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Title: Evaluation of DNA methylation status of Toll-like receptors 2 and 4 promoters in Behcet's disease

Running Title:
DNA methylation status of TLRs 2 and 4 promoters in Behcet's disease

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Abstract

Background: Altered innate immune function plays an important role in the initiation of inflammatory response in Behcet's disease (BD). Toll-like receptors (TLRs) are the master regulators of the innate immune system. Owing to the role of TLRs remains unknown in the pathogenesis of BD, this study aimed to evaluate the expression levels and methylation status of TLR2 and TLR4 promoters in patients with BD.

Material and Methods: In this study, Iranian Azeri BD patients (n=47) with active (n=22) and inactive (n=25) period, and healthy controls (n=61) matched according to age, sex and ethnic were recruited. TLR2 and TLR4 genes promoter CpG islands were predicted with eukaryotic promoter database. Methylated DNA immunoprecipitation (MeDIP) was carried out.

Results: Our results showed that mRNA of TLR4 was significantly increased in the peripheral blood mononuclear cells (PBMCs) of BD patients with active phase compared with the control group. Differences in mRNA of TLR4 between inactive BD and control groups were not significant. Differences in TLR2 mRNA levels in the PBMCs of active and inactive phase BD and control groups were not significant. The methylation rate of TLR4 gene promoter was significantly lower in the active and inactive BD groups than the control group. The difference between the active and inactive BD groups was not significant. There was no significant difference in the methylation rates of TLR2 gene between studied groups.

Conclusion: Our preliminary findings suggest that the hypomethylation of TLR4 gene may be involved in the pathogenesis of BD *via* increasing TLR4 expression.

Keywords: Behcet's disease; Toll like receptor; DNA methylation; epigenetic**.**

Introduction

Behcet's disease (BD) is a chronic refractory multi-system autoimmune disorder characterized by oral aphthous ulcers, genital ulceration, erythema nodosum, pseudofolliculitis and uveitis. The etiology of BD is not understood completely; however, it is suggested that inflammatory process in BD can be triggered by environmental parameters like microorganisms [1], vitamin D deficiency [2] and probably smoking [3] in participants who are genetically susceptible [4-6]. Altered innate immune function has an essential function in the initiation of inflammatory process in BD [1]. Toll-like receptors (TLRs) have a crucial activity in the innate immune system function. The expression of these receptors are on the antigen presenting cells (APCs) e.g. dendritic cells and macrophages, recognize structurally conserved molecules like lipopolysaccharides, lipoproteins, lipopeptides, lipoteichoic acids and peptidoglycan derived from microbes. Furthermore, they bind to the endogenous heat shock proteins (HSPs) [7]. There are 13 types of TLRs in invertebrates and vertebrates, 10 of which have been found in humans (TLR1-10) [8]. TLR2 discerns a broad range of microorganisms' molecules such as bacteria, viruses, fungi and parasites. TLR4 recognizes components of the outer membrane structure of Gram-negative bacteria [8]. This causes transcription of cytokine-encoding genes to the secretion of several cytokines, such as interleukin-1β (IL-1β), IL-6, IL-23, and tumor necrosis factor-α (TNF-α), which can cause differentiation of naive CD4+T cells into T-helper 17 (Th17) cells [9,10]. Altered TLR

pattern and especially the increased activity of TLR 2 and 4 have been depicted in active phase BD [11-16].

Epigenetics is a relatively new science that refers to heritable changes in a chromosome that affects the gene activity without altering in the DNA sequence. DNA methylation, histone modification, micro RNAs (miRNAs) and chromatin remodeling are the major epigenetic mechanisms [17]. These changes cause alteration in immune system function and development of inflammatory diseases like BD [17-21]. Epigenetic mechanisms, especially DNA methylation control TLR-related immunity. Hypermethylation of TLR promoter region is usually associated with reduction of TLR expression, while hypomethylation causes TLR overexpression [17,22]. Thus, maintaining the proper level of the TLR gene methylation is vital for ensuring the proper protective function of immune system. Limited studies have been performed on the DNA methylation status of the promoter region of the TLRs gene in inflammatory diseases, and to the best of our knowledge, no studies concerning the methylation of gene promoter encoding TLR2 and TLR4, have yet been performed on this topic in BD.

