Association of interleukin-2, interleukin-4 and transforming growth factor-beta gene polymorphisms with Behçet's disease

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ABSTRACT

Behçet's disease (BD) is a chronic immune-mediated disease, characterised by oral and genital lesions and ocular inflammation. As cytokines seem to have important roles in the pathogenesis of BD and production of cytokines could be affected by genetic polymorphisms, this study was performed to investigate gene polymorphisms of a number of cytokines in the patients with BD in comparison with control subjects.

One hundred and fifty patients with BD were enrolled in this study. Interleukin (IL)-2 (-330, +166), IL-4 (-1098, -590, -33), IL-10 (-1082, -819, -592), IL-12 (-1188), IFN-γ (5644), transforming growth factor (TGF)-β (codon 10, 25), and IL-4RA (+1902) typing were performed by polymerase chain reaction with sequence-specific primers.

In the patients with BD, there were significantly increased frequency of IL-2 (-330) GG genotype ($p<0.001$), IL-4 (-33) CC genotype ($p<0.001$), and TGF-β (codon 10) CC genotype ($p=0.004$). Meanwhile a significant decrease in the frequency of IL-4 (-33) TC genotype ($p<0.001$) was detected in the patient group in comparison with normal controls. The genotype CC of TGF-β at codon 10 was also significantly over-represented in the patient group ($p=0.004$). Haplotype frequencies of IL-4 (-1098, -590, -33) showed that the frequency of TTC haplotype was significantly increased in the patients ($p<0.001$), whereas TTT haplotype was significantly decreased in this group of patients ($p<0.001$). There was not any significant difference in allele and genotype frequencies of IL-10, IL-12, IFN-γ, and IL-4RA between patient and control groups.

Cytokine single nucleotide polymorphisms could play a role in the pathophysiology of BD. The results of this study could suggest a tendency towards higher production of IL-2 and lower production of IL-4 in the patients with BD.

Introduction

Behçet's disease (BD) is a chronic systemic disease, manifested by oral and genital lesions and ocular inflammation. Although the pathophysiology of disease is not clearly understood, an immune-mediated origin is considered on the basis of the vasculitic nature of the disease (1).

Cytokines seem to have important roles in the pathogenesis of BD (2-6). Inflammatory response is mediated in part by cytokines associated with the T-helper (Th)1 subset of T lymphocytes, which seems to be correlated with the progression of BD (1, 7, 8). Measurement of cytokine levels in aqueous humor and peripheral blood from patients with Behçet uveitis revealed higher levels of interferon gamma (IFN-γ) and lower concentrations of interleukin (IL)-4 and IL-10 in the patient group (2).

Genetic polymorphisms within the critical promoter or other regulatory regions of cytokine genes, can affect gene transcription resulting in inter-individual variation in levels of cytokine production. Differences in the genetic regulation of the immune processes could explain susceptibility of some individuals to a number of diseases (9-13). Considering some Th1 and Th2 cytokine discrepancies in patients with BD, especially evidences on increased serum concentrations of Th1 cytokines and decreased serum concentrations of Th2 cytokines, this study was performed for the first time in Iranian patients with BD to investigate cytokine gene polymorphisms profile of patients in comparison with normal individuals.

Patients and methods

Participants

One hundred and fifty patients with a diagnosis of BD according to International Study Group (ISG) criteria, referred to the BD outpatient clinic (Rheumatology Research Centre at Shariaty Hospital,

Tehran, Iran) were enrolled in this study. Healthy unrelated sex and age matched individuals (n=140) were selected as normal controls (14). This study was approved by local Ethics Committee and informed consent was obtained from all participants before the study.

Sampling and genotyping

Ten mL blood samples were collected with ethyleneediaminetetraacetic acid (EDTA), as anticoagulant, by venepuncture from all subjects. Genomic DNA was extracted using a 'salting out' method.

