

Molecular Analysis of Single-Nucleotide Polymorphisms of TRAF5 Gene in Patients with Behçet's Disease from the Azeri Population of Northwest Iran

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Received date: June 17, 2021; **Accepted date:** August 11, 2021; **Published date:** August 20, 2021

Citation: R Hosseinzadeh, L Saremi, S Babaniamansour, Mohammad E Ghaffari, Mohammad K Kondori. (2021) Molecular Analysis of Single-Nucleotide Polymorphisms of TRAF5 Gene in Patients with Behçet's Disease from the Azeri Population of Northwest Iran. International Journal of Clinical Case Reports and Reviews. 8(2); DOI:10.31579/2690-4861/148

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Abstract

Background: Behçet's disease (BD) is a chronic inflammatory disease with unknown causes. The geographical distribution is mostly consistent with the historic Silk Road. The role of tumor necrosis factor receptor-associated factor 5 (TRAF5) gene in the inflammation signaling pathway leads more attention to the potency of different polymorphisms of the TRAF5 gene, in the development of BD.

Methods: This was a case-control study conducted among the Azeri Turk ethnic group (50 BD patients and 50 persons with no history of autoimmune disease), Tabriz, Iran. Four different TRAF5 gene polymorphisms were assessed via the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism technique. Data were analyzed by SPSS Version 20.

Results: Genotypic distributions of four studied Single nucleotide polymorphisms (SNPs) had no significant difference between case and control groups (rs6540679 (p=0.50), rs12569232 (p=0.86), rs10863888 (p=0.14), and rs7514863 (p=0.24). There were also found no significant differences in SNP allelic frequencies between cases and controls (all $P > 0.05$).

Conclusions: Although the role of the TRAF5 gene polymorphisms in inflammatory diseases has been proved, our study did not show a significant association between the TRAF5 gene polymorphisms with BD.

Keywords: behçet syndrome; genetic polymorphism; human traf5 protein; polymerase chain reaction

Background

Behçet's disease (BD) is a multifactorial inflammatory and systemic uveitis autoimmune disease, which is associated with recurrent skin lesions, oral aphthae, and genital ulcers with unknown causes [1-6]. The highest incidence of BD belongs to Turkey (80-370 patients in 100,000 persons), whereas it has a low rate in North America (0.2 patients in 100,000 people) [7]. The onset age of the disease is usually between 25

and 30 years old [8]. The disease is equally distributed between genders, but the previous studies reported higher severity in males than females for unknown reasons. Although numerous studies have been done, the main causes of the BD are still unclear [9].

Recently, the role of various active oxygen derivatives (free radicals) including superoxide, hydroxyl peroxide, hydroxyl radicals, and nitric oxide as well as the activity of antioxidant enzymes (dismutase oxide and

glutathione peroxidase) in the pathogenesis of BD were examined. These elements have been considered as transcription activators of pro-inflammatory cytokines [10, 11]. Additionally, a wide range of mutations and polymorphisms in association with BD patients have been reported, such as human leukocyte antigen (HLA-B) serotype 5, chemokine receptor 5, heat shock protein 60, anti-tumor necrosis factor (TNF) α , Interleukin-6 (IL-6), and Interferon-gamma (IFN- γ), Cluster of differentiation 40 (CD40), signal transducer and activator of transcription 3 (STAT3), STAT4, and Janus kinase 2 (JAK2) [9, 12], Intercellular adhesion molecule-1, small ubiquitin-like modifier 4, IL-23R-IL12RB2, IL10, Monocyte chemoattractant protein-1, and Fc receptor-like 3 [13].

Moreover, dominant immune responses in the patients are considered important factors in its pathogenesis. The physicians choose Corticosteroids, Colchicine, Azathioprine, Cyclosporine, Thalidomide, Cyclophosphamide, Interferon- α , or TNF α inhibitors as the BD treatment based on the severity of the organ involvement and clinical symptoms [14].

