Cytokine Gene Polymorphisms in Iranian Patients with Beta-Thalassemia Major

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ABSTRACT

Background: β-thalassemia as a hereditary disease is defined as defective synthesis of β-globin chains, resulting in erythropoiesis abnormalities and severe anemia. Different studies have shown that cytokines and cytokine gene polymorphisms play a major role in the pathogenesis of β-thalassemia. Single nucleotide polymorphisms (SNPs) within the promoter region or other regulatory sequences of cytokine genes lead to overall production of cytokines. **Objective:** To analyze the genetic profile of Th1 and Th2 cytokines in Iranian patients with β-thalassemia major. **Methods:** Allelic and genotype frequencies of cytokine genes were determined in 30 thalassemia patients and 40 healthy subjects using PCR-SSP assay. Allele and genotype frequencies were calculated and compared with those of normal controls. **Results:** The results of our study show a significant decrease in A allele at position UTR 5644 IFN-γ, G alleles at position -238 TNF-α and 166 IL-2, and C allele at position -590 IL-4. TGF-β haplotype TG/TG increased whereas TGF-β haplotype CG/CG and IL-10 haplotype GCC/ACC decreased significantly in all patients. **Conclusion:** Data of this investigation suggest that variations among cytokine gene polymorphisms may contribute to the disease susceptibility. A finding which needs to be fairly clarified in other ethnic groups.

Keywords: β-thalassemia, Cytokine Gene Polymorphism

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INTRODUCTION

β-thalassemia as a hereditary disease is defined as defective synthesis of β-globin chains, resulting in erythropoiesis abnormalities and sever anaemia. Resent studies have shown that the ineffective erythropoiesis present in β-thalassemia is the result of an increased rate of apoptosis of the marrow erythroid cells. Transfusions lead to iron overload exerting a negative effect on the functional integrity of the immune system in multi-transfused patients with β-thalassemia. There are large numbers of immune abnormalities in β-thalassemia, including defective function of polymorphonuclear neutrophils and monocytes, decrease of $CD4^+$ cells and increase of $CD8^+$ cells. Abnormalities may explain the tendency for severe or unusual infections (1). Evidence suggests that cytokines and cytokine gene polymorphisms may play a major role in the pathogenesis of β-thalassemia. Single nucleotide polymorphisms (SNPs) within the promoter region or other regulatory sequences of cytokine genes lead to overall production of cytokines (2). In present study, we analyzed the genetic profile of Th1 and Th2 cytokines in 30 Iranian patients with β-thalassemia and 40 healthy subjects.

PATIENTS AND METHODS

Thirty Iranian patients with β-thalassemia candidate for bone marrow transplantation and 40 healthy subjects were randomly selected. DNA was isolated from whole blood collected in EDTA as anticoagulant, using a "Salting out" method (3). Cytokine typing was performed in a polymerase chain reaction with sequence specific primers (PCR-SSP) assay, which uses identical amplification and detection conditions, enabling rapid and cost-efficient analysis of polymorphisms. Amplification was carried out using a PCR 9600 apparatus (Applied Biosystems, Fostercity, CA) under the following conditions: initial denaturation at 94^{o}C, 2 min; denaturation at 94^{o}C, 10s; annealing + extension at 65^{o}C, 1 min (10 cycles); denaturation at 94^{o}C, 10s; annealing at 61^{o}C, 50s; extension at 72^{o}C, 30s (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 for interpretation and documentation 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-1β (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). Allele frequencies were estimated by direct gene counting. In order to test Hardy-Weinberg equilibrium, all the frequencies of various genotypes were compared using the chi-square test.

RESULTS

Allelic and genotype frequencies of patients and controls are reported in Table 1. Almost all allele and genotype frequencies were in Hardy-Weinberg equilibrium: the exceptions were A allele at position UTR 5644 IFN-γ (20% *vs.* 52.5%), TGF-β haplotype CC/TG (13.3% *vs.* 25%, P < 0.0001) and IL-10 haplotype GCC/ACC (16.66% *vs.* 52.5%) decreased in our patients, whereas G alleles at position -238 TNF-α (93.3% *vs.* 72.5%) and 166 IL-2 allele G at +166 (80% *vs.* 52.5%), C allele at position -590 IL-4 (50% *vs.* 12.5%), TGF-β haplotype TG/TG (50% *vs.* 12.5%, P<0.0001) increased in our patients comparing to control subjects.