All cytokine typing were performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) assay. The PCR-SSP kit used was the Heidelberg cytokine gene polymorphism SSP kit (Heidelberg University, Heidelberg, Germany). Amplification was carried out using a PCR Techne Flexigene apparatus (Rosche, Cambridge, UK) under the following conditions: initial denaturation 94°C, 2 min; denaturation 94°C, 10s; annealing + extension 65°C, 1 min (10 cycles); denaturation 94°C, 10s; annealing 61°C, 50s; extension 72°C, 30s (20 cycles). The presence or absence of PCR products was visualised by 2% agarose gel electrophoresis. When the quality of agarose gel was not acceptable, the result was excluded. After electrophoresis, the gel was placed on a UV transilluminator and a picture for interpretation and documentation was taken. Each of the primer mixes contained a control primer pair that amplified either a part of the β-globin gene or a part of the C-reactive protein gene. The β-globin control primers produce a 89-bp fragment, while the primer pairs amplifying the CRP gene produced a 440-bp amplicon. The allele and genotype frequencies of the following cytokine genes were determined: IL-2 (T/G -330, G/T +166), IL-4 (T/G -1098, T/C -590, T/C -33), IL-10 (G/A -1082, C/T -819, C/A -592), IL-12 (C/A -1188), IFN-γ [A/T untranslated region (UTR) 5644], transforming growth factor (TGF)-β (C/T codon 10, G/C codon 25), and IL-4RA (G/A +1902).

Statistical analysis

Associations with disease were ana-

Table I. Comparisons of alleles and genotypes frequencies between patients with BD and controls.

Cytokine	Position	Alleles/ Genotypes	Patients with BD (n=147) n. (%)	Controls (n=140) n. (%)	*p*-value	Odds ratio (95% confidence interval)
IL-2	-330	G	144 (49)	110 (39.6)	0.029	1.47 (1.04–2.07)
		T	150 (51)	168 (60.4)	0.029	0.68 (0.48–0.96)
		GG	31 (21.1)	8 (5.8)	<0.001*	4.38 (1.83–10.80)
		GT	82 (55.8)	94 (67.6)	0.053	–
		TT	34 (23.1)	37 (26.6)	0.585	–
IL-2	+166	G	229 (77.9)	219 (78.8)	0.876	–
		T	65 (22.1)	59 (21.2)		
		GG	87 (59.2)	82 (59)	0.930	–
		GT	55 (37.4)	55 (39.6)	0.801	–
		TT	5 (3.4)	2 (1.4)	0.449	–
IL-4	-1098	G	93 (31.6)	84 (30.2)	0.783	–
		T	201 (68.4)	194 (69.8)		
		GG	2 (1.4)	1 (0.7)	0.546	–
		GT	89 (60.5)	82 (59)	0.883	–
		TT	56 (38.1)	56 (40.3)	0.796	–
IL-4	-590	C	153 (52)	149 (53.6)	0.773	–
		T	141 (48)	129 (46.4)		
		CC	7 (4.8)	10 (7.2)	0.536	–
		TC	139 (94.6)	129 (92.8)	0.714	–
		TT	1 (0.6)	0 (0)	0.514	–
IL-4	-33	C	239 (81.3)	200 (71.9)	0.011*	1.69 (1.12–2.56)
		T	55 (18.7)	78 (28.1)	0.011*	0.59 (0.39–0.89)
		CC	96 (65.3)	61 (43.9)	<0.001*	2.41 (1.45–3.99)
		TC	47 (32)	78 (56.1)	<0.001*	0.37 (0.22–0.61)
		TT	4 (2.7)	0 (0)	0.123	–
IL-4RA	+1902	A	258 (87.8)	242 (87.7)	0.919	–
		G	36 (12.2)	34 (12.3)		
		AA	113 (76.9)	106 (76.8)	0.898	–
		GA	32 (21.8)	30 (21.7)	0.891	–
		GG	2 (1.3)	2 (1.5)	0.665	–
IL-10	-1082	A	197 (67)	181 (64.6)	0.611	–
		G	97 (33)	99 (35.4)		
		AA	58 (39.5)	53 (37.8)	0.875	–
		GA	81 (55.1)	75 (53.6)	0.887	–
		GG	8 (5.4)	12 (8.6)	0.419	–
IL-10	-819	C	206 (70)	199 (71.1)	0.863	–
		T	88 (30)	81 (28.9)		
		CC	71 (48.3)	71 (50.7)	0.771	–
		CT	64 (43.5)	57 (40.7)	0.715	–
		TT	12 (8.2)	12 (8.6)	0.929	–
IL-10	-592	A	90 (30.6)	81 (28.9)	0.727	–
		C	204 (69.4)	199 (71.1)		
		AA	12 (8.2)	12 (8.6)	0.929	–
		CA	66 (44.9)	57 (40.7)	0.551	–
		CC	69 (46.9)	71 (50.7)	0.602	–
IL-12	-1188	A	198 (67.3)	204 (72.9)	0.177	–
		C	96 (32.7)	76 (27.1)		
		AA	65 (44.2)	72 (51.4)	0.269	–
		CA	68 (46.3)	60 (42.9)	0.645	–
		CC	14 (9.5)	8 (5.7)	0.322	–
IFN-γ	UTR5644	A	165 (56.9)	140 (50.7)	0.165	–
		T	125 (43.1)	136 (49.3)		
		AA	52 (35.6)	43 (31.2)	0.503	–
		AT	63 (43.2)	54 (39.1)	0.570	–
		TT	31 (21.2)	41 (29.7)	0.132	–
TGF-β	Codon 10	C	161 (54.8)	131 (47.5)	0.097	–
		T	133 (45.2)	145 (52.5)		
		CC	43 (29.2)	20 (14.5)	0.004*	2.44 (1.30–4.61)
		CT	75 (51)	91 (65.9)	0.015	0.54 (0.32–0.89)
		TT	29 (19.7)	27 (19.6)	0.909	–
TGF-β	Codon 25	C	40 (13.6)	21 (7.6)	0.029	1.91 (1.06–3.46)
		G	254 (86.4)	255 (92.4)	0.029	0.52 (0.29–0.94)
		CC	4 (2.7)	2 (1.5)	0.685	–
		CG	32 (21.8)	17 (12.3)	0.050	–
		GG	111 (75.5)	119 (86.2)	0.032	0.49 (0.25–0.95)