Tumor necrosis factor receptor-associated factors (TRAFs) are an intracellular family of adaptor proteins that share a common structural domain

at the C-terminus and directly attach to TNF receptors (TNF-R) [15, 16]. There are seven known types of TRAF family (TRAF1 to 7) in mammals [17]. All TRAFs, except TRAF1, consist of an N-terminal ring finger domain followed by the various number of zinc fingers [16, 18]. The ring finger consists of one histidine residue and seven cysteine residues forming a single folded domain binding two zinc ions. In ubiquitin ligases, ring finger motifs are vital for ubiquitin molecule transfer to substrates or ring finger motifs themselves. TRAFs are related to various diseases such as immunodeficiency, cancer pathogenesis, autoimmune, and neurodegenerative [19].

The TRAF5 identified as a CD40 binding protein and a lymphotoxin-b receptor-interacting protein (LTBR), locates on the humans' chromosome 1q32.2. (Figure 1) It significantly expresses in the thymus gland, lungs, spleen, kidneys as well as peripheral blood cells and plays an important role in cell signaling [20]. TRAF5 using the yeast two-hybrid system, and LBR, by PCR using degenerate primers corresponding to conserved amino acids in the TRAF domain of TRAF1-3 [21].

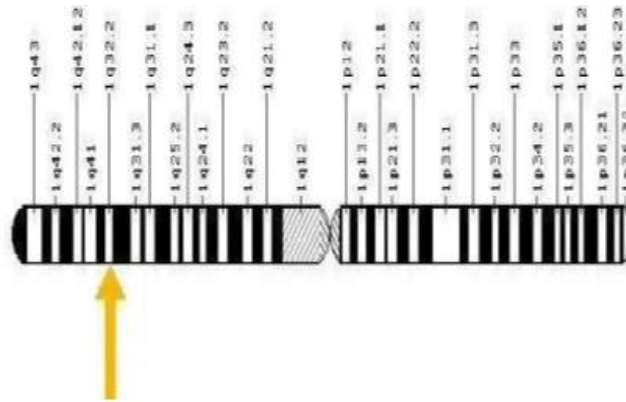


Figure 1: Chromosomal location of TRAF5.

Single nucleotide polymorphism (SNP) in TRAF is related to autoimmune diseases such as lupus erythematosus, rheumatoid arthritis (RA) [22-25]. Xiang et al. demonstrated that TRAF5 polymorphisms are related to susceptibility to autoimmune diseases such as RA. An SNP (rs7514863) upstream of TRAF5 that affects a transcriptional factor binding site is associated with RA [26]. A major dominant mutation in TRAF3 was reported in a young child with a herpes simplex virus (HSV1) encephalitis history [27]. A TRAF6 heterozygous mutation has been recently reported in a patient with Hypohidrotic Ectodermal Dysplasia [28]. In 2014, the association between rs10863888 and BD was observed in a Chinese population [20].

We selected four SNPs (rs1256932, rs6540679, rs10863888, and rs7514863) for the investigation of TRAF5 polymorphisms based on reports in psoriasis and RA [29], linkage disequilibrium patterns in Han Chinese, and Japanese data in the Hap Map database.

Methods

Study design

This was a case-control study conducted at the Molecular-Medical Genetic department of Islamic Azad University East Azarbaijan Science and Research Branch, Tabriz, Iran. The executive protocol of the study was conducted under the Declaration of Helsinki (seventh revision 2013). All participants had written consent before the study.

Participants

In this study, the case group included 50 BD patients from the Azeri Turk ethnic group, diagnosed by a rheumatologist (26 males and 24 females in each group). The control group included 50 persons with neither history of autoimmune disease nor a family history of autoimmune disease.

Genomic deoxyribonucleic acid (DNA) extraction using the Salting out method

A venous blood sample (5 cc) was obtained from all participants and collected in Ethylenediaminetetraacetic acid (EDTA, Merck Company, Germany)-containing falcon (SARSTEDT Company, Germany). The samples were shaken slowly to mix and prevent the formation of blood clots. DNA was extracted from blood samples based on the Nasiri et. al, methods [30]. The quantity and quality of extracted DNA were determined using spectrophotometry and agarose gel (Kosar Company, Iran) electrophoresis, respectively.

Polymerase Chain Reaction (PCR)

PCR (ABI Company, USA) was performed to amplify specific DNA sequences using specific primers (manufactured by Biorad Company, Germany). Amplification conditions for PCR reactions were as follows: one cycle at 95 °C for 5 min, 30 cycles at 94 °C for 25 s, 63 °C for 20 s and 72 °C for 20 s, followed by a final extension at 72 °C for 5 min. PCR

products were electrophoresed on agarose gels (2%), stained with the ethidium bromide (manufactured by CinnaGen, Iran), and documented.