Considering the function of TLRs in the pathology of BD and introducing the function of epigenetic mechanisms in the pathology of inflammatory diseases, current study designed to evaluate the expression levels and methylation alterations of TLR2 and TLR4 promoters in peripheral blood mononuclear cells (PBMCs) of patients with BD.

Materials and Methods

Participants

We recruited consecutively 47 Azeri patients with BD from the BD clinic of Connective Tissue Diseases Research Center (CTDRC) between April 2017 and December 2018. All patients met the International Criteria for Behcet's Disease (ICBD) [23]. Sixty-one healthy

controls matched according to age, sex and ethnicity were included. The participants were not related. The study design was approved by the Ethics Board of Tabriz University of Medical Sciences (TUOMS) (Ethic code: IR.TB2MED.REC.1395.67). Written informed consents were obtained from all the study participants. We measured BD activity using the Iranian Behcet's Disease Dynamic Activity Measure (IBDDAM), Total Inflammatory Activity Index (TIAI) [24,25] and Behcet's Disease Current Activity Form (BDCAF) [26]. BDCAF ≥ 1 was considered active, meaning that the patient had at least one of the clinical symptoms of BD in the past month [25].

Sample processing and mRNA expression analysis

The expression of TLR2 and TLR4 was assessed by MIC real-time instrument (Bio Molecular Systems, AUSTRALIA). PBMCs were isolated from the blood samples of participants who were shed in tubes containing ethylene diamine tetra acetic acid (EDTA) by Ficoll (Lymphodex, Inno-Train, Germany), density-gradient centrifuged and stored at −80 °C till analysis. Genomic DNA was isolated by the rapid genomic DNA extraction (RGDE) procedure. Total RNA was extracted from the PBMCs using TRIzol Reagent (Invitrogen, USA). Complementary DNA (cDNA) synthesis was performed; using random hexamer primers and RNase H-reverse transcriptase (Thermo Fisher Scientific, USA). Then, we estimated the total RNA level by nanodrop ND1000 and assessed RNAs purity at 260-280 nm. TLR2 and TLR4 primers data are demonstrated in Table 1. Relative expression levels of TLR2 and TLR4 were calculated *via* ΔΔCt.

Methylated DNA immunoprecipitation assessment (MeDIP)

We used eukaryotic promoter database (EPD) for predicting CpG islands of TLR2 and TLR4 gene promoters. We designed a pair of primers *via* the Primer Quest Tool and MethMarker (PREMIER Biosoft, CA, USA) to augment CpG islands of transcription start site (TSS)

upstream. EpiQuik™ MeDIP Ultra Kit (Epigentek, Farmingdale, NY, US) was used for MeDIP. We used the BANDELIN sonicator (UVV: 3200, Germany) with 15 cycles of 20s on/20s off to sonicating extracted DNA to produce random fragments ranging in size from 200 to 800 bp. Electrophoresis was performed on a 1.5% agarose gel to confirm fragment size. After MeDIP enrichment, 1µl of DNA was used. We diluted 5 micrograms of fragmented genomic DNA to 400 µl in Tris-EDTA (TE) buffer. DNA was denatured at 95 °C for 5 min. Thereafter, immediately cooling was performed on ice for 5 minutes. In the final process, the assays were performed in three replicates. The fold change of DNA methylation was measured for each sample with the Fold Enrichment percentage (FE%). This formula [FE% = 2(IgG CT – Sample CT) × 100%] calculates FE%.

Primer design

The primer pairs were designed for mRNA sequences of TLRs using Primer Quest Tool and OLIGO7 software (Molecular Biology Insights, Inc., Cascade, CO., USA) (Table 1). We predicted the CpG islands of the TLRs gene promoter with the Eukaryotic Promoter Database (EPD). One pair of TLRs primer was also designed using Primer Quest software to augment CpG islands upstream (Figure 1).

