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Association of TNF-alpha, TNF-beta, IFN-gamma and IL-1Ra Gene Polymorphisms with Graves' Disease in the Thai Population

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SUMMARY Cytokines play a key role in the regulation of immune and inflammatory responses. Therefore, cytokine genes are potentially related to susceptibility to Graves' disease (GD). The aim of this study was to investigate the putative functional polymorphisms within tumor necrosis factor–alpha (TNF-α), tumor necrosis factor-beta (TNF-β), interferon-gamma (IFN-γ), and interleukin-1 receptor antagonist (IL-1Ra) genes, in patients with GD (n = 137) compared to a healthy Thai control group (n = 137). The results showed no statistically significant difference between the study groups for TNF-β (*Nco*I site in intron 1), IFN-γ (+874 in intron 1), and IL-1Ra (variable numbers of tandem repeats in intron 2) gene polymorphisms. Only the –863A allele within the promoter region of the TNF-α gene, which may affect the affinity of the promoter nuclear factor (NF)-κb interaction, was found to be increased in GD patients compared to the controls (p = 0.009, OR =1.8, 95% CI =1.15 to 2.84). The effect of the –863A allele of the TNF-α gene was similar to the autosomal dominance mode of inheritance (p = 0.01, OR = 2, 95% CI = 1.16 to 3.44). This polymorphism may be involved in the susceptibility to GD in part through its higher promoter activity of TNF-α production.

Graves' disease (GD) is an organ-specific autoimmune disease of the thyroid gland characterized by hyperthyroidism, diffuse goitre, ophthalmopathy and rarely dermopathy.[1,2] The disease is mediated by autoantibodies that bind to the thyrotropin (TSH) receptor and stimulate thyroid hormone production. GD is a multifactorial disease that develops as a result of a complex interaction between genetic susceptibility genes and environmental factors.[3] Evidence for the role of the genes is shown by the incidence of the disease within families and twins. Concordance rates of GD in monozygotic twins, at around 30-40%, are much higher than in dizygotic twins which supports a genetic contribution to the disease.[4] The lack of a clear pattern of inheritance suggests that multiple genes are involved in influencing the autoimmune events in GD.[5] During the last decade, much effort has been put into the characterization of the genetic background of GD. Several candidate genes have been examined. These included the human leukocyte antigen (HLA) genes, cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene, vitamin D

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receptor, Ig heavy chain allotype, T cell receptor β-chain and TSH receptor.[6] However, each of these candidate genes is likely to contribute no more than 5% to the overall genetic susceptibility.[7] The understanding of the mechanisms underlying the development of GD is crucial to the prevention and control of this disease in the future. Much interest has been focused on cytokine genes because cytokines participate in the induction and effector phases of the immune and inflammatory response and are therefore likely to play a critical role in the development of autoimmune thyroid disease.[8-12] However, these associations are still controversial and await confirmation by other studies.

The role of cytokines in the pathogenesis of GD has been extensively investigated. In patients with GD, these molecules can be found in both the thyroid and in extrathyroidal sites with complications of the disease.[13] Cytokines in the thyroid gland also have a role in regulating antigen presentation and lymphocyte trafficking by enhancing the expression of HLA class II and adhesion molecules on thyroid follicular cells.[14] The production of cytokines varies among individuals and correlates with the polymorphism in the cytokine gene. This study was designed to investigate the polymorphisms of various cytokine genes in patients with GD compared to a Thai control group by selecting markers that have been suggested to affect the expression level of each cytokine.

MATERIALS AND METHODS

Subjects

One hundred and thirty-seven Thai patients with GD (female:male ratio = 121:16; mean age ± SD = 38.6 ± 12.6 years) were recruited for the study after obtaining their informed consent. One hundred and thirty-seven healthy controls (female:male ratio = 100:37; mean age ± SD = 23 ± 12.3 years) were recruited from Thai volunteers from the same geographic area. The healthy individuals and patients were unrelated. Patients were followed up in an outpatient clinic at the Department of Endocrinology, Faculty of Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The diagnosis of Graves' disease was based on clinical features, diffuse enlargement of thyroid gland and the elevation of free thyroxine or triiodothyronine levels for more than 3 months with either positive thyroid autoantibodies (thyroid binding inhibitory immunoglobulins or thyroid peroxidase), thyroid eye disease or a diffuse increase in uptake on radionuclide scanning. The study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University.