Digestion of PCR-products with restriction enzymes (PCR - RFLP)

PCR-Restriction Fragment Length Polymorphism (RFLP) is commonly used to examine the various alleles of a gene in a population. The sequence diversity of the gene corresponding alleles creates different digestion locations for the restriction enzyme, and segments with different lengths are obtained. Induced mutations in the detection site of restriction

enzymes (TRUII-SacI-TSP509I-APOI-hinfI, manufactured by Fermentas company, Germany) and resulted polymorphism can be detected by its RFLP pattern. After amplification of the mentioned DNA segments, PCR products were digested using restriction enzymes. Each reaction tubes contain 9 µl ddH₂O, 1 µl buffer 10 x, 0.5 µl restriction enzyme, and 5 µl PCR product, then incubated overnight at 37 °C. The position information of each of the polymorphisms, restriction enzymes, and DNA fragment size is listed in table 1. Finally, the incubated reactions were electrophoresed on agarose 3% and documented.

Gene	SNPID	Primer	Restriction Enzyme	Fragment Size (bp)
TRAF5	rs6540679	5-TGTGTCTAAAGGGCCATGTG-3	XmiI (AccI)	233 (133-100)
		5-CTTAGAGACCCTGCTCACCC-3		
	rs12569232	5-AGATGAAACAACCTGAGGCACA-3	MvaI (BstNI)	305 (220-85)
		5-ACTGCCTTTGGTTTGACAGTC-3		
	rs10863888	5-ATCTCATCTGATTAACCTC/GTC-3	Mae III	311 (288-24)
		5-CTCTTCAGAGACTGAGTCA-3		
	rs7514863	5-CCATCTCATCTGATTAACCTCTC-3	Tsp45I	254 (231-23)
		5-TTTGATGACTGTCTCGCTGG-3		

Table 1: Primers, enzymes, and the size of the digested fragments using digestion enzymes

Statistical analysis

A Chi-square test with continuity correction was used to examine the Hardy-Weinberg equilibrium. After it was found that Hardy-Weinberg equilibrium is established in all polymorphisms, Chi-square and Fisher's exact tests were used to compare the frequency of alleles and genotypes between case and control groups. To perform calculations, R version 3.4.3 and SPSS version 20 (SPSS Inc., Chicago, IL, USA) were used to analyze the data. P<0.05 was considered statistically significant.

Results

The present study was conducted among 100 participants. The mean age of participants in the case and control groups were 34.02±7.39 (range 11 to 65) and 34.42±8.27 (range 21 to 76) years, respectively. Patients in the case group had no family history of BD. The frequency of the positive Pathergy test in the case and control groups were 14(28%) and zero, respectively. The clinical characteristic of the case group is shown in table 2.

Variables		Case group (N=50)
Oral Aphthosis		47(94)
Genital Aphthosis		22(44)
Skin manifestations	Erythema nodosum	5(10)
	Pseudolliculitis	11(22)
Ocular	Anterior uveitis	27(54)
	posterior uveitis	31(62)
	Retinal vasculitis	5(10)
	Cataract	14(28)
Vascular involvement		6(12)
Neurological involvement		1(2)
Arthritis		6(12)

Data were described as frequency (percent).

Table 2: Clinical characteristics of case group.

In this study, four polymorphisms (rs12569232, rs6540679, rs7514863, and rs10863888) of the TRAF5 gene were evaluated. To investigate the polymorphism of rs6540679 (SNP: rs6540679 Chr.1: 211491152) a segment about 233 bp was amplified using PCR followed by digestion using the XmiI (ACC I) enzyme, which PCR products that contain allele

(G) was not digested but a product with allele (A) digested and fragments with 100 and 133 bp were produced. The people who were heterozygous for the given site showed three bands with lengths of 133, 100, and 233 bp. (Figure 2)

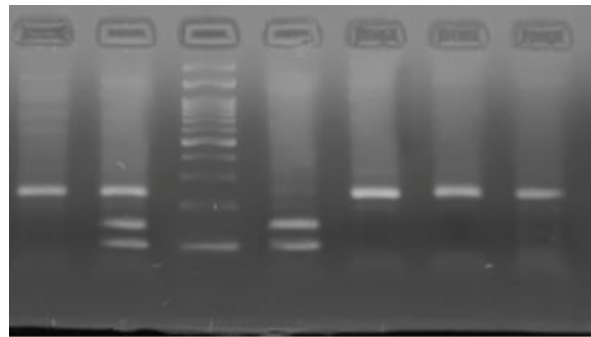


Figure 2. Polymorphism of rs6540679.

