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CLINICAL STUDY

Genetic susceptibility to Graves' ophthalmopathy: the role of polymorphisms in proinflammatory cytokine genes

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Abstract

Purpose **In order to investigate the underlying genetic mechanisms of Graves' ophthalmopathy (GO), we examined the association between single-nucleotide polymorphisms in five important proinflammatory cytokines, namely IL-12, TNF-α, IFN-γ, IL-2, and IL-6, with GO in a sample of Iranian adults.**

Methods **A total of 57 patients with Graves' disease without GO, 50 patients with GO, and 140 healthy controls were enrolled. Patients were recruited consecutively from the outpatient endocrine clinic of a large university general hospital. Genotype and allele frequencies of the following proinflammatory cytokines were compared between the groups: IL-12 (−1188A/C), TNF-α (−308A/G, −238A/G), INF-γ (UTR 5644A/T), IL-2 (-330T/G, 166G/T), and IL-6 (−174C/G, nt565A/G). A corrected (for multiple testing) *P*-value (P_c) less than 0.05 was considered statistically significant.**

Results **The IL-12 −1188C allele (odds ratio (OR) = 2.65, $P_c < 0.01$) and CC genotype (OR = 7.58, $P_c < 0.01$) were significantly more common in patients with GO than in patients without GO. The TNF-α −238A allele was more frequent in patients with GO than in patients without GO (OR = 2.99, $P_c < 0.05$). The frequency of the IFN-γ UTR 5644T allele (OR = 2.67, $P_c < 0.05$), AT genotype (OR = 13.33, $P_c < 0.05$), and TT genotype (OR = 18.46, $P_c < 0.01$) was significantly higher among patients with GO than patients without GO. No significant association was found for other polymorphisms.**

Conclusions **We demonstrated that specific polymorphisms in IL-12, IFN- γ, and TNF-α genes are associated with susceptibility to GO in the Iranian population. Our results open a new perspective to genetic correlates of GO.**
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Keywords: Grave's disease; ophthalmopathy; interleukin; gene; polymorphism

Introduction

Graves' ophthalmopathy (GO) is an autoimmune inflammatory process in the orbit, closely associated with Graves' disease (GD).[1] As the major extrathyroidal manifestation of GD, GO is associated with substantial morbidity. GO presents by proptosis, orbital inflammation, and oedema.[2] Recent studies point to both genetic and environmental factors in GO pathogenesis.[3] Although the production of antibodies against the thyroid-stimulating hormone receptor is the best known underlying cause of GD, the precise aetiology of GO remains largely unclear.[3,4]

Extraocular muscles, surrounding connective tissues and the retrobulbar fat, are targets of the inflammatory process during GO development,[5,6] with the orbital fibroblasts being the major effector cells. Activation of these cells is mediated by certain cytokines, most importantly those belonging to the proinflammatory family.[7–9] Production of cytokines is under genetic control, and certain single-nucleotide polymorphisms in cytokine

genes may be associated with higher or lower cytokine production rates in immunologic processes. Hence, such polymorphisms might be predictive of susceptibility to certain diseases or clinical outcomes.[10]

As proinflammatory cytokines are known to have important roles in the development of GO,[9,11,12] we investigated the hypothesis that specific polymorphisms in proinflammatory cytokine genes might contribute to the development of GO. Previous reports on the topic are limited,[13–19] and further studies are necessary to elucidate the issue.[20] In this study, the association of GO with the following polymorphisms in proinflammatory cytokines were evaluated in Iranian GD patients with and without GO: IL-12 (−1188A/C), TNF-α (−308A/G, −238A/G), INF-γ (UTR 5644A/T), IL-2 (−330T/G, 166G/T), and IL-6 (−174C/G, nt565A/G).

Materials and methods

Participants

This case–control hospital-based study was conducted between February 2005 and September 2008, and comprised a total of 50 unrelated patients (16 males) diagnosed with GO, 57 patients (17 males) without GO, and 140 healthy controls. Patients were enrolled from the outpatient endocrine clinic of a large university general hospital, and controls were selected from the healthy staff with no clinical evidence or family history of any type of autoimmune disorders. All participants were Iranian. The local ethics committee of our university approved the study protocol, and written informed consent was obtained from all participants. All applicable institutional and governmental regulations regarding the ethical use of human volunteers were followed during this research.

