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ORIGINAL ARTICLE

Role of cytokine gene (IFN-γ, TNF-α, TGF-β1, IL-6, and IL-10) polymorphisms in pathogenesis of acute rheumatic fever in Turkish children

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Abstract Acute rheumatic fever (ARF) is a delayed immunologically mediated sequela of throat infection by group A β-hemolytic streptococci. Inflammatory cytokines may play a pathogenic role in ARF. The objective of this study was to investigate the potential associations between interferon (IFN)-γ, interleukin (IL)-6, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β1, and IL-10 gene polymorphisms and childhood ARF. Thirty-eight ARF patients and 40 age- and sex-matched healthy controls were analyzed for eight polymorphisms in five different cytokine genes [IFN-γ (+874), IL-6 (−174), TNF-α (−308), TGF-β1 (+10, +25), and IL-10 (−1082, −819, −592)]. Cytokine genotyping was performed by polymerase chain reaction sequence-specific primer methods. Patients with ARF had significantly higher frequencies of IFN-γ (+874) polymorphism in both TT genotype (p=0.0002) and T allele (p=0.0004). No statistically significant differences were observed in genotypes, haplotypes, and allele frequencies of IL-6, TNF-α, TGF-β1, and IL-10 genes between ARF and control groups (p>0.05). GG genotype frequency of TNF-α gene (low expression) was higher in patients who had previous ARF history (p=0.006). High expression of TGF-β1 (TT/GG, TC/GG) was more frequent in patients with CRP positivity (p=0.034). IL-6 CC genotype (low expression) frequency was higher in patients with tricuspid valve insufficiency (p=0.002), while IFN-γ TT genotype (high expression) frequency was higher in patients with mitral valve prolapse (p=0.049). *Conclusion:* High expression of the IFN-γ gene may carry a higher risk for ARF in Turkish children, while IL-6, TNF-α, and TGF-β1 may have an impact in mediating some clinical and laboratory manifestations of the disease.

Keywords Acute rheumatic fever · Cytokine genotyping · Cytokine expression · PCR-SSP

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Introduction

Acute rheumatic fever (ARF) is a delayed immunologically mediated autoimmune sequela of throat infection by group A β-hemolytic streptococci, and it is a multisystem inflammatory disease that can affect different tissues including synovial joints and cardiac valves [2, 4, 21]. The clinical features of the disease are carditis, arthritis, chorea, subcutaneous nodules, and erythema marginatum [4]. Since clinical and laboratory findings are specific for the diagnosis of ARF, the diagnosis can be established by the Jones criteria when a patient fulfills two major, or one major and two minor criteria plus evidence of antecedent group A streptococcal infection (with supporting evidence as well as showing the presence of streptococcal infection) [4, 11]. Carditis, which causes heart damage followed by permanent valvular

lesions, can evolve to rheumatic heart disease (RHD), which represents one of the most severe manifestations in ARF patients [3, 18]. Recently, it has been shown that inflammatory cytokines may play a pathogenic role in RHD [5, 18, 21, 31]. Cytokines, as the products of host response to inflammation, play an important role in the defense against infections [1]. Interindividual variation in levels of cytokine production may be due to single-nucleotide polymorphisms (SNPs) in the regulatory regions of cytokine genes [32]. An association has also been reported between the cytokine gene polymorphisms affecting cytokine production and secretion and infectious, allergic, autoimmune, and malignant diseases, both at the stage of disease formation as well as in the course of disease and in the patient's response to treatment [26].