*Significant at 5% level, adjusted for Bonferroni multiple testing correction.

lysed by both allelic frequency and haplotype analysis using chi-square (^{2} test with Yates's correction) or Fisher's exact tests. Results are expressed as odds ratios (OR) with 95% confidence intervals (95% CI). The data were tested for their fit to Hardy-Weinberg equilibrium. A *p*-value of less than 0.05 was considered significant. For multiple testing corrections, Bonferroni correction was performed.

Table II. Comparisons of haplotypes frequencies between patients with BD and controls.

Cytokine	Haplotype	Patients with BD (n=147) n. (%)	Controls (140 subjects) n. (%))	*p*-value	Odds ratio (95% confidence interval
IL-2	GG	141 (48)	107 (38.8)	0.033	1.46 (1.03–2.06)
(-330, +166)	TG	89 (30.2)	112 (40.6)	0.013	0.64 (0.44–0.91)
	TT	62 (21.1)	56 (20.3)	0.895	–
	GT	2 (0.7)	1 (0.3)	0.524	–
IL-4	TTC	94 (32)	51 (18.3)	<0.001*	2.09 (1.39–3.15)
(-1098, -590, -33)	GCC	88 (29.9)	83 (30)	0.943	–
	TTT	45 (15.3)	76 (27.3)	<0.001*	0.48 (0.31–0.74)
	TCC	56 (19)	65 (23.4)	0.244	–
	TCT	4 (1.4)	2 (0.7)	0.687	–
	GTT	1 (0.3)	1 (0.3)	0.736	–
	GCT	3 (1)	0 (0)	0.249	–
	GTC	3 (1)	0 (0)	0.249	–
IL-10	GCC	94 (32)	99 (35.4)	0.442	–
(-1082, -819, -592)	ACC	111 (37.7)	100 (35.7)	0.674	–
	ATA	84 (28.6)	81 (28.9)	0.998	–
	ACA	5 (1.7)	0 (0)	0.061	–
TGF-β	CG	122 (41.5)	110 (39.9)	0.754	–
(codon 10, codon 25)	TG	134 (45.6)	145 (52.5)	0.115	–
	CC	38 (12.9)	21 (7.6)	0.052	–

*Significant at 5% level, adjusted for Bonferroni multiple testing correction.

Results

IL-2 gene polymorphisms

Two polymorphisms at positions -330 and +166 of IL-2 gene were investigated. In the BD patients, GG genotype at -330 was significantly higher than control group (21% *vs*. 6%, $p<0.001$, OR: 4.38, 95%CI: 1.8–10.8). There was not any significant difference between allele and genotype frequencies of patients and healthy controls at +166 (Table I).