Diagnosis of GD and GO

Graves' disease and GO were diagnosed by an endocrinologist (AE) with substantial experience in thyroid diseases. Diagnosis was based on suggestive history, compatible physical examination, and confirmatory laboratory tests including sensitive TSH, free T4, total T3, and anti-thyroglobulin antibody. To exclude thyrotoxicosis not caused by hyperthyroidism, 24-h radioactive iodine uptake was performed. All patients had diffuse uptake in their iodine uptake scan. GO was defined as class 3 or higher in the American Thyroid Association mnemonic NOSPECS scheme.[21] Patients without GO were selected from those who did not have any features of ophthalmopathy (including features of class 1–2).

DNA analysis

Cytokine typing was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) assay (PCR-SSP kit, Heidelberg University, Heidelberg, Germany). Briefly, amplification was carried out using a thermal cycler Techne Flexigene apparatus (Rosche, Cambridge, UK). The presence or absence of PCR products was visualised by using 2% agarose gel electrophoresis. The gel was placed on a UV transilluminator after electrophoresis 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, whereas the primer pairs amplifying the CRP gene produce a 440-bp amplicon. The allele and genotype frequencies of the following cytokine genes were determined: TNF-α (−308A/G, −238A/G), IL-2 (−330T/G, 166G/T), IL-12 (−1188A/C), INF-γ (UTR 5644A/T), IL-6 (−174C/G), and (nt565A/G).

Statistical analysis

The statistical package SPSS 16 (Chicago, IL, USA) was used for analysis. Continuous variables are expressed as mean ± SEM. Prevalence of genotypes and alleles were determined in each group and the corresponding odds ratios (ORs) with 95% confidence intervals (95% CI) were calculated. Two-sided *P*-values were determined by χ^2 or Fisher's exact tests as appropriate, and were then corrected for multiple testing using the Bonferroni method. All *P*-values are reported as corrected values (P_c) and a P_c-value smaller than 0.05 was considered statistically significant.

Results

Eight polymorphisms in five genes were analysed. Allele and genotype distributions were in Hardy–Weinberg equilibrium. There were no significant differences between patients with and without GO with respect to family history of GD (32.0 *vs* 35.1%, respectively), age of onset of disease (28.50 ± 0.79 *vs* 29.57 ± 0.97 years, respectively), and duration of GD (3.94 ± 0.48 *vs* 4.28 ± 0.59 years, respectively). The results of allele and genotype analysis are presented in Tables 1 and 2. The IL-12 –1188C allele (OR = 2.65, $P_c < 0.01$) and CC genotype (OR = 7.58, $P_c < 0.01$) were significantly more common in patients with GO than patients without GO. The TNF-α −238A allele was more common in patients with GO than those without GO (OR = 2.99, $P_c < 0.05$). The frequency of the IFN-γ UTR 5644T allele (OR = 2.67, $P_c < 0.05$), AT genotype (OR = 13.33, $P_c < 0.05$), and TT genotype (OR = 18.46, $P_c < 0.01$ respectively) was

Table 1 Association between eight polymorphisms and Graves' ophthalmopathy (GO) in 247 subjects

Cytokine	*Position*	*Alleles*	*Ctrl* (n = *140*) N (%)	*Without GO* (n = *57*) N (%)	*With GO* (n = *50*) N (%)	*OR (95% CI)*[a]	*OR (95% CI)*[b]
IL-12	−1188	A	204 (72.9)	77 (67.5)	44 (44.0)	1[c]	1
		C	76 (27.1)	37 (32.5)	56 (56.0)	1.29 (0.80–2.07)	2.65 (1.52–4.62)**
TNF-α	−308	G	235 (85.8)	90 (80.4)	66 (67.3)	1	1
		A	39 (14.2)	22 (19.6)	32 (32.7)	1.47 (0.83–2.62)	1.54 (0.82–2.86)
TNF-α	−238	G	215 (78.5)	102 (89.5)	74 (74.0)	1	1
		A	59 (21.5)	12 (10.5)	26 (26.0)	0.43 (0.22–0.83)	2.99 (1.42–6.30)*
IFN-γ	UTR 5644	A	140 (50.7)	49 (43.0)	22 (22.0)	1	1
		T	136 (49.3)	65 (57.0)	78 (78.0)	1.37 (0.88–2.12)	2.67 (1.47–4.88)*
IL-2	−330	T	168 (60.4)	59 (51.8)	37 (37.0)	1	1
		G	110 (39.6)	55 (48.2)	63 (63.0)	1.42 (0.92–2.21)	1.83 (1.06–3.16)
IL-2	166	G	219 (78.8)	88 (77.2)	67 (67.0)	1	1
		T	59 (21.2)	26 (22.8)	33 (33.0)	1.10 (0.65–1.85)	1.67 (0.91–3.05)
IL-6	−174	G	177 (63.7)	60 (52.6)	37 (37.0)	1	1
		C	101 (36.3)	54 (47.4)	63 (63.0)	1.58 (1.01–2.45)	1.89 (1.09–3.27)
IL-6	nt 565	G	228 (82.0)	85 (74.6)	77 (77.0)	1	1
		A	50 (18.0)	29 (25.4)	23 (23.0)	1.56 (0.92–2.62)	0.88 (0.47–1.64)