The pathogenic mechanisms involved in the development of ARF are due to exaggerated immune response of the susceptible host against the specific bacterial agents [4]. Autoimmunity induced by antigenic mimicry between group A streptococcal glycoprotein and human cardiac, articular , and central nervous system proteins may be responsible for the pathogenesis of ARF in susceptible hosts with genetic predisposition [18]. In the present study, we aimed to investigate: (1) whether there were any differences between the ARF patient group and the healthy controls in terms of the cytokines [tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), interleukin-10 (IL-10), IL-6, and interferon-gamma (IFN-γ)], known to be related to autoimmunity and inflammation, and (2) the association between the identified genotypes/expressions and their clinical features in childhood ARF.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from 38 unrelated Turkish children affected by ARF, followed in the Pediatric Cardiology Clinic of Gaziantep University, School of Medicine, and 40 age- and sex-matched healthy controls (relatives of ARF patients were not included as healthy controls). The age- and sex-matched controls were selected from among those referring to the Well-Child Outpatient Clinic of Gaziantep University, School of Medicine for routine health checkups. The medical records of all children with ARF were reviewed. All of the patients fulfilled the Jones criteria for the diagnosis of ARF [4, 11]. The children were diagnosed with ARF in the Pediatric Cardiology Clinic of Gaziantep University, School of Medicine, and all of them were evaluated by a pediatric cardiologist. We confirmed that they met the Jones criteria from their medical records available in the hospital. Other data collected from these records included informations about age, gender, prior history of ARF and antecedent upper respiratory tract infection, major and minor manifestations, and evidence of streptococcal infection. Fourteen patients (36.8%) had a previous ARF history. The other children (24, 63.2%) were entered into the study when they were diagnosed with ARF. Children with carditis were followed in our clinic for ARF + RHD (36, 94.7%), while the other two patients without carditis (5.3%) were followed for ARF. Peripheral blood samples for antistreptolysin O (ASO), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) levels were obtained at the time of ARF diagnosis. For RHD patients, ASO, CRP, and ESR values at the time of diagnosis were used. Therefore, these levels reflect acute illness for all patients.

The study was approved by the local ethical committee. All parents of the children were informed about the study, and informed consents were obtained from all of them.

Cytokine genotyping

Genomic DNA was extracted from whole blood by standard salting-out method [24]. Patients and controls were analyzed for eight SNPs in five different genes. The analyzed SNPs in the present study were: TNF-α (−308, rs1800629), IFN-γ (+874, rs2430561), IL-6 (−174, rs1800795), TGF-β1 (+10, rs1982073 and +25 rs1800471), and IL-10 (−1082, rs1800896, −819, rs1800871, and −592, rs1800872). These specific SNPs were chosen because they affect the gene expressions of cytokines at different levels, as high, intermediate, or low expression. Cytokine genotyping was performed by polymerase chain reaction sequence-specific primer method using a commercially available kit according to the manufacturer's instructions (One Lambda, Inc, Canoga Park, CA).

Statistical analysis

Statistical analysis was performed using the computer software Statistical Package for the Social Sciences (SPSS) for Windows (version 18.0; SPSS Inc, Chicago, IL, USA). Results are given as mean±SD, while allele frequencies and the distribution of genotypes are given as percentage. Clinical characteristics and cytokine gene polymorphisms were compared using the chi-square test and Fisher's exact test when needed. Statistical significance was considered at $p<0.05$. The results of genotyping were statistically analyzed by calculating odds ratios (ORs) and 95% confidence intervals (CIs), using chi-square and Fisher's exact test (GraphPad Instat Version 3). Hardy–Weinberg equilibrium (HWE) was calculated using the de Finetti program [22].

Results

Clinical characteristics

The age of patients ranged between 5 and 15 years (mean 10.71±2.51 years). Fourteen patients (36.8%) had a previous ARF history. Demographic data and clinical features of the patients are shown in Table 1. In the echocardiographic evaluation, nine patients (23.7%) had mitral valve prolapse (MVP), while all of the patients (100%) had mitral valve insufficiency. Thirteen patients (34.2%) had aortic valve and 12 patients (31.6%) had tricuspid valve insufficiency.

Genotype frequencies of cytokine genes

The distribution of genotypes and allele frequencies of TNF-α, IFN-γ, and IL-6 are shown in Table 2. The frequency of a high IFN-γ (+874)-producing TT genotype was significantly higher in patients compared to the controls (p=0.0002). The frequency of IFN-γ (+874) T allele was also found significantly higher in patients (p=0.0004). No statistically significant differences were observed between groups in genotype and allele frequencies of IL-6, TNF-α, IL-10, and TGF-β1 (p>0.05, Table 2). There were also no statistically significant differences in the haplotypes of TGF-β1 (+10, +25) and IL-10 (−1082, −819, −592) between the ARF patients and controls (p>0.05, Table 3). According to the HWE, observed genotype counts did not deviate from expected genotypes (p>0.05) except for IFN-γ (+874) (p=0.047).

