Cytokine single nucleotide polymorphisms in Iranian patients with pulmonary tuberculosis

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ABSTRACT. ***Background.*** **Several genes coding for different cytokines may affect host susceptibility to tuberculosis.** ***Methods.*** **In the present study, the allele and genotype frequencies of a number polymorphic genes coding for cytokines or cytokine receptors were investigated in Iranian patients with pulmonary tuberculosis (PTB).** ***Results.*** **From the IL-1 cluster, a positive, significant difference was found at position -889, where the T/T genotype was over represented in PTB patients (p = 0.01); a positive, significant increase was found in the IL1R PstI 1970 C/C genotype, where the C allele was over represented in the PTB patients (p = 0.01). A significant negative association at codon 10 TGF-β, T allele, was shown in our patients and the C allele and C/C genotype were over represented in the PTB patients (P<0.005). For TNF-α at position -238, we found a negative association for the G/A genotype and a positive association for the G/G genotype (p = 0.0009). Significant negative associations at position -590 IL-4, T allele and the T/T genotype were shown in our patients (p = 0.0007); also, the C allele and T/C genotype were significantly increased in our patients (P<0.05). With IL-6 at -174, G/G increased and G/C decreased significantly in the patients (P<0.005).** ***Conclusion.*** **Pro-inflammatory cytokines such as TNF-α and TGF-β seem to be decreased, and IL-6 increased in PTB patients.**

Keywords: cytokine gene, Iran, polymorphisms, pulmonary tuberculosis

Tuberculosis is one of the oldest diseases known to affect humans. Pulmonary tuberculosis (PTB) has recently re-emerged as a major problem in developed countries, with the increase in the number of immune-compromised, HIV-infected individuals and the increase in the emergence of multi-drug resistance (MDR) bacilli [1-3]. The burden of TB on mankind continues to be enormous, one-third of the world's population being infected with the bacillus, the vast majority of which resides in developing countries like Iran, with an incidence of 24 tuberculosis-positive smears-per 100,000 and more than 15,000 patients with PTB-positive smears [3-6].

Cytokines, their genes and receptors have been implicated in the protective immunity, pathophysiology and development of tuberculosis [7-15]. Gene polymorphisms associated with cytokine production may differ in different types of tuberculosis [8]. A recent study by Henao *et al*., indicated the role of the low-IL-10 producer polymorphism and high-IFN-γ producer polymorphism in pleural tuberculosis [8]. The study by Hussain *et al*., showed that PTB patients have significantly suppressed T-cell-derived IFN-γ, but greater monocyte-derived IL6 and IL10 production in response to culture filtrate proteins in comparison with healthy controls [9]. TNF-α (-238 and -308) gene polymorphism studies were carried out by Selvaraj *et al*., in an Indian population. They suggested that TNF-α/β gene variants are not independently associated with susceptibility to PTB [10]. Mandernelo *et al*., in a Spanish population, investigated IFN-γ and IL-10 gene polymorphisms, and showed that homozygous, IFN-γ (+874) A allele individuals had a 3.75-fold increased risk of developing tuberculosis. In contrast, the IL-10 polymorphism did not affect susceptibility to tuberculosis [11]. Bellamy *et al*., investigated the IL-1 gene cluster in a group of Gambian PTB patients and suggested that susceptibility to tuberculosis in Gambian patients may be partly determined by genes in the IL-1 gene cluster on chromosome 2 [12]. The TGF-β codon 10 polymorphism was investigated by Niimi *et al*., who found no significant differences in TGF-β genotypes between healthy controls and tuberculosis patients [13]. Polymorphism of the TNF-α gene at -308 position was investigated by Bikmaeva *et al*., in a Bashkorstan population from Russia, with infiltrative tuberculosis; they found that the frequency of the TNF-α allele in tuberculosis patients was significantly higher than in controls (p = 0.001) [14]. Lopez-Maderuelo *et al*., investigated the relationship of the single base change polymorphic variants with cytokine production by peripheral blood