*$P_c < 0.05$, **$P_c < 0.01$.
[a]When comparing patients without GO and normal controls.
[b]When comparing patients with and without GO.
[c]The first allele is considered as the reference allele.

significantly higher among patients with GO than those without GO. No significant association was found for other polymorphisms.

Haplotypic analysis (Table 3) showed significantly higher frequency of the TNF-α AA haplotype in patients with GO than those without GO (OR = 38.61, $P_c < 0.01$). We found no significant associations for other haplotypes.

Discussion

The hypothesis that there might be immunogenetic differences between patients with GO and those without GO was first tested in 1999 by Vaidya *et al*,[22] who showed that cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene polymorphism confers susceptibility to GO. We recently showed that certain CTLA-4 gene polymorphisms are associated with GO in our country;[23] however, this association does not seem to exist in all populations.[20] We have also shown significant associations between GO and IL-1α and IL-1RA gene polymorphisms in Iranian patients.[24] This study revealed significant associations between GO and certain polymorphisms in INF-γ, TNF-α, and IL-12 genes.

Orbital involvement during the course of GD seems to be the ultimate outcome of the effects of cytokines released by inflammatory cells.[9,11] The potential role of proinflammatory cytokines in this process is of high importance as they are among the most likely initiators of thyroid-related autoimmune reactions in preorbital tissues.[4] These cytokines stimulate the expression of immunologically relevant molecules such as adhesion molecules and heat-shock proteins in orbital fibroblasts.[25,26] They also promote the proliferation of orbital fibroblasts and modulate glycoseaminoglycan synthesis.[7,27] These effects may lead to the increase in the orbital tissue volume and impairment of extraocular muscle function, thus contributing to the mechanical complications of GO.[7] IL-23R gene polymorphisms were recently correlated in a Caucasian population with GO with the suggested mechanism that these polymorphisms change the expression and/or function of IL-23 receptor and promote a proinflammatory signalling cascade.[19] However, this result did not hold in another study in a Japanese population.[28]

Experimental studies have shown the important role of IFN-γ and TNF-α in the development of GO.[27,29] These cytokines can induce or enhance the expression of HLA-DR molecules in orbital fibroblasts. This effect is more prominent on cultured retroocular fibroblasts derived from patients with GO than on those derived from normal individuals.[30] It has also been demonstrated that TNF-α and IFN-γ enhance surface expression of intercellular adhesive molecule-1 (ICAM-1) in orbital fibroblast of patient with GO.[25] These adhesion molecules are known to activate T cells and facilitate antigen recognition, amplifying the cellular immune process.