Lane C: Ladder 100 bp, Lane A, E, F and G: GG genotype (rs6540679) (233 bp), lane D: AA genotype (rs6540679) (100, 133 bp), Lane B: AG genotype (rs6540679) (100, 133, 233 bp).

To assess the polymorphism of rs12569232 (SNP: rs12569232 Chr.1: 211553064) a segment, about 305 bp was amplified using PCR followed by digestion using the Mva I (BstNI) enzyme, which PCR products contain allele (C) was not digested but a product with allele (G) digested

and fragments 220 and 85 bp were produced. The people who were heterozygous for the given site showed three segments with lengths of 220, 85, and 305 bp. (Figure 3)

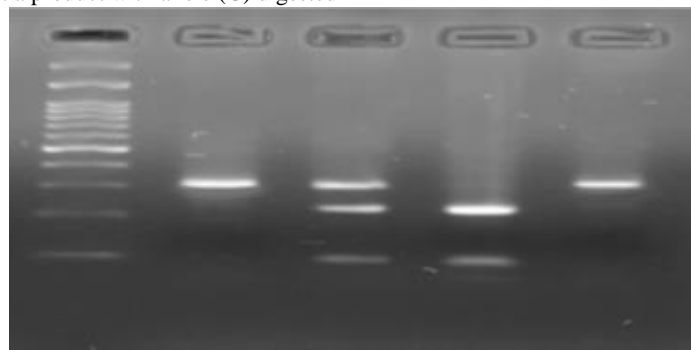


Figure 3. Polymorphism of rs12569232.

Lane A: Ladder 100 bp, Lane B and E: CC genotype (rs12569232) (305 bp), Lane D: GG genotype (rs12569232) (85, 220 bp), Lane C: CG genotype (rs12569232) (85, 220, 305 bp).

To study the polymorphism of rs10863888 (SNP: rs10863888 Chr.1: 211502769) a fragment about 311 bp was amplified using PCR followed by digestion using the Mae III enzyme, which PCR products that contain

allele (A) digested, but allele (G) was not digested and fragments with 288 and 24 bp produced. The heterozygous people for the given site showed three bands with lengths of 288, 24 and, 311 bp. (Figure 4)

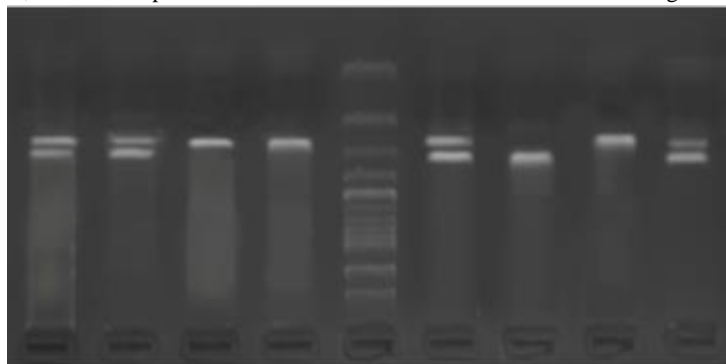


Figure 4. Polymorphism of rs10863888. Lane E: Ladder 100 bp.