Genotyping methodology

DNA was isolated from buffy coat collected with ethylenediaminetetraacetic acid (EDTA) as anticoagulant, using a salting out method.[15] The variable numbers of tandem repeats (VNTR) within the interleukin-1 receptor antagonist (IL-1Ra) gene were amplified with specific primers described by Arnalich and colleagues.[16] Polymorphism at –863 in the promoter region of the tumor necrosis factor–alpha (TNF-α) gene,[17] and the *Nco*I polymorphism in intron 1 of the tumor necrosis factor-beta (TNF-β) gene were identified using the polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method.[18] Polymorphisms at +874 within intron 1 of the interferon-gamma (IFN-γ) gene were identified using the PCR-sequence specific primer (SSP) method described by Pravica and colleagues.[19] Additionally, selected PCR products were analyzed by DNA sequencing to confirm the PCR-SSP and PCR-RFLP results.

Statistical analysis

Allele and genotype frequencies were determined by direct counting and then divided by the number of chromosomes to produce an allele frequency, or by the number of subjects to produce the genotype frequency. The goodness of fit to the Hardy-Weinberg equilibrium, calculating the expected frequencies of each genotype and comparing them with the observed values, was performed using a chi-square test. Allele and genotype frequencies were compared between groups using the Chi-square (χ^2) test or Fisher's exact probability test, where appropriate. A p value of < 0.05 was considered significant. Odds ratios (OR) with a 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6 (http://www.CDC.gov/epiinfo/Epi6/ei6.htm).

RESULTS

Genotype and allele frequencies for the TNF-α (-863), TNF-β (*Nco*I polymorphism in intron 1), IFN-γ (+874) and IL-1Ra (VNTR in intron 2) gene in healthy controls and GD patients were shown in Table 1. All control genotype frequencies were in Hardy-Weinberg equilibrium. The –863A allele of TNF-α gene was found to be significantly increased in GD patients as compared with healthy controls (GD = 24% and control = 15%; $p = 0.009$, OR = 1.8, 95% CI = 1.15 to 2.84). The effect of –863A allele of TNF-α gene was compatible with the autosomal dominance mode of inheritance (Table 2). The presence of one A allele (AA or AC) conferred the significant OR of 2 ($p = 0.01$, 95% CI = 1.16 to 3.44). There was no statistically significant difference between the study groups for the other cytokine gene polymorphisms.

DISCUSSION

This study was designed to analyze the potential association between cytokine gene polymorphisms and GD in Thai population. Out of 4 cytokine genes (TNF-α, TNF-β, IFN-γ, and IL-1Ra) analyzed in this study, only the –863 polymorphism in the TNF-α gene promoter was proved to be a marker for susceptibility to GD in Thai population. This polymorphism has been associated with predispose to the development of ophthalmopathy in Japanese patients with GD.[20] Previous studies also reported an association of susceptibility to GD with other polymorphic markers within TNF-α gene in Tunisia (-308A), Polish (–308A) and UK Caucasians (-238A).[9,21,22] Several polymorphisms in the promoter region of the TNF-α gene have been proposed to be important for TNF-α gene expression and protein production. However, the results are still controversial and vary from one study to the other. Functional studies of the TNF polymorphism at position -863 (C/A) has been extensively investigated. The transcriptional activity by luciferase assay of the –863A polymorphism in response to ConA was two times greater than that of the –863C allele. Moreover, TNF-α production by peripheral blood mononuclear cells in response to ConA from donors possessing these –863A allele were about 1.8 times greater than the levels from donors with the –863C allele.[23] In 2000, Udalova and co-workers demonstrated a clear effect of this nucleotide change on the relative binding affinities of different forms of the NF-κB complex. It was shown that the p50-p50 homodimeric form of this complex had significantly decreased affinity to its DNA binding site for -863A. As the p50-p50 homodimer acts as a transcriptional repressor on

Table 1 Genotype and allele frequencies for polymorphic markers within TNF-α, TNF-β, IFN-γ and IL-1Ra gene in healthy controls and GD patients