Table 1 Clinical characteristics of the children with acute rheumatic fever (ARF)

Demographic and clinical findings	ARF patients, *N*/total (%)
Age	10.71±2.51 years (5–15)
Sex	
Male	21/38 (55.3)
Female	17/38 (44.7)
Major manifestations	
Carditis	36/38 (94.7)
Migratory polyarthritis	25/38 (65.8)
Sydenham chorea	7/38 (18.6)
Erythema marginatum	0/38 (0.0)
Subcutaneous nodules	0/38 (0.0)
Minor manifestations	
Clinical features	
Arthralgia	16/38 (42.1)
Fever	12/38 (31.6)
Laboratory findings	
Elevated acute phase reactants	
C-reactive protein	21/38 (55.3)
Erythrocyte sedimentation rate	16/38 (42.1)
Prolonged PR interval	12/17 (70.6)
Supporting evidence of antecedent group A streptococcal infection	
Positive throat culture	12/38 (31.5)
Increased streptococcal antibody titer	28/38 (73.7)

Table 2 Genotype and allele frequencies of TNF-α (−308), IFN-γ (+874) and IL-6 (−174) polymorphisms in children with acute rheumatic fever (ARF) and controls

	Genotype/ Allele	ARF, n^a (%)	Control, n^b (%)	OR (95% CI)	p
TNF-α (−308)	GG[c]	28 (73.7)	33 (82.5)	0.593 (0.199–1.766)	0.2522[d]
	AG[e]	10 (26.3)	6 (15)	2.024 (0.654–6.26)	0.1695[d]
	AA[e]	0 (0)	1 (2.5)	0.342 (0.013–8.66)	0.5128[d]
	G allele	66 (86.8)	72 (80)	0.733 (0.273–1.970)	0.3570[d]
	A allele	10 (13.2)	8 (10)	1.364 (0.507–3.663)	0.3570[d]
IFN- γ (+874)	TT[e]	18 (47.4)	4 (10)	8.100 (2.406–27.266)	0.0002[d]
	TA[f]	12 (31.5)	21 (52.5)	0.417 (0.165–1.052)	0.0501[d]
	AA[c]	8 (21.1)	15 (37.5)	0.444 (0.162–1.219)	0.0891[d]
	T allele	48 (63.2)	29 (36.3)	3.015 (1.571–5.787)	0.0004
	A allele	28 (36.8)	51 (63.7)	0.331 (0.172–0.636)	0.0004
IL-6 (−174)	GG[e]	21 (55.3)	23 (57.5)	0.913 (0.372- 2.236)	0.4211
	GC[e]	12 (31.6)	15 (37.5)	0.769 (0.301–1.963)	0.2914
	CC[c]	5 (13.1)	2 (5)	2.879 (0.523–15.842)	0.1948[d]
	G allele	54 (71.1)	61 (76.3)	0.764 (0.374–1.563)	0.2305
	C allelle	22 (28.9)	19 (23.7)	1.308 (0.639–2.674)	0.2305

[a] n=38

[b] n=40

[c] Low expression

[d] Fisher's exact test

[e] High expression

[f] Intermediate expression

Association between the identified genotypes and clinical/ laboratory parameters of patients

In the present study, we investigated the correlations of cytokine genotypes with clinical and laboratory parameters of ARF such as carditis, polyarthritis, fever, CRP level, ESR, and increased streptococcal ASO. Frequency of GG genotype of TNF-α gene (low expression) was higher in patients who had previous ARF history (p=0.006, χ^2=7.917). Frequencies of TGF-β1 haplotypes (TT/GG, TC/GG), which are associated with high cytokine production, were higher in patients with CRP positivity (p=0.034, χ^2=6.743). Frequency of CC genotype of IL-6 (low expression) was higher in patients with tricuspid valve insufficiency (p=0.002, χ^2=12.745). Frequency of TT genotype of IFN-γ (high expression) was higher in patients with MVP (p=0.049, χ^2=6.034). There was no relationship between cytokine genotypes and other clinical and laboratory findings.

Table 3 Haplotype analysis of IL-10 (−1082, −819, −592) and TGF-β1 (T/C10, C/G 25) in children with acute rheumatic fever (ARF) and controls

	ARF, *n* (%)	Control, *n* (%)	OR (95% CI)	*p*
IL10 (−1082, −819, −592)				
GCC/GCC[a]	9 (23.7)	8 (20)	1.241 (0.422–3.645)	0.4520[b]
GCC/ACC, GCC/ATA[c]	12 (31.6)	17 (42.5)	0.624 (0.246–1.580)	0.1592
ACC/ACC, ACC/ATA, ATA/ATA[d]	17 (44.7)	15 (37.5)	1.349 (0.545–3.335)	0.2580
Total	38 (100.0)	40 (100.0)		
TGFβ1 (codon 10, codon 25)				
TT/GG, TC/GG[a]	24 (63.2)	26 (65)	0.923 (0.365–2.330)	0.4327
TC/GC, CC/GG, TT/GC[c]	13 (34.2)	12 (30)	1.213 (0.468–3.145)	0.3452
CC/GC, CC/CC, TT/CC, TC/CC[d]	1 (2.6)	2 (5)	0.513 (0.044–5.911)	0.5195[b]
Total	38 (100.0)	40 (100.0)		