mononuclear cells and tuberculosis susceptibility in a Spanish population. They found that individuals homozygous for the IFN-γ (=874) A allele had a 3.75-fold increased risk of developing tuberculosis (p = 0.001) [11]. The frequency of the functional polymorphisms of the genes coding for TNF-α (-308) and IL-10 (-1082) were analyzed in a group of Sicilian patients affected by chronic lung tuberculosis by Scola *et al*. They reported fewer -308 G/G TNF homozygous individuals in the affected subject group [15]. Although family-based genetic linkage studies and population-based case-control analyses have been used to identify candidate genes for susceptibility to tuberculosis in some regions, especially in West Africans [12], there has been no comparable study in the Eastern Mediterranean region. In the present study, the genotype frequencies of a number polymorphic genes coding for cytokines or for cytokine receptors were investigated in a case-control study involving a group of 41 Iranian, PTB patients and 123 healthy individuals.

METHODS

Study population

Forty one Iranian patients with PTB and 123 healthy subjects were randomly selected. Diagnosis of the patients was based on the finding of acid-fast bacilli (AFB) on microscopic examination of a specimen such an expectorated sputum smear. Chest X-ray (CXR) was also used as a definitive diagnostic procedure when the classic picture of TB-like, upper lobe infiltrations and cavities was present. We did not use PPD (Mantoux test) as a diagnostic procedure because most Iranians are vaccinated with the BCG vaccine at birth. Healthy control subjects were selected randomly from blood donors at Iranian blood transfusion organizations. Informed consent, approved by the Ethical Committee of Tehran University of Medical Sciences, was obtained from the patients and healthy donors at the time of sampling.

Genotyping

DNA was isolated from whole blood collected with EDTA as anticoagulant, using a "salting out" method [16]. Cytokine typing was performed in a polymerase chain reaction with a sequence specific primers (PCR-SSP) assay, using Heidelberg University cytokine genotyping kits. Amplification was carried out using a PCR 9600 apparatus (applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation 94°C, 2 min; denaturation 94°C, 10 s; annealing+extension 65°C, 1 min (10 cycles); denaturation 94°C, 10 s; annealing 61°C, 50 s; extension 72°C, 30 s (20 cycles). The presence or absence of PCR products was visualized by 2% agarose gel electrophoresis. After electrophoresis, the gel was placed on a UV transilluminator, and a Polaroid picture was taken. Each of the primer mixes contained as a control, a primer pair that amplified either a part of the β-globin gene or a part of the C-reactive protein (CRP) gene. The β-globin control primers produce an 89-bp fragment, while the primer pairs amplifying the CRP gene produce a 440-bp amplicon. The allele and genotype frequencies of the following cytokine genes were determined: IL-1α (T/C -889), IL-1β (C/T -511, T/C +3962), IL-12 (C/A-1188), IFN-γ (A/T UTR 5644), TGF-β (C/T codon 10, G/C codon 25), TNF-α (G/A -308, G/A -238), IL-2 (T/G -330, G/T +166), IL-4 (T/G -1089, T/C -590, T/C-33), IL-6 (G/C-174, G/A nt565), IL-10 (G/A-1082, C/T -819, C/A-592), IL-1R (C/T pst11970), IL-1RA (T/C mspa111100), IL-4RA (G/A +1902).

Statistical analysis

Allele frequencies were estimated by direct gene counting. In order to test the Hardy-Weinberg equilibrium, all frequencies of various genotypes were compared using the chi-square test. The odds ratio and P-value were calculated for each allele in the patient and control groups. The odds ratios (OR) were calculated and a P-value of less than 0.05 was considered significant with 95% confidence intervals (CI).

RESULTS

Genotype frequencies in PTB patients and healthy control subjects are shown in *tables 1-3*.

From the IL-1 cluster (pro-inflammatory cytokines), a positive significant difference was found at position -889, where the T/T genotype was over represented in PTB patients (25% *versus* 9.3%, p = 0.01); also, the C/C genotype at IL1-R PstI 1970 was significantly over represented in the PTB patients (58.5% *versus* 36.4%, p = 0.01). The frequency of IL1-β -511, +3962, and IL1-RA Mspa11100 alleles did not show any significant difference between PTB patients and control subjects *(table 1)*.

For TNF-α at position -238, we found a significant negative association with the G/A genotype and a significant positive association with the G/G genotype (p = 0.0009); the G allele was over represented in PTB patients (95% *versus* 81.1%, p = 0.002). The frequency of IL-2 -330, +160, IL-12 -1188, and TNF-α -308 alleles did not show any significant difference between PTB patients and control subjects *(table 2)*.