To assess the polymorphism of rs7514863 a fragment of about 254 bp was amplified and followed by digestion using the Tsp 451 enzyme, which PCR products contain allele (A) digested but a product with allele (G) was

not digested and fragments 231 and 23 bp were produced. The heterozygous individuals for the given site showed three segments with lengths of 254, 231, and 23 bp. (Figure 5)

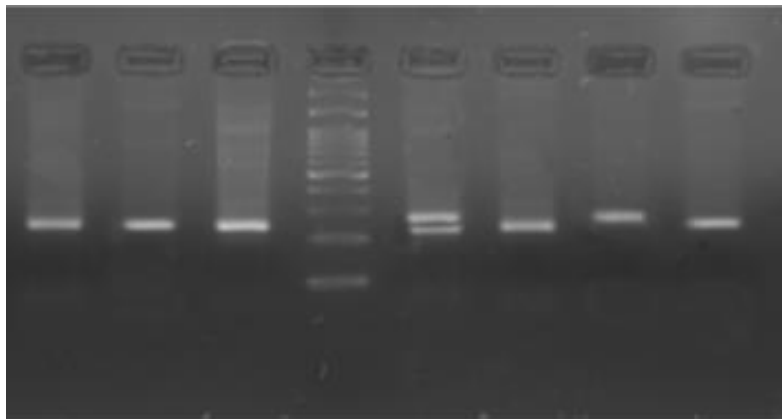


Figure 5. Polymorphism of rs7514863. Lane D: Ladder 100 bp.

Tables 3 and 4 show the frequency of allele and genotype in four different polymorphisms of the TRAF5 gene between case and control groups. The results showed no significant difference for rs6540679 polymorphism (P=0.92) (OR=0.895, 0.41-1.94), rs12569232 (P=0.75, OR=1.17, 0.61-

2.24), rs10863888 (P=0.088; OR= 1.76, 0.97-3.21) and rs7514863 (P=0.65, OR= 0.674, 0.23-1.967) between case and control groups. (Table 5).

SNP	rs6540679		P-value	OR (95% CI)	SNP	rs12569232		P-value	OR (95% CI)
	A	G				G	C		
Case	14	86	0.780	0.895 (0.412-1.94)	Case	25	75	0.627	1.17 (0.61-2.24)
Control	16	88			Control	23	81		
SNP	rs10863888		P-value	OR (95% CI)	SNP	rs7514863		P-value	OR (95% CI)
	A	G				A	G		
Case	37	63	0.064	1.76 (0.966-3.21)	Case	6	94	0.468	0.674 (0.23-1.967)
Control	26	78			Control	9	95		

p-value refers to the association of the frequency of alleles between case and control groups, based on Pearson Chi-Square. SNP: Single Nucleotide Polymorphism; OR: Odds Ratio; CI: Confidence Interval.

Table 3: The frequency of each allele between case and control groups

SNP	rs6540679			P-value	SNP	rs12569232			P-value
	AA	AG	GG			CC	CG	GG	
Case	1	12	37	0.509 ^b	Case	4	17	29	0.860 ^b
Control	0	16	36		Control	3	17	32	
SNP	rs10863888			P-value	SNP	rs7514863			P-value
	AA	AG	GG			AA	AG	GG	
Case	7	23	20	0.141 ^a	Case	1	4	45	0.236 ^b
Control	5	16	31		Control	0	9	43	

p-value refers to the association of the frequency of alleles between case and control groups. ^a refers to the analysis based on Pearson Chi-Square. ^b refers to the analysis based on Fisher's Exact Test. SNP: Single Nucleotide Polymorphism.

Table 4: The frequency of each genotype between case and control groups.

SNPs	Genotype						P-value	Allele				P-value	OR (95% CI)
	mm		mM		MM			M		M			
	BD	CON	BD	CON	BD	CON		BD	CON	BD	CON		
rs6540679	1	0	12	16	37	36	0.50	14	16	86	88	0.780	0.895 (0.41-1.95)
rs12569232	4	3	17	17	29	32	0.86	25	23	75	81	0.627	1.17 (0.61-2.24)
rs10863888	7	5	23	16	20	31	0.14	37	26	63	78	0.064	1.76 (0.97-3.21)
rs7514863	1	0	4	9	45	43	0.24	6	9	94	95	0.468	0.674 (0.23-1.967)

BD: Behçet Disease; CON: Control group; M: normal allele; m: mutant allele; MM: normal-normal genotype; mm: mutant-mutant genotype; mM: normal-mutant genotype. p-value refers to the association of the frequency of alleles between case and control groups, based on Pearson Chi-Square. SNP: Single Nucleotide Polymorphism.

Table 5: The frequency of gene polymorphisms (allele and genotype)

Discussion

In this study, the relationship between common polymorphisms of the TRAF5 gene and BD in the Azari-Iranian population was studied. Although the cause of BD has not been fully characterized, however, it seems that genetic and environmental factors contribute to the disease. For example, HLA-B51 is commonly found in BD patients around the Silk Road.