[a]High expression
[b]Fisher's exact test
[c]Intermediate expression
[d]Low expression

Discussion

Although the etiology of ARF is not well known, genetic and environmental factors are considered to have a role in its development. The pathogenesis of the disease seems to result from overt immune response to specific bacterial agents in susceptible individuals triggered by group A streptococci, and innate/adaptive immune responses may be associated with the development of ARF [4, 16, 18]. The autoimmune response-causing ARF probably is triggered by molecular mimicry between streptococcal antigens and some specific human tissues, mainly heart tissue proteins, in susceptible individuals with genetic predisposition [4, 18]. Guilherme et al. identified three immunodominant regions of the streptococcal M protein as M5 (1-25), M5 (81-103), and M5 (163-177). It has been shown that these proteins preferentially recognized by both intralesional T-cell clones and peripheral T-cells, and displayed cross-reactivity with heart-derived proteins. The structural and immunological similarities between streptococcal M protein and cardiac myosin seem essential to the development of rheumatic carditis. They established the significance of molecular mimicry between beta hemolytic streptococci and heart tissue by assessing the T-cell repertoire leading to local tissue damage in RHD, while on the periphery the M5 (81-96) peptide was preferentially recognized by DR7/DR53-positive severe RHD patients. Known data in the literature support the idea that T cells sensitized in the periphery by M protein during streptococcal infection migrate to the heart and initiate heart tissue damage after activation, due to cross-reactive recognition of the relevant heart antigen [16].

After the antigen stimulation, a series of events occurs. Lymphocytes and macrophages differentiate into cytokine-producing cells, while stimulated lymphocytes can also stimulate macrophages either by direct cell contact or by producing certain cytokines [19].

Cytokines such as TNF-α, TGF-β1, IL-10, IL-6, and IFN-γ are important signals following an infection as they trigger effective immune responses in individuals [17]. Since cytokines can play an important role in triggering immunologic and inflammatory reactions in ARF [31], we thought that searching the relationship between ARF and some cytokine gene polymorphisms may be informative in understanding the pathogenesis of the disease.

Guilherme et al. identified a large number of mononuclear cells in the heart tissue (myocardium and valves) of ARF and RHD patients that were able to secrete inflammatory cytokines (TNF-α and IFN-γ) and the regulatory cytokine (IL-10). They suggested that, in RHD patients, TNF-α and IFN-γ produced by heart-infiltrating mononuclear cells can lead to local inflammation [14]. Interestingly, it has also been shown that, during the acute phase of streptoccocal infection, inflammatory acute phase proteins, such as cytokines (IL-6 and TNF-α), were produced by peripheral blood mononuclear cells [18] and tonsilar mononuclear cells [15].

Tumor necrosis factor-α is a potent immunomodulatory and proinflammatory cytokine that mediates inflammatory diseases [10]. The presence of a G or A nucleotide in position −308 of the promoter region was analyzed for TNF-α, and found that AA and AG genotypes represent the potential to produce high levels of TNF-α, whereas the GG genotype represents the potential to produce low levels [33]. In the literature, different results have been reported about the relationship between TNF-α gene polymorphism and ARF. Hernandez-Pacheco et al. reported increased frequencies of TNF-α -308 A allele and AG genotype in RHD patients [21], while Ramaswmy et al. reported that both −308 A and −238 A alleles of the TNF-α gene were associated with RF/RHD [28]. Settin et al. demonstrated that all RHD cases showed a significantly higher frequency of homozygote AA genotype of TNF-α −308 [31]. Recently, Sallakci et al. found higher TNF-α −308 A allele frequency in Turkish ARF patients [29], while Berdeli et al. from Turkey showed no association between TNF-α −308 genotype and ARF [2], consistent with our findings. However, differently from the above studies, we observed higher frequency of the GG genotype of TNF-α gene (low

expression) in patients who had previous ARF history. Guilherme et al. showed that the frequencies of autoreactive T-cells that produced significant amounts of inflammatory cytokines, such as TNF-α, were higher in ARF patients than the post-RF and chronic RHD patients (67%, 20%, and 27%, respectively) [13]. We speculate that GG genotype of the TNF-α gene may cause decreased inflammatory response and therefore the recurrence of streptococcal infection in our patients. Although the differences between current studies in literature may be partially related to ethnicity, different results in same ethnic groups underline the complex influence of TNF-α gene in ARF.