Table 1. Allele and genotype frequencies in Iranian patients with ß-Thalassemia

Cytokine	Position	Allele	Genotype	Thalassemia		Control		P-value
				%	F	F	%	
IFNγ	UTR5644	A	AA	24	6	21	52.5	0.04
		T	AT	36	9	1	2.5	0.004
			TT	40	10	18	45	
TGFβ	codon 10,25	CTGC	TG/TG	50	15	5	12.5	0.005
			TG/CG	36.66	11	17	42.5	
			CC/TG	13.33	4	1	2.5	0.00005
TNFα	-308	A	AA	0	0	1	2.5	
		G	GA	20	6	13	32.5	
			GG	80	24	26	65	0.2
	-238	A	AA	0	0	0	0	
		G	GA	6.66	2	11	27.5	0.05
			GG	93.33	28	29	72.5	0.05
IL 2	-330	G	GG	10 .3	3	1	2.5	
		T	GT	58.9	17	23	57.5	
			TT	31	9	16	40	
	+166	G	GG	85.7	24	21	52.5	0.009
		T	GT	10 .7	3	19	47.5	0.003
			TT	3.5	1	0	0	
IL 4	-1098	G	GG	0	0	0	0	
		T	GT	25	7	22	55	0.02
			TT	75	21	18	45	
	-590	C	CC	51.7	15	5	12.5	0.001
		T	CT	41.3	12 35	87.	5	0.0001
			TT	6.9	2	0	0	
	-33	C	CC	85	23	22	55	0.02
		T	CT	14.8	4	18	45	0.02
			TT	0	0	0	0	
IL 6	-174	C	CC	6.66	2	3	7.5	
		G	CG	33.33	10	21	52.5	
			GG	60	18	16	40	0.15
	nt565	A	AA	3.33	1	0	0	
		G	GA	36.66	11	20	50	
			GG	60	18	20	50	
IL 10	-1082,-819,-592	GCTA	GCC/ACC	16.66	5	21	52.5	0.0001

DISCUSSION

Significant increase in cytokines has a negative effect on hematopoiesis which is detected in multi-transfused patients with β-thalassemia. The relationship between cytokine genotype and *in vivo* production of some of the cytokines are well known (Table 2) (4,5,6).

Table 2. Cytokine production by different genotypes

Cytokine	position	Genotype	Production
TNF-α	-308	G/G	Low
		G/A	High
		A/A	High
TGF-β	codon10,25	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
IL-10	-1082, -819,-590	GCC/GCC	High
		GCC/ACC	Intermediate
		GCC/ATA	Intermediate
		ACC/ACC	Low
		ACC/ATA	Low
		ATA/ATA	Low
IL-6	-174	G/G	High
		G/C	High
		C/C	Low
IFN-γ	Intron 1	T/T	High
		T/A	Intermediate
		A/A	Low

* For more details see references 4-6.

In healthy subjects, a certain level of production (High, Intermediate, or Low) has been found more frequently for some cytokines. A possible reason for a high frequency of a genotype associated with a given rate of production is the selection over the course of evolution, increasing the frequency of the most advantageous genotype. In our study the frequency of A allele at position UTR 5644 IFN-γ (20% *vs.* 52.5%), TGF-β haplotype CC/TG (13.3 *vs.* 25%, $P < 0.0001$) and IL-10 haplotype GCC/ACC (16.66% *vs.* 52.5%) decreased in the patients, whereas G alleles at position -238 TNF-α (93.3% *vs.* 72.5%) and 166 IL-2 allele G at +166 (80% *vs.* 52.5%), C allele at position -590 IL-4 (50% *vs.* 12.5%), TGF-β haplotype TG/TG (50% *vs.* 12.5%, $P < 0.0001$) increased in our patients comparing to control subjects.

Interleukin 4 (IL-4) is a cytokine of T helper 2 (Th2) subtypes, which is mainly produced by activated T cells and mast cells and is a pleiotropic cytokine that affects the cells of multiple lineages. IL-4 is an important mediator of Th2 immune responses