The results of this study showed that there was no relationship between polymorphisms of rs6540679, rs12569232, rs10863888, and rs7514863 with the susceptibility to the BD.

The relationship between autoimmune diseases like BD and the TRAF5 gene polymorphisms was assessed in the previous studies but they showed contradictory results. Potter et al. conducted a study among 351 BD patients and 368 persons (as a control group) in England. They examined 44 markers of the TRAF gene such as rs7514863 and showed a strong relationship between the TRAF5 gene polymorphism (rs7514863) and RA. Potter suggested that this marker can be a candidate polymorphism for RA [26].

Xiang et al. studied markers of rs12569232, rs6540679, and rs10863888 in 450 acute anterior uveitis autoimmune patients, 458 pediatric uveitis patients, and 1601 persons as a control group in China. There was a significant relationship between polymorphism of rs12569232 and disease, but no significant relationship was observed for rs6540679 and rs10863888 [31].

In the study on 789 BD and 940 patients suffering Vogt-Koyanagi-Harada Syndrome in China, rs10563888 marker was significantly associated with BD, while there was no significant association for rs6540679 and rs12569232 [20]. Studies stated that TNF- α and MEFV polymorphisms had a significant association with BD in Iranian Azeri Turkish [32, 33]. High expression of JAK1 and TLR2 in patients with BD confirmed the important role of this gene in its development [34].

A study among BD patients in China showed that AG and GG alleles of rs10863888 polymorphism had a significant difference. We thought that if the number of patients and the control group considered on a larger scale the significance of this polymorphism would be very high. Although the P-value obtained for rs10863888 polymorphism in this study is 0.088, confirms our hypothesis that this polymorphism may be meaningful in the case of a larger patient and control community.

Differences in the results of various studies on the association between the TRAF5 gene polymorphism and BD can be justified for several reasons. First, this difference can be due to the genetic variation of studied populations in various studies, which indicates the presence of different polymorphism levels in different populations. On the other hand, this inconsistency could be due to the low number of surveyed people and used techniques. Thus, it is recommended to carry out more extensive studies with more samples and more precise techniques, including sequencing of the fragments using DNA sequencing techniques.

Conclusions

Although the role of the TRAF5 gene polymorphisms in inflammatory diseases has been proved in many investigations, Our study did not show a significant relationship between the TRAF5 gene polymorphisms and BD.

List of abbreviations

BD: Behçet's Disease; TRAF: Tumor Necrosis Factor Receptor-Associated Factor; SPSS: Statistical Package for the Social Sciences; SNP: Single Nucleotide Polymorphism; HLA: Human Leukocyte Antigen; TNF: Tumor Necrosis Factor; IL: Interleukin; IFN: Interferon; CD: Cluster of Differentiation; STAT: Signal Transducer and Activator of Transcription; JAK: Janus Kinase; LTBR: Lymphotoxin-b Receptor-Interacting Protein; RA: Rheumatoid Arthritis; HSV: Herpes Simplex Virus; DNA: Deoxyribonucleic Acid; EDTA: Ethylenediaminetetraacetic Acid; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism.

Declaration:

Ethic approval: The implementation of the project was approved by the ethics committee of Islamic Azad University East Azarbaijan Science.

Consent to participate: Informed consent was obtained from all the participants prior to the study.

Consent for publication: Not applicable.

Availability of data and material: Data sharing is available upon the author's request.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.

Competing Interests: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Authors' contributions

Leila Saremi, Sepideh Babaniamansour, Reza Hosseinzadeh and Mohammad Khalaj-Kondori were in charge of samples collection, experimental design and PCR. Leila Saremi, Mohammad Khalaj-Kondori, and Mohammad Ebrahim Ghaffari contributed equally performed and designed experiments, analyzed data, and generated figures. Reza Hosseinzadeh, and Sepideh Babaniamansour were in charge of idea and concept of the paper. Mohammad Ebrahim Ghaffari, Sepideh Babaniamansour and Mohammad Khalaj-Kondori assisted in supervision and generated figures and edited the manuscript All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Aziz Khorrami for his excellent technical assistance and contributions in this study.

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