Interferon-γ has immunomodulatory, antimicrobial, antiproliferative, and antifibrotic activities that also modulate the production or activities of several cytokines and chemokines that are produced by immune cells [30]. To our knowledge, no studies have investigated the possible role of IFN-γ in patients with ARF. IFN-γ (+874) AA genotype is associated with low production, TA with intermediate production, and TT with high production of the cytokine [27]. In our study, ARF patients had significantly higher frequencies of IFN-γ (+874) polymorphism in TT genotype (high expression). Interestingly, high expression of IFN-γ +874 was associated with MVP. MVP appears to be the most common cardiac valvular disorder, and the pathogenesis of the MVP remains uncertain [20]. It has been shown that the basic architecture of extracellular matrix proteins remains unaltered in patients with rheumatic MVP, and these proteins are disrupted only at the sites of inflammatory damage, possibly secondary to the reparative process [25]. Guilherme et al. demonstrated that IFN-γ, TNF-α positive cells were consistently predominant in both myocardium and valvular tissue [15, 18]. Therefore, we suggest that IFN-γ may play a possible role in the inflammatory damage of valves in ARF. Although these findings support our hypothesis, we certainly agree that different detailed study designs are necessary to evaluate the role(s) of this cytokine in ARF.

Interleukin-6 is a proinflammatory cytokine that plays a crucial role in acute and chronic inflammation, affecting acute phase proteins and other systems such as the endocrine, central nervous, and cardiovascular systems [9]. The presence of a single-nucleotide modification in position −174 was examined for the IL-6 promoter. Both the GG and GC genotypes are associated with increased levels of IL-6, while the CC genotype leads to decreased expression of the cytokine [8]. In the present study, we found no relationship between the frequency of IL-6 −174 polymorphic (G/C) genotypes and ARF patients, similar to the study of Settin et al. [31]. However, frequency of the CC genotype of IL-6 (low expression) was higher in our patients with tricuspid valve insufficiency, suggesting that IL-6 may have a role in the valvular damage in ARF.

As a regulatory cytokine, IL-10 has an anti-inflammatory activity. It has emerged as a key immunoregulator during infection, and is mainly produced by macrophages, monocytes, lymphocytes, and heart tissue infiltrating cells from the myocardium and valvular tissue [7]. IL-10 antagonizes the proinflammatory cytokine response by inhibiting production of IFN-γ and TNF-α [15]. Settin et al. reported that the homozygous GG genotype of IL-10 −1082 possibly contributes to RHD susceptibility, and the homozygous AA genotype of IL-10 −1082 showed a possible susceptibility for multivalvular affection and form of disease severity among cases of RHD [31]. In another study, no association was found between the IL-10 C627A polymorphism and RHD and severity of disease [6].We found no relationship between IL-10 (−1082, −819, −592) gene polymorphism and the clinical and laboratory findings of ARF. This diversity between studies may be partially explained by racial and ethnic differences in various populations.

Transforming growth factor-β1 is an anti-inflammatory cytokine, and increased TGF-β1 production inhibits autoimmune and chronic inflammatory disease [23]. Sallakci et al. did not find any difference in the distribution of TGF-β1 SNP (G915C, codon 25) between ARF patients and controls [29]. However, Chou et al. reported a significant difference in the distribution of genotypes between RHD patients and control groups for TGF-β1 C509T and T869C polymorphisms [5]. In the present study, high expression of TGF-β1 was more frequent in patients with CRP positivity. It has been shown that levels of high-sensitivity CRP were significantly higher in RHD patients [12]. Considering this correlation between TGF-β1 and increased CRP positivity in our patients, we suggest that TGF-β1 may have a role in the progression and/or severity of RHD.

One of the major limitations of our study comes from the relatively small sample size. Therefore, the statistical power was limited. The weakness of the study is to evaluate single SNPs for TNF-α, IFN-γ, and IL-6 genes. A multi-SNP analysis would be more appropriate to link the genotype to a phenotype in children with ARF.

Conclusion

High expression of the IFN-γ gene may carry a higher risk for ARF in Turkish children, while IL-6, TNF-α, and TGF-β1 may have an impact in mediating some clinical and laboratory manifestations of the disease. However, further detailed study designs are necessary to evaluate the role(s) of these cytokines in ARF.

Conflict of interest The authors do not have any financial relationship with the organization that sponsored the research.

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