Several studies have reported a role for TNF-α gene polymorphisms in the development of GD.[14,15,18,31–33] However, the association between TNF-α polymorphisms and GO has remained controversial. The TNF-α -238A allele is associated with GO in Polish patients,[14] and polymorphisms at position −1031T/C and −863C/A are reported to have a role in Japanese patients.[18] Our data

Table 2 Association between eight polymorphisms and Graves' ophthalmopathy (GO) in 247 subjects

Cytokine	*Position*	*Genotype*	*Ctrl* (n = *140*) N *(%)*	*Without GO* (n = *57*) N *(%)*	*With GO* (n = *50*) N *(%)*	*OR (95% CI)*[a]	*OR (95% CI)*[b]
IL-12	−1188	AA	72 (51.4)	26 (45.6)	8 (16.0)	1[c]	1
		AC	60 (42.9)	25 (43.9)	28 (56.0)	1.15 (0.60–2.20)	3.64 (1.40–9.50)
		CC	8 (5.7)	6 (10.5)	14 (28.0)	2.08 (0.66–6.56)	7.58 (2.19–26.27)**
TNF-α	−308	GG	98 (71.5)	35 (62.5)	21 (42.9)	1	1
		AG	39 (28.5)	20 (35.7)	24 (49.0)	1.44 (0.74–2.79)	1.33 (0.60–2.95)
		AA	0 (0)	1 (1.8)	4 (8.1)	8.32 (0.33–209.23)	5.33 (0.56–50.85)
TNF-α	−238	GG	79 (57.7)	45 (78.9)	27 (54.0)	0.37 (0.18–0.76)	1
		AG	57 (41.6)	12 (21.1)	20 (40.0)	0.58 (0.02–14.61)	2.78 (1.18–6.57)
		AA	1 (0.7)	0 (0.0)	3 (6.0)	0.37 (0.18–0.76)	11.58 (0.58–232.98)
IFN-γ	UTR 5644	AA	43 (31.2)	14 (24.6)	1 (2.0)	1	1
		AT	54 (39.1)	21 (36.8)	20 (40.0)	1.19 (0.54–2.62)	13.33 (1.60–111.05)*
		TT	41 (29.7)	22 (38.6)	29 (58.0)	1.65 (0.74–3.65)	18.46 (2.25–151.25)**
IL-2	−330	TT	37 (26.6)	12 (21.1)	5 (10.0)	1	1
		TG	94 (67.6)	35 (61.4)	27 (54.0)	1.15 (0.54–2.45)	1.85 (0.58–5.90)
		GG	8 (5.8)	10 (17.5)	18 (36.0)	3.85 (1.24–12.00)	4.32 (1.18–15.83)
IL-2	166	GG	82 (59)	33 (57.9)	20 (40.0)	1	1
		TG	55 (39.6)	22 (38.6)	27 (54.0)	0.99 (0.52–1.88)	2.02 (0.92–4.47)
		TT	2 (1.4)	2 (3.5)	3 (6.0)	2.49 (0.34–18.39)	2.47 (0.38–16.12)
IL-6	−174	GG	42 (30.2)	13 (22.8)	4 (8.0)	1	1
		CG	93 (66.9)	34 (59.6)	29 (58.0)	1.18 (0.57–2.47)	2.77 (0.81–9.44)
		CC	4 (2.9)	10 (17.5)	17 (34.0)	8.08 (2.17–30.12)*	5.53 (1.41–21.66)
IL-6	nt 565	GG	93 (66.9)	33 (57.9)	28 (56.0)	1	1
		AG	42 (30.2)	19 (33.3)	21 (42.0)	1.28 (0.65–2.50)	1.30 (0.59–2.90)
		AA	4 (2.9)	5 (8.8)	1 (2.0)	3.52 (0.89–13.92)	0.24 (0.03–2.14)

*$P_c < 0.05$, **$P_c < 0.01$.
[a]When comparing patients without GO and healthy controls.
[b]When comparing patients with and without GO.
[c]The first allele is considered as the reference allele.

showed a significant association for the −238A allele. Considering the higher serum levels of TNF-α in the hyperthyroid phase of GD, and its overexpression in the orbital connective tissues of patients with GO,[3] our results are in agreement with a previous observation that the −238A allele was associated with higher TNF-α production.[34] We did not find a significant association between the −308G/A polymorphism and GO. The TNF-α −308A allele has been suggested to be a player in the pathogenesis of GD in Polish and Tunisian patients.[14,15] However, no independent association of this allele with GD was observed when the groups were adjusted for their DRB1*03 status.[14]

IFN-γ has critical roles in modulating the cytokine network pathway and in augmenting TNF-α activity.[35] Given the importance of TNF-α in GO pathogenesis,[12] IFN-γ gene polymorphisms may potentially have a role as well. The significantly increased frequencies of the UTR 5644T allele and AT and TT genotypes in our GO patients are in support of the hypothesis. This polymorphism is involved in pathogenesis of Behcet's disease[36] and lichen planus,[37] but to our knowledge this study is the first to suggest the involvement of this polymorphism in GO.