Statistical analysis

We used the chi-square test, independent-samples t-test and Mann-Whitney U test with SPSS 16 software. Normal distribution of data was assessed by Kolmogorov Smirnov test. Continuous and categorical variables were expressed as means ± standard deviations (SD), and frequency and percentage, respectively. Pearson correlation analysis was used for assessing possible correlations. Statistical significance was defined as P-values lower than 0.05.

Results

The study included a total of BD patients (n=47), with active (n=22) and inactive (25) periods and healthy controls (n=61). BD was active in 22 and inactive in 25 patients. Demographic characteristics of the study participants and clinical information of the case group are presented in Table 2. We analyzed TLR2 and TLR4 mRNA levels of the studied groups and the findings depicted that mRNA of TLR4 was considerably enhanced in BD patients with active phase in comparison to the controls (Figure 2a). Differences between the inactive phase BD and control participants were not remarkable (Figure 2a). The methylation rate of TLR4 gene promoter was significantly lower in the active and inactive BD groups compared with the controls (Figure 2b). However, no significant difference was observed between the active and inactive BD groups (Figure 2b). Although the TLR2 mRNA levels in the PBMCs of active and inactive BD groups were higher than the control group, these differences did not reach to a significant level (Figure 2c). There was no significant variation in the methylation rates of TLR2 gene between the study groups (Figure 2d).

We additionally analyzed the correlation between TLR2 and TLR4 expression rates and their promoter's methylation status with disease activity (Table 3). No considerable correlation was noticed between these parameters with BD activity.

Discussion

Microbial agents have a crucial function in the pathology of BD. Heat shock proteins (HSPs) and lipopolysaccharides (LPS) of microbial agents were frequently notified in the involved organs of BD patients [27-30]. It is suggested that microorganisms' HSPs and LPS cross-reaction with human antigens leads to the innate immune activation and inflammatory response [31,32]. TLR4 can involve in the pathology of BD by recognizing HSPs and LPS [15]. The present research illustrated a higher expression of TLR4 in patients with active BD. No significant difference was found in the TLR2 expression.

Our results confirmed previous observations that showed an increase in TLR4 expressions in active BD patients [11-16]. Studies by Liu et al. [11] on 16 Chinese patients with active ocular BD demonstrated a higher TLR4 expression. Seoudi et al. [12] studied 55 BD patients and reported a higher expression of TLR4 in the oral mucosa of patients with active BD in comparison to inactive BD and healthy groups. Horie et al. [15] showed that the number of TLR4 TAGCGGTAA haplotype in BD patients with positive HLA-B51 was more than three times higher in comparison with healthy controls.

Our current study showed hypomethylation of TLR4 promoter in BD. Despite the relatively large number of studies on the expression of TLRs in patients with BD, no studies have been performed on the methylation status of TLRs promoters in BD. However, several studies reported DNA methylation abnormalities in some other genes in BD, including IL-6 [33], IL-10 [34] and suppressor of cytokine signaling 1 (SOCS1) [35] gene. Alipour et al. [33] noted a higher IL-6 gene expression and hypomethylation of IL-6 gene promoters in BD patients. Furthermore, Alipour et al. [34] in another investigation on 51 BD patients noticed hypermethylation of IL-10 gene leading to decreased generation of IL-10. Abdi et al. [35] studied the methylation status of SOCS1 gene. This gene plays a role in the negative modulation of cytokines that signal *via* the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. They showed SOCS1 gene promoter hypermethylation and decreased expression of this gene in BD patients [35]. Abolhasani et al. [36] compared methylation status of IL-6, IL-8, and IL-10 in patients with severe and mild BD and did not find any significant differences. In addition, Takahashi et al. [37] in a study on human epithelial colonic carcinoma cell lines showed that DNA methylation led to a lower expression of TLR4. Kim et al. [38] in another research on gastric cancer cells showed that epigenetic mechanisms *via* DNA methylation regulated TLR4 expression.

Our study had important limitations. First, the number of our patients was low. Second, we did not study other epigenetic mechanisms of controlling gene expression, including histone modifications, and noncoding ribonucleic acid regulation (miRNAs).

In summary, our findings for the first time suggest that hypomethylation of TLR4 gene may have a role in the pathology of BD through increasing TLR4 expression. Further studies with more cases are needed to validate our results and evaluate the role of other epigenetic mechanisms in controlling TLR gene expression.