Gene (position)	**Genotype**	**Healthy controls** n = 137 (%)	**GD patients** n = 137 (%)	**Allele**	**Healthy controls** n = 274 (%)	**GD patients** n = 274 (%)
TNF- α (-863)	A/A	5 (3.7)	9 (6.6)	A	41 (15)	66 (24)[a]
	A/C	31 (22.6)	48 (35)	C	233 (85)	208 (76)
	C/C	101 (73.7)	80 (58.4)			
TNF-β (intron 1)	1/1 (G/G)	27 (19.7)	29 (21.2)	1 (G)	123 (44.9)	131 (47.8)
	1/2 (G/A)	69 (50.4)	73 (53.3)	2 (A)	151 (55.1)	143 (52.2)
	2/2 (A/A)	41 (29.9)	35 (25.5)			
IFN-γ (+874)	A/A	83 (60.6)	72 (52.5)	A	212 (77.4)	200 (73)
	A/T	46 (33.6)	56 (40.9)	T	62 (22.6)	74 (27)
	T/T	8 (5.8)	9 (6.6)			
IL-1Ra (intron 2)	1/1	105 (76.6)	108 (78.8)	1	240 (87.6)	241 (88)
	1/2	29 (21.2)	25 (18.3)	2	33 (12)	31 (11.3)
	1/4	1 (0.7)	1 (0.7)			
	2/2	2 (1.5)	3 (2.2)			

[a] $p = 0.009$, OR = 1.8, 95% CI = 1.15 to 2.84

binding to its regulatory site in the promoter region of the TNF gene. Decreased binding is thought to result in an inadequate down-regulation of TNF gene expression, and thus increased TNF-α production.[24] These results suggest that the –863A allele may be involved in susceptibility to GD in part through their higher promoter activity of TNF-α production. Indeed, the presence of TNF-α has been reported in thyroid tissues from patients with GD using reverse transcriptase PCR technology and immunohistochemistry.[25-27] TNF-α is also secreted by thyroid epithelial cells, fibroblasts and lymphocytes within the thyroid.[14, 26] TNF-α can induce the expression of adhesion molecules, regulatory molecules and HLA class II molecules on thyroid epithelial cells, allowing these cells to present antigens to activated T cells.[28] This presentation of antigen might exacerbate the autoimmune processes involved in the pathogenesis of GD or other autoimmune thyroid diseases.[29] However, further studies are necessary to clarify the exact mechanism of how this TNF-α gene polymorphism results in increased susceptibility to GD.

Although the above explanation supports a pathophysiological mechanism for the association of this TNF-α variant with GD, it is also possible that this association is not due to the TNF-α gene, but to another gene in linkage disequilibrium. The human TNF-α gene is located right within the class III region of the major histocompatibility complex (MHC) on the short arm of chromosome 6.[20] Other genes crucial for the immune response and immunoregulatory including MHC, complement components (C2, C4, factor B), lymphotoxin (LTα and β), heat shock proteins (HSP), transporters associated with antigen processing (TAP) are all in close proximity.

The other cytokine gene polymorphisms evaluated in this study did not reveal any significant associations with GD, demonstrating that the VNTR in intron 2 of IL-1Ra gene, SNP at +874 within intron 1 of the IFN-γ gene and *Nco*I restriction site polymorphisms in intron 1 of the TNF-β gene are not a marker for genetic susceptibility of GD in Thai population. However, since only one position within the gene was analyzed in this study, other polymorphic markers in these cytokine genes might play a role in the genetic susceptibility of GD in Thai population. Thus, further study by scanning more polymorphic markers or haplotype analysis is required.

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Table 2 Risk of GD associated with TNF-α (-863C/A) genotype according to different models of inheritance

	Healthy controls n = 137	GD patients n = 137
A dominance, C wild type		
C/C	101 (73.7%)	80 (58.4%)
A/A or A/C	36 (26.3%)	57 (41.6%)[a]
A recessive, C wild type		
C/C or A/C	132 (96.4%)	128 (93.4%)
A/A	5 (3.6%)	9 (6.6%)[b]

[a] $p = 0.01$, OR = 2, 95% CI = 1.16 to 3.44
[b] $p = 0.41$

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