Previous reports have indicated that IL-12 is overproduced during the active phase of GD and GO.[38,39] The presence of IL-12 is necessary for induction of T-helper1 response. The IL-12 −1188A/C polymorphism has been examined in immune-mediated diseases,[40,41] and may alter the susceptibility to a number of autoimmune diseases such as multiple sclerosis and type I diabetes mellitus.[42,43] A previous study in Japan failed to show this association with GD or GO.[13] In contrast, we revealed in an Iranian population a significant increase in the frequency of the 1188C allele and CC genotype among patients with GO. Ethnic differences might explain a part of this inter-population inconsistency.

Although the presence of significantly higher levels of IL-2 are reported in the patients with GO,[39] the gene polymorphisms of this cytokine have not been investigated in GO. We did not find a significant association between GO and IL-2 –330T/G or 166G/T polymorphisms. The lack of an association between the −330T/G polymorphism and rheumatoid arthritis and Behcet's disease has been reported before.[36,44] Our patients with GO were not significantly different from those without GO in allele and genotype frequencies of IL-6 at positions −174 and nt565. In line

Table 3 The results of haplotypic analysis

Cytokine	*Haplotypes*	*Control* N *(%)*	*GO(−)* N *(%)*	*GO(+)* N *(%)*	*Control vs GO(−) OR (95% CI)*	*GO(+) vs GO(−) OR (95% CI)*
TNF-α	GG	176 (64.2)	78 (69.7)	56 (57.1)	1.28 (0.80–2.05)	0.58 (0.33–1.03)
	AG	39 (14.2)	22 (19.6)	18 (18.4)	1.47 (0.83–2.62)	0.92 (0.46–1.84)
	GA	59 (21.5)	12 (10.7)	10 (10.2)	0.44 (0.23–0.85)	0.95 (0.39–2.30)
	AA	0 (0)	0 (0.0)	14 (14.3)	—	38.61 (2.27–656.86)[a]
IL-2	TG	112 (40.6)	37 (32.5)	17 (17.0)	0.70 (0.44–1.12)	0.43 (0.22–0.82)
	GG	107 (38.7)	51 (44.7)	50 (50.0)	1.28 (0.82–1.99)	1.24 (0.72–2.12)
	GT	1 (0.4)	4 (3.5)	13 (13.0)	10.00 (1.11–90.52)	4.11 (1.29–13.05)
	TT	56 (20.3)	22 (19.3)	20 (20.0)	0.94 (0.54–1.63)	1.05 (0.53–2.06)
IL-6	GG	173 (62.2)	58 (50.9)	37 (37.0)	0.63 (0.40–0.98)	0.57 (0.33–0.98)
	CG	55 (19.8)	27 (23.7)	40 (40.0)	1.25 (0.74–2.10)	2.15 (1.19–3.87)
	GA	4 (1.4)	2 (1.7)	0 (0.0)	1.22 (0.22–6.78)	0.22 (0.01–4.72)
	CA	46 (16.6)	27 (23.7)	23 (23.0)	1.57 (0.92–2.67)	0.96 (0.51–1.82)

[a]$P_c < 0.01$.

with previous studies, polymorphisms in this cytokine do not seem to have an important role in GO.[16]

In conclusion, we explored the relationship between GO and polymorphisms of various proinflammatory cytokine genes. It was shown that the IL-12 –1188C allele, TNF-α 238A allele, and IFN-γ UTR 5644A allele are associated with increased susceptibility to GO in Iranian patients with GD. These associations need to be further evaluated in other populations. Our results open a new perspective to genetic correlates of GO. Some of our negative results might have been affected by our small sample size and the resulting low statistical power. This potential limitation in our study needs to be addressed in studies with larger samples.

Summary

What was known before

- Graves' ophthalmopathy (GO) is the major extrathyroidal manifestation of Graves' Disease. The underlying genetic mechanisms of GO is not known completely.

What this study adds

- This study demonstrated that specific polymorphisms in IL-12, IFN-γ, and TNF-α genes are associated with susceptibility to GO.

Conflict of interest

The authors declare no conflict of interest.

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