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Conflicts of interest

No conflicts of interests were declared by the authors.

Author contributions

SK, AK, ES and ZB designed the study plan. SK, NR, AMM, JF, AK, ES, NB, ZB, and MJFP performed research and analyzed data. SK, AMM, AK, and ES supervised research and wrote the paper. MAE reviewed and finalized the manuscript.

Ethical statement and patients' consent

The study design was approved by the Ethics Board of Tabriz University of Medical Sciences (TUOMS) (Ethic code: IR.TB2MED.REC.1395.67). Written informed consents were obtained from all the study participants.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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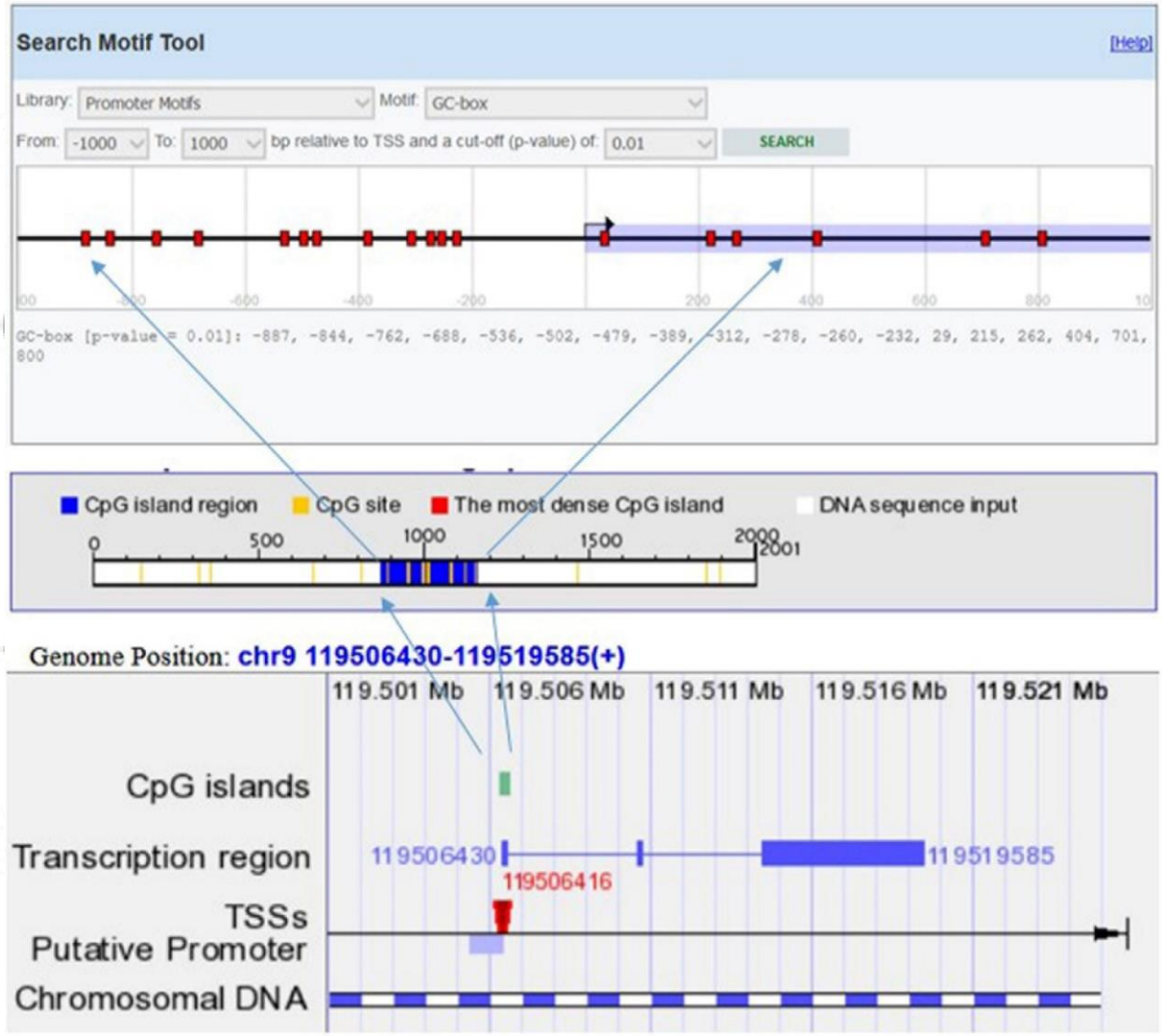

