alleles in the studied population, which also reduces the probability of finding a homozygous mutant genotype, could be due to the small size of the sample being studied. Therefore, by studying a large sample size with consideration of these factors, significance or insignificance of these polymorphisms with BD in Iranian patients can be argued with greater certainty.

3. Iran consists of a large population with various ethnic groups, and each ethnic groups can have a special

⁶ Vogt-Koyanagi-Harada

allele frequency. Therefore, ignoring this issue can sometimes show false results. Moreover, the role of the interaction of genes on the each other and the environment cannot be ignored.

Conclusion

According to the results of this study, it can be concluded that rs2910164 and rs57095329 polymorphisms in the studied population have no significant association with BD in Iran.

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