A significant negative association at position -590 in the IL-4 T allele was shown in our patients, where the T/T genotype was not detected. However, 23.1% of the normal subjects expressed this genotype (p = 0.0007); in addition, the C allele was significantly over represented in our patients (p = 0.03) and the T/C genotype was significantly increased in our PTB patients (85.4% *versus* 66.1%, p = 0.01). For IL-6 at -174, the G/G genotype was significantly higher (57.5% *versus* 31.9%, p = 0.004) and the G/C genotype significantly lower (32.5% *versus* 59.7%, p = 0.002) in PTB patients; the G allele was over represented in the patients. A significant negative association at codon 10 of the TGF-β T allele was shown in our patients, whereas the T/T genotype was not detected in our patients; 15.5% of the control subjects expressed this genotype (p = 0.006); also in addition, the C allele was significantly over represented in the PTB patients (p = 0.002) and the C/C genotype was significantly increased (36.6% *versus* 13.6%, p = 0.001). There were no significant differences found in the frequency of IL-4 -1098, -33, IL4-RA +1902, IL-6 nt565, IL-10 -1082, -819, -592, and TGF-β codon 25 alleles between PTB patients and control subjects *(table 3)*.

Table 1
Interleukin-1 gene polymorphisms in Iranian PTB patients and normal controls

Cytokines	Position	Allele	Genotype	PTB (%)	Normal (%)	P-value	Odds ratio (95%CI)
IL1-α	-889		C/C	19 (47.5%)	60 (50.9%)	0.71	0.40 (0.87-1.90)
			T/C	11 (27.5%)	47 (39.8%)	0.16	0.24 (0.57-1.34)
			T/T*	**10 (25%)**	**11 (9.3%)**	**0.01**	**1.14 (3.24-9.23)**
		C		49 (61.3%)	167 (70.8%)	0.11	0.37 (0.65-1.15)
		T		31 (38.7%)	69 (29.2%)	0.11	0.87 (1.53-2.69)
IL1-β	-511		C/C	12 (30.0%)	30 (24.8%)	0.51	0.55 (1.30-3.07)
			T/C	17 (42.5%)	69 (57.0%)	0.11	0.25 (0.56-1.22)
			T/T	11 (27.5%)	22 (18.2%)	0.20	0.68 (1.71-4.23)
		C		41 (51.3%)	129 (53.3%)	0.74	0.54 (0.92-1.58)
		T		39 (48.7%)	113 (46.7%)	0.74	0.63 (1.09-1.86)
	+3962		C/C	17 (41.5%)	59 (48.8%)	0.41	0.34 (0.74-1.61)
			C/T	22 (53.7%)	56 (46.3%)	0.41	0.62 (1.34-2.90)
			T/T	2 (4.8%)	6 (4.9%)	1.00	0.13 (0.98-5.73)
		C		56 (68.3%)	174 (71.9%)	0.53	0.47 (0.84-1.50)
		T		26 (31.7%)	68 (28.1%)	0.53	0.67 (1.19-2.11)
IL1-R	PstI1970		**C/C***	**24 (58.5%)**	**44 (36.4%)**	**0.01**	**1.13 (2.47-5.43)**
			C/T	15 (36.6%)	60 (49.6%)	0.14	0.27 (0.59-1.29)
			T/T	2 (4.9%)	17 (14.0%)	0.16	0.05 (0.31-1.52)
		C		**63 (76.8%)**	**148 (61.2%)**	**0.01**	**1.15 (2.11-3.90)**
		T		**19 (23.2%)**	**94 (38.8%)**	**0.01**	**0.26 (0.47-0.87)**
IL1-RA	Mspa11100		C/C	3 (7.3%)	3 (2.5%)	0.16	0.48 (3.13-20.51)
			T/C	12 (29.3%)	53 (43.4%)	0.10	0.23 (0.54-1.23)
			T/T	26 (63.4%)	66 (54.1%)	0.29	0.67 (1.47-3.25)
		C		18 (21.9%)	59 (24.2%)	0.68	0.46 (0.88-1.67)
		T		64 (78.1%)	185 (75.8%)	0.68	0.60 (1.13-2.16)