Fig. 1. CpG islands upstream for TLRs. TLRs gene promotor CpG islands were predicted with EPD and database of CpG island tools.

TLRs, Toll-like receptors.

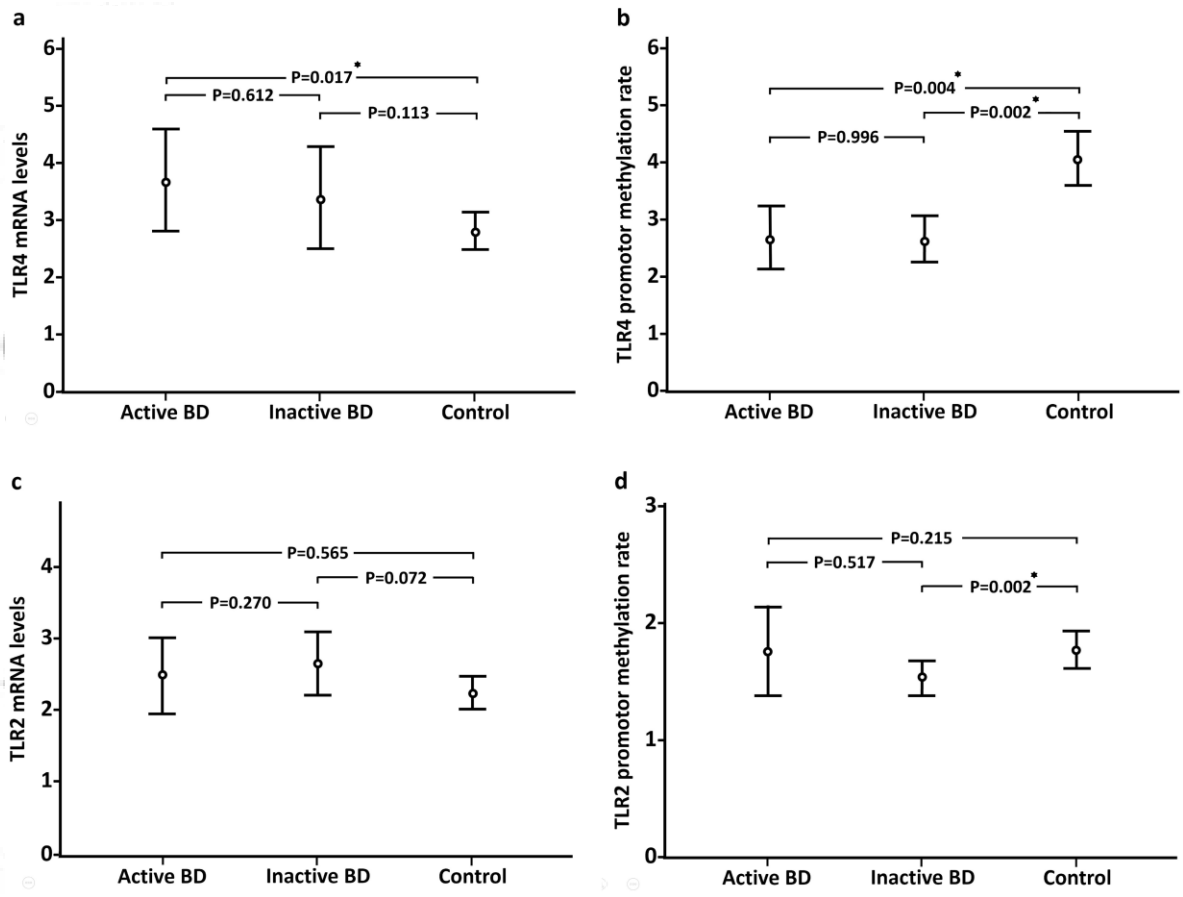

Fig. 2. TLR2 and TLR4 expression and promoters methylation rates in active BD, inactive BD and control groups.