IL-4 gene polymorphisms

IL-4 gene polymorphisms at -33 revealed that the genotype CC was significantly over-represented in the patient group (65% *vs*. 44%, $p<0.001$), whilst a significant decrease in the frequency of genotype TC at the same position was detected in the patients with BD (32% *vs*. 45%, $p<0.001$) (Table I). Haplotype frequencies of IL-4 at -1098, -590 and -33 showed that the frequency of TTC haplotype was significantly increased in the patients (32% *vs*. 18%, $p<0.001$), whereas TTT haplotype was significantly decreased in this group of patients (15% *vs*. 27%, $p<0.001$) (Table II). There was not any significant difference in allele and genotype frequencies of IL-4RA at +1902 between patient and control groups (Table I).

TGF-β gene polymorphisms

The genotype CC of TGF-β at codon 10 was significantly over-represented in the patient group (29% *vs*. 15%, $p=0.004$, OR: 2.44, 95%CI: 1.3–4.6). No difference was seen in codon 25 of TGF-β gene (Table I).

Other cytokine gene polymorphisms

Comparison of IL-10 gene polymorphisms between patients and controls did not show any significant difference at -1082, -819, and -592. AT genotype was the most common genotypes of IFN-γ at position UTR5644 in both group of patients and controls. There was no significant difference in alleles and genotypes frequencies at this position. Similarly, there was not any significant difference in IL-12 gene polymorphisms at -1188 between patient and control groups (Table I).

Discussion

This study was performed to investigate constellation of cytokines that overall profile of their gene expression could affect their production. A number of cytokines gene polymorphisms were studied in a group of patients with BD and compared with normal controls.

Two single nucleotide polymorphisms in the IL-2 gene, at positions -330 and +166, relative to the transcription start site, have been reported. Studies on a T to G polymorphism at -330 of IL-2 gene promoter region have demonstrated that T lymphocytes from subjects with G/G genotype were able to produce *in vitro* higher amount of IL-2, than those with TG or TT genotypes (15). However, G allele seems to be associated with lower expression of IL-2. We found an association between GG high producer IL-2 and BD, but the basis underlying this association remains unknown. We need to take into account the whole polymorphic gene cytokine profiles. Aqueous and serum samples from patients with uveitis showed higher levels of IL-2 than those of controls (3,4,6), while other studies did not confirm it (2).

In the 5' flanking region of the IL-4 gene, there is C to T transversion located at position -33 from the first nucleotide of exon 1. Serum IL-4 level depends on gene polymorphism at this region and T allele associates with increased IL-4 production (16). We found significant lower frequency of allele T in BD patients than controls, indicating a tendency toward low production of this cytokine in the patients with BD. Previous study showed that aqueous IL-4 levels were lower in patients with Behçet uveitis, compared to control group (2).

Polymorphisms detected in the first exon of TGF-β1 gene are at position +869 (T/C) resulting in the substitution of leucine by proline at codon 10, and at position 915 (G/C) that results in the substitution of arginine by proline at codon 25. A significant rise in

incidence of CC homozygotes of TGF-β (codon 10) was found in the BD patients at the expense of lowering the incidence of C/T carriers. It should be noted that the frequency of TGF-β1 GG genotype was found lower in BD patients compared to control subjects, which is similar to findings in the Turkish population (17).

Although we did not find any significant difference on IL-10 gene polymorphisms between patients and controls, there are some evidences indicating an association between this cytokine and BD (18, 19).

Due to controversies in different studies, an independent study on patients with BD is needed to correlate serum levels and genotypes. Moreover, further *in vitro* studied on T cells isolated from BD patients and stimulation with anti-CD3/anti-CD28 antibodies would help to test such hypothesis. We should keep in mind that polymorphisms do not exist in isolation, and it could be the combination of several substitutions in different parts of whole cytokine gene, *i.e.* the haplotype, that influences function. In this regard, interpretation of results just based on single changes is oversimplification and could be misleading. On the other hand, cytokines work in coordinated network and it would be better to analyse their increased and decreased levels in the integrated profile. Regarding this, we investigated extended array of cytokines gene polymorphism to avoid such complexities.

Conclusion

Cytokine single nucleotide polymorphisms could play a role in pathophysiology of BD. Considering higher frequency of IL-2 (-330) GG genotype and IL-4 (-33) CC genotype and lower frequency of IL-4 (-33) TC genotype in the patients with BD, a tendency towards higher production of IL-2 and lower production of IL-4 could be expected in the patients with BD.

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