Table 2
Interleukin 2, 12 and TNF-α gene polymorphisms in Iranian PTB patients and normal controls

Cytokines	Position	Allele	Genotype	PTB (%)	Normal (%)	P-value	Odds ratio (95%CI)
IL-2	-330		G/G	3 (8.8%)	9 (7.6%)	0.73	0.23 (1.17-5.15)
			G/T	16 (47.1%)	73 (61.9%)	0.12	0.24 (0.55-1.26)
			T/T	15 (44.1%)	36 (30.5%)	0.13	0.77 (1.80-4.22)
		G		22 (32.4%)	91 (38.6%)	0.35	0.41 (0.76-1.40)
		T		46 (67.6%)	145 (61.4%)	0.35	0.71 (1.31-2.42)
	+160		G/G	22 (53.7%)	75 (61.5%)	0.37	0.33 (0.73-1.57)
			G/T	16 (39.0%)	45 (36.9%)	0.80	0.50 (1.10-2.41)
			T/T	3 (7.3%)	2 (1.6%)	0.10	0.61 (4.74-42.37)
		G		60 (73.2%)	195 (79.9%)	0.20	0.37 (0.69-1.28)
		T		22 (26.8%)	49 (20.1%)	0.20	0.78 (1.46)
IL-12	-1188		A/A	26 (65%)	62 (51.2%)	0.12	0.79 (1.77-3.97
			C/A	11 (27.5%)	50 (41.3%)	0.11	0.23 (0.54-1.25)
			C/C	3 (7.5%)	9 (7.5%)	1.00	0.20 (1.01-4.38)
		A		63 (78.8%)	174 (71.9%)	0.22	0.76 (1.45-2.78)
		C		17 (21.2%)	68 (28.1%)	0.22	0.36 (0.69-1.31)
TNF-α	-308		A/A	0	1 (0.8%)	1.00	0.00 (0.00-53.94)
			G/A	8 (20%)	33 (26.8%)	0.38	0.26 (0.68-1.75)
			G/G	32 (80%)	89 (72.4%)	0.33	0.60 (1.53-4.01)
		A		8 (10%)	35 (14.2%)	0.33	0.27 (0.67-1.59)
		G		72 (90%)	211 (85.8%)	0.33	0.63 (1.49-3.67)
	-238		A/A	0	0	-	-
			G/A*	**4 (10%)**	**46 (37.7%)**	**0.0009**	**0.05 (0.18-0.59)**
			G/G*	**36 (90%)**	**76 (62.3%)**	**0.0009**	**1.70 (5.45-19.34)**
		A		**4 (5%)**	**46 (18.9%)**	**0.002**	**0.07 (0.23-0.69)**
		G		**76 (95%)**	**198 (81.1%)**	**0.002**	**1.46 (4.41-14.97)**

DISCUSSION

Host genetic factors may be important determinants of susceptibility to tuberculosis, and several candidate gene polymorphisms have been shown to date. In fact, several genes coding for different cytokines may affect host susceptibility to tuberculosis [7, 8, 11].

For IL-1 receptor (IL-1R), a significant positive association was found with position PstI 1970 C/C polymorphism, where the C allele was significantly over represented in the PTB patients. A previous report by Bellamy *et al.*, in Gambian PTB patients has confirmed a positive association with IL-1 cluster genes, which is compatible with our data [12]. A significant negative association with position

Table 3
Interleukin 4, 6 10, and TGF-β gene polymorphisms in Iranian PTB patients and normal controls