and also stimulates the growth and differentiation of Th2 lymphocytes and mast cells. IL-4 down-regulates the Th1 activity by inhibiting the production of Th1 cytokines and induces tolerance (7). The -590 (C→T) polymorphism of IL-4 gene is associated with high levels of IgE in asthmatic families (8). Our studies show that C allele at position -590 at IL-4 gene was increased significantly in patients and it is suggestive that our thalassemic patients will produce more IL-4 and probably more IgE response.
TNF-α (low production alleles are more frequent in healthy individuals) shows a wide spectrum of biological activities. TNF-α is a potent pro-inflammatory cytokine causing the cytolysis of many tumor cell lines *in vitro* and is a powerful promoter of angiogenesis *in vivo*. Although TNF-α is required for normal immune responses, its over-expression may have severe pathological consequences. The most common studies in TNF polymorphism are the SNPs at -308 position which involves a G/A substituation (9). It has been shown that an "A" at position -308 was associated with increased transcription and production of TNF-α (10,11). Patients with either renal or cardiac transplants with a high producing TNF-α genotype were found to be at increased risk of rejection (12-15). We found a significant decrease in G alleles at position -238 TNF-α. in our patients.
TGF-β (high production is more frequent in healthy individuals), as a multifactorial cytokine, is the strongest known growth inhibitor of normal and transformed cells. At high concentrations, it stimulates the growth of the smooth muscle cells, fibroblasts, and chondrocytes. TGF-β may be effective in treatment of osteoporosis, and TGF-β local application accelerates wound repair and its sustained production regulates the development of tissue fibrosis (16). Two SNPs in the first exon of TGF-β, have been described at position +869 (T/C) and at position +915 (G/C). These polymorphisms result in the change of codon 10 from leucine (T) to proline (C) and in the change of codon 25 from arginine (G) to proline (C) (17). In vitro, the presence of leucine or arginine in part has been shown to lead to a higher production of TGF-β (18,19). As it is shown in Table 2, the homozygous genotype CC either at codon 10 or at codon 25 is strongly associated with low production, while the homozygous genotype GG at codon 25 is associated with high production. This might explain the significant excess of GG homozygous observed in our patients, as reported in Table 1. When both GG and CC genotypes are present together, an intermediate production is observed, indicating an interaction or dose effect of the two alleles. The results of our research show that TGF-β haplotype TG/TG increased whereas TGF-β haplotype CG/CG decreased significantly among patients.
IL-6 (high production was more frequent in healthy individuals) is a pleiotropic cytokine with a central role in host defense. Its functions include stimulation of the hepatic acute phase response and differentiation or activation of macrophages, B and T cells. IL-6 is produced by many different cell types and although initially thought to be a pro-inflammatory cytokine having additional anti-inflammatory and immunosuppressive properties (20). It has been shown that IL-6 derived from APC cells is able to initiate the polarization of native $CD4^+$ T cells to effector TH2 cells, thereby antagonizing differentiation to Th1 cells (20) because Th1 dependent effector mechanisms are held responsible for allograft rejection. Recently, a biallelic polymorphism within the promoter region of the IL-6 gene at position -174 G→C was detected, and the C

allele was found to be associated with lower *in vitro* and *in vivo* production of IL-6. Even through the relevance of the recipient's genotype for acute rejections is still controversial, the -174 G allele has recently been associated with Reno protection (20).
IL-10 (intermediate production is more frequent in healthy individuals) as an anti-inflammatory cytokine induces the secretion of IgG, IgA and IgM. This effect is synergized by IL-4 while the synthesis of immunoglobulins induced by IL-10 is antagonized by TGF-β, IL-10 is also a cytotoxic T cell differentiation factor. Three SNPs in the promoter region of IL-10 are most commonly studied in the context of transplantation: -1082 (G/A), -819 (C/T) and -592 (C/A) upstream from the transcriptional start site (21). The presence of an "A" at -1082 and at –592 has been related to low IL-10 production in the cultured cells (19,22). This low producer allele has been correlated with increased incidence of rejection in the heart and kidney graft rejection (12,23). The results of our study show that IL-10 haplotype GCC/ACC decreased significantly in patients.
IFN-γ (intermediate production is more frequent in healthy individuals) microsatellite has two common alleles, 2 and 3, which exhibits significant differences in IFN-γ production *in vitro*; allele 2 is associated with greater IFN-γ production than other alleles from mitogen-stimulated peripheral blood mononuclear cells. IFN-γ Intron 1 microsatellite has shown association with a variety of autoimmune and alloimmune disease states, including lung transplant fibrosis and renal transplant rejection (24). IFN-γ has an immunomodulatory activity unlike the other interferons which are mainly antiviral. IFN-γ also influences cell mediated mechanisms of cytotoxicity and is a modulator of T cell growth and functional differentiation (24). This research shows a significant decrease in A allele at position UTR 5644 IFN-γ in patients.
In conclusion, the genetic study of cytokine gene polymorphisms is likely to provide information on disease susceptibility in different ethnic groups. As a further step, research on associations between cytokine genotypes and immunological phenomena may explain basic biological events and indicate clinical ways to predict, prevent or manage harmful situations in disease. Also, it may help increase the donor pool by haplo-identical transplants.

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