BD, Behcet's disease; TLR, Toll-like receptor.

* $P < 0.05$ indicates statistically significant differences.

Table 1. PCR primers and product size

Target	Primer		Target size
TLR2	Forward:	GGAAGCTGGTGGCAATAA	126
	Reverse:	ACATGGGATGGAGAGTCAC	
TLR4	Forward:	GTCACTCGATGTCATTCC	150
	Reverse:	GCCTCTAGCCCAAATTGT	
TLR2-MeDIP	Forward:	ATGTGAGGACACAGCAACAA	94
	Reverse	GGCTGGGAAGTCTAAGATCAAG	
TLR4-MeDIP	Forward:	CTTTCACTTCCTCTCACCCTTT	89
	Reverse:	GGCACTGGTGTCTTCTCTTC	

PCR, polymerase chain reaction; TLR, Toll-like receptor; MeDIP, Methylated DNA Immunoprecipitation.

Table 2. Demographic, clinical and laboratory characteristics, and medications of participants

Characteristics	BD patients (N=47)	Healthy controls (N=61)	P-value*
Age (mean ± SD) years	38.1 ± 10.3	37.4 ± 8.5	NS
Gender (male/female)	29/18 (1.6)	37/24 (1.5)	NS
Oral aphthous ulcer (%)	45 (95.7)	-	-
Uveitis (%)	34 (72.3)	-	-
Genital ulcer (%)	23 (48.9)	-	-
Positive Pathergy (%)	19 (40.4)	-	-
Pseudofolliculitis (%)	11 (23.4)	-	-
Arthritis (%)	9 (19.1)	-	-
Erythema nodosum (%)	8 (17)	-	-
Phlebitis (%)	5 (10.6)	-	-
CNS involvement	1 (2.1)	-	-
HLA-B5 (%)	27 (57.4)	-	-
HLA-B51 (%)	25 (53.2)	-	-
Medications used at the time of the study			
Colchicine	25 (53.2)	-	-
Prednisolone	23 (48.9)	-	-
AZA	22 (46.8)	-	-
MTX	15 (31.9)	-	-
NSAIDs	12 (25.5)	-	-

TNFis	3 (6.4)	-	-
IFN-α	2 (4.3)	-	-
CYC	2 (4.3)	-	-
CSA	1 (2.1)	-	-
SSZ	1 (2.1)	-	
Treatment regimens			
No IMS (on colchicine or no medication)	8	-	-
IMS = 1	29	-	-
IMS (AZA combined with CSA or CYC) ≥2	5	-	-
Biologics (interferon-α or TNFis)	5	-	-

BD, Behcet's disease; NS, non-significant; CNS, central nervous system; NSAIDs, Non-steroidal anti-inflammatory drugs; AZA, azathioprine, MTX, methotrexate; TNFis, Tumor necrosis α inhibitors; IFN-α, interferon; CSA, cyclosporine A; SSZ, sulfasalazine; IMS, immunosuppressants.

*P values indicate comparison between groups (independent-sample t test or Chi-squared test, as appropriate).

Table 3. Correlation between the TLR2 and TLR4 expression and promoters methylation rates with BD activity

	BDCAF		IBDDAM		TIAI	
	r	P-value*	r	P-value*	r	P-value*
TLR2 expression rate	0.187	0.220	0.225	0.058	-0.238	0.120
TLR2 methylation rate	-0.286	0.056	-0.052	0.732	-0.117	0.451
TLR4 expression rate	-0.028	0.856	0.138	0.361	0.058	0.707
TLR4 methylation rate	0.046	0.764	0.164	0.277	-0.026	0.866

TLR, Toll-like receptor; BD, Behcet's disease; BDCAF: Disease Current Activity Form; IBDDAM: Iranian Behcet's Disease Dynamic Activity Measure; TIAI: Total Inflammatory Activity Index.

* Pearson correlation analysis.