Cytokines	Position	Allele	Genotype	PTB (%)	Normal (%)	P-value	Odds ratio (95%CI)
IL-4	-1098		G/G	0	0	-	-
			G/T	22 (53.7%)	69 (56.6%)	0.74	0.41 (0.89-1.92)
			T/T	19 (46.3%)	53 (43.4%)	0.74	0.52 (1.12-2.43)
		G		22 (26.8%)	69 (28.3%)	0.80	0.51 (0.93-1.69)
		T		60 (73.2%)	175 (71.7%)	0.80	0.59 (1.08-1.96)
	-590		C/C	6 (14.6%)	13 (10.8%)	0.57	0.44 (1.42-4.43)
			T/C*	**35 (85.4%)**	**80 (66.1%)**	**0.01**	**1.09 (2.99-8.65)**
			T/T*	**0**	**28 (23.1%)**	**0.0007**	**0.00 (0.00-0.42)**
		C		**47 (57.3%)**	**106 (43.8%)**	**0.03**	**1.01 (1.72-2.95)**
		T		**35 (42.7%)**	**136 (56.2%)**	**0.03**	**0.34 (0.58-0.99)**
	-33		C/C	22 (55%)	55 (46.2%)	0.33	0.65 (1.42-3.11)
			C/T	18 (45%)	64 (53.8%)	0.33	0.32 (0.70-1.53)
			T/T	0	0	-	-
		C		62 (77.5%)	174 (73.1%)	0.43	0.67 (1.27-2.41)
		T		18 (22.5%)	64 (26.9%)	0.43	0.42 (0.79-1.49)
IL4-RA	+1902		A/A	31 (77.5%)	88 (72.8%)	0.55	0.52 (1.29-3.28)
			G/A	5 (12.5%)	27 (22.3%)	0.17	0.15 (0.50-1.50)
			G/G	4 (10%)	6 (4.9%)	0.26	0.47 (2.13-9.19)
		A		67 (83.8%)	203 (83.9%)	0.97	0.48 (0.99-2.08)
		G		13 (16.2%)	39 (16.1%)	0.97	0.48 (1.01-2.10)
IL-6	-174		C/C	4 (10%)	10 (8.4%)	0.75	0.30 (1.21-4.57)
			G/C*	**13 (32.5%)**	**71 (59.7%)**	**0.002**	**0.14 (0.33-0.74)**
			G/G*	**23 (57.5%)**	**38 (31.9%)**	**0.004**	**1.30 (2.88-6.43)**
		C		21 (26.3%)	91 (38.2%)	0.05	0.31 (0.57-1.04)
		G		59 (73.7%)	147 (61.8%)	0.05	0.96 (1.74-3.18)
	nt565		A/A	4 (10%)	9 (7.4%)	0.73	0.34 (1.40-5.38)
			G/A	13 (32.5%)	42 (34.4%)	0.82	0.40 (0.92-2.09)
			G/G	23 (57.5%)	71 (58.2%)	0.93	0.44 (0.97-2.13)
		A		21 (26.3%)	60 (24.6%)	0.76	0.59 (1.09-2.02)
		G		59 (73.7%)	184 (75.4%)	0.76	0.50 (0.92-1.70)
IL-10	-1082		A/A	7 (17.5%)	18 (17.6%)	0.98	0.34 (0.99-2.82)
			G/A	31 (77.5%)	79 (77.5%)	0.99	0.39 (1.00-2.64)
			G/G	2 (5%)	5 (4.9%)	1.00	0.13 (1.02-6.32)
		A		45 (56.3%)	115 (56.4%)	0.98	0.57 (1.00-1.73)
		G		35 (43.7%)	89 (43.6%)	0.98	0.58 (1.00-1.75)
	-819		C/C	19 (46.3%)	62 (50.4%)	0.65	0.39 (0.85-1.83)
			C/T	20 (48.8%)	52 (42.3%)	0.46	0.60 (1.30-2.80)
			T/T	2(4.8%)	9 (7.3%)	0.73	0.09 (0.65-3.44)
		C		58 (70.7%)	176 (71.5%)	0.88	0.54 (0.96-1.73)
		T		24 (29.3%)	70 (28.5%)	0.88	0.58 (1.04-1.87)
	-592		A/A	2 (5%)	9 (7.3%)	1.00	0.09 (0.67-3.54)
			C/A	20 (50%)	52 (42.3%)	0.39	0.63 (1.37-2.97)
			C/C	18 (45%)	62 (50.4%)	0.55	0.37 (0.80-1.75)
		A		24 (30%)	70 (28.5%)	0.79	0.60 (1.08-1.94)
		C		56 (70%)	176 (71.5%)	0.79	0.52 (0.93-1.67)
TGF-β	Codon 10		**C/C***	**15 (36.6%)**	**15 (13.6%)**	**0.001**	**1.46 (3.65-9.17)**
			C/T	26 (63.4%)	78 (70.9%)	0.37	0.31 (0.71-1.62)
			T/T*	**0**	**17 (15.5%)**	**0.006**	**0.00 (0.00-0.71)**
		C		**56 (68.3%)**	**108 (49.1%)**	**0.002**	**1.27 (2.23-3.95)**
		T		**26 (31.7%)**	**112 (50.9%)**	**0.002**	**0.25 (0.45-0.79)**
	Codon 25		C/C	2 (5%)	2 (1.6%)	0.25	0.21 (3.18-33.03)
			C/G	2 (5%)	13 (10.6%)	0.36	0.07 (0.45-2.22)
			G/G	36 (90%)	108 (87.8%)	1.00	0.36 (1.25-4.79)
		C		6 (7.5%)	17 (6.9%)	0.85	0.37 (1.09-3.08)
		G		74 (92.5%)	229 (93.1%)	0.85	0.32 (0.92-2.71)

