No Association between an Interleukin 4 Gene Promoter (-589) Polymorphism and Graves' Disease in Thai Patients

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Cytokines play a key role in the regulation of immune and inflammatory responses and therefore are potential candidate genes for autoimmune thyroid disease. Polymorphisms in cytokine genes may effect gene transcription, causing individual variations in cytokine production. Several investigators have linked the interleukin-4 (IL-4) gene and autoimmune disease. The present population-based study was to investigate the polymorphisms of IL-4 gene promoter (-589C/T) in GD patients compared with a control group and determine the association with GD in a Thai population. The subjects included 137 GD patients and 137 healthy control subjects with similar ethnic and geographic backgrounds. The IL-4 gene polymorphism at position -589 in the promoter was analyzed using the PCR-RFLP. The protective effect of the -589T allele as suggested by Hunt et al in a Caucasian population was not observed in the present study. The -589T allele frequencies were similar between patients and control subjects (69% vs 69.3%) suggesting that this polymorphism can not be used as a genetic marker for GD susceptibility in Thais.

Keywords: *Interleukin 4, Graves' disease*

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Graves' disease (GD) is an organ-specific autoimmune disease of the thyroid gland characterized by hyperthyroidism, diffuse goitre and ophthalmopathy. The disease is mediated by autoantibodies that bind to the thyroid-stimulating hormone (TSH) receptor and stimulate thyroid hormone production. These stimulatory antibodies belong predominantly to the IgG class and act as TSH agonists [1]. This disease is influenced by both environments and genetic factors. Evidence supporting the genetic effect comes from family and twin studies [2-5]. Several potential susceptibility loci have recently been identified by linkage analysis both in sib-pair and multiplex family. Important genetic susceptibility loci with high lod score have been identified on chromosomes 5q31[6], Xp11 [7], 14q31 [8], 20q11-2 [9,10], 18q21 [11], 2p21 [12], 2q33 [13]. However, the major susceptibility genes contributing to the development of GD haven't been revealed yet. Candidate gene study is another approach that is very effective in detecting susceptibility genes as well as genes important for disease progression [14]. In GD, candidate gene approach, in both family and case control data sets, has focused on immune genes such as HLA and CTLA-4 genes that confer susceptibility to GD [15]. However, each of these candidate genes is likely to contribute no more than 5% to the overall genetic susceptibility [3]. 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 [16-19]. For example, IFN-γ, TNF-α, TNF-β, and IL-1Ra have been reported to be associated with GD [20-23]. However, these associations are still controversial and await confirmation by other studies.

IL-4 gene is located on chromosome 5q31-33, which is one of the candidates susceptibility loci for GD. IL-4 is an important candidate gene within this region due to its role in the regulation of the differentiation of precursor T helper cells into those of the Th2 subset that mediate humoral immunity and modulate antibody production [24]. In humans, Th1 cytokines (IFN-γ, IL-2 and TNF-β) stimulate the production of

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an IgG1 isotype response which is the predominant pathogenic TSH receptor autoantibody seen in GD [25-27]. IgG1 isotypes are also prevalent among thyroglobulin and peroxidase antibodies[28, 29]. Hence, in autoimmune thyroid disease, lower IL-4 activity may result in a propensity to develop IgG1 autoantibodies along with polarizing the immune response toward cell-mediated immunity. Supporting this mechanism, IL-4 has been shown to inhibit organ-specific autoimmune disease in animals[30, 31].

The IL-4 gene contains three widely typed markers, -589C/T single nucleotide polymorphism (SNP) in promoter region, -33C/T SNP on exon 1 and 70 bp variable number tandem repeat (VNTR) at intron 3 [32, 33]. IL-4 promotor polymorphism at position -589 is a novel binding site for NFAT located upstream of all the known control elements of IL-4, such as the negative regulatory element, the positive regulatory element, the NF-Y recognition sequence, the OAP40 recognition sequence, the NF-P recognition sequence, and the TATA box [34], and affects IL-4 transcriptional activity [35, 36]. IL-4 -589T allele was associated with the increased promoter activity for IL-4 transcription and elevated levels of serum IgE in asthmatic families [35]. Previous characterization of IL-4 polymorphism with GD has controversial result. Hunt et al suggested that the -589T allele or a gene in linkage disequilibrium with this allele, may confer modest protection against the development of GD in Caucasians [37]. However, Heward et al reported that this polymorphism did not play a role in the genetic susceptibility to GD in another group of Caucasians [38]. The present study aimed to investigate the role of this proven functional polymorphism -589C/T of the IL-4 gene as a genetic marker for susceptibility to GD in a Thai population.

Material and Method

Subjects

A total of 137 patients with GD (121 females and 16 males) and 137 healthy individuals (100 females and 37 males) were examined after obtaining informed consent. All healthy individuals and patients were unrelated. Patients were followed up in an outpatient clinic at the Department of Endocrinology, Faculty of Medicine, Chulalongkorn Hospital, Bangkok, Thailand. The diagnosis of Graves' disease was based on 2 clinical features, diffuse enlargement of the thyroid gland and the elevation of free thyroxine or triio-dothyronine levels for more than 3 months. Healthy controls were recruited from volunteer unrelated healthy donors from the same geographic area. The present study was reviewed and approved by the Ethics Committees of the Faculty of Medicine, Chulalongkorn Hospital, Chulalongkorn University.

PCR-restriction fragment-length polymorphism (RFLP) of IL-4

Genomic DNA was extracted from whole blood by using a standard salting-out method [39]. Polymorphism at -589C/T in the promoter region of the IL-4 gene were identified using the PCR-RFLP method. The genomic DNA of 137 patients with GD and 137 healthy controls were amplified with the use of the IL-4 gene specific primers described by Michel et al [40]. The reaction volume for the amplification reaction was 30 µl, containing 50 ng/µl genomic DNA, 0.15 µl of 5.0 U/µl Taq polymerase (Promega or Gibco), 3 µl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 1.8 µl of 25 mM $MgCl_2$, 0.6 µl of 10 mM deoxynucleotide triphosphates and 1.5 µl (20 pmol) of each primer. Amplification was performed in Perkin Elmer/GeneAmp PCR system 2400 or Applied Biosystems/GeneAmp PCR system 9600. The PCR protocol consisted of an initial denaturation at 94 C for 2 minutes, followed by 30 cycles of denaturation (94 C, 20 seconds), annealing (59 C, 50 seconds) and extension (72 C, 20