-590 in the IL-4 T/T genotype has been shown in our patients, where the C allele was significantly over represented in our patients.

For TNF-α, a significant negative association with the G/A genotype and a significant positive association with the G/G genotype were found; where the G allele was over represented in the patients. Moreover, an insignificant tendency was found for position -308 G/G polymorphism, where the G allele was carried by 90% of cases and 85.8% of controls. At this position, the A allele is associated with high TNF-α production and the G allele is associated with low level TNF-α production. The relation between genotype and cytokine production is shown in *table 4* [17-22]. In healthy subjects, a certain level of production (high, intermediate, or low) has been found more frequently for some cytokines. A possible reason for the high frequency of a genotype associated with a given rate of production is selection over the course of evolution, increasing the frequency of the most advantageous genotype [23]. It is suggested that TNF-α production in our PTB patients has

Table 4
Cytokine production by different genotypes [17-23]

Cytokine	Position	Genotype	Production
IL-4	-590	C/C	Low
		T/C	Intermediate
		C/T	High
IL-6	-174	G/G	High
		G/C	High
		C/C	Low
IL-10	-1082, -819, -592	GCC/GCC	High
		GCC/ACC	Intermediate
		GCC/ATA	Intermediate
		ACC/ACC	Low
		ACC/ATA	Low
		ATA/ATA	Low
TGF-β	Codon 10, 25	TG/TG	High
		CG/TG	High
		CC/TG	Intermediate
		CG/CG	Intermediate
		TG/TC	Intermediate
		CG/CC	Low
		CC/CC	Low
		TC/TC	Low
		TC/CC	Low
TNF-α	-308	G/G	Low
		G/A	High
		A/A	High

been decreased. Although the study by Scola *et al*., demonstrated a reduction of low-producer -308 G/G TNF homozygous individuals in the affected group [15], and the study of Bikmaeva *et al*., revealed an association of the high-producer A allele with a higher risk of pulmonary tuberculosis [14], several studies have not confirmed the TNF association in tuberculosis [8, 10].

For IL-4, a significant negative association with position -590 IL-4 T/T polymorphism has been shown in our patients, where the C allele was significantly over represented and was associated with low production of IL-4 [22]. For IL-6, a significant positive association with position -174 G/G polymorphism has been shown in our patients, where the G allele was significantly over represented and associated with a high production of IL-6. This finding is in contrast with a recent study by Henao *et al*.; they did not find any significant differences in IL-6 -174 alleles between patients and controls [8]. In fact, this is the first report, indicating the role of IL-6 polymorphisms and PTB.

For IL-10, several studies on TB patients have produced different results [8, 11, 15]. In a recent study by Henao *et al*., they did not find any significant difference in IL-10 -819 and -592 between patients and controls, which is similar to our study; however, in contrast to our study, they suggested an association of the IL-10 -1082 low-producer polymorphism in the patient group [8].

A significant negative association with the codon 10 TGF-β T/T polymorphism was also shown in our patients, where the C allele was significantly over represented and associated with high TGF-β production. However, the study by Henao *et al*., did not confirm this TGF association in tuberculosis [8].

There is the suggestion that in our PTB patients TNF-α and TGF-β cytokines are decreased and IL-6 increased, however, further studies of cytokine gene polymorphisms in PTB patients using a larger sample size are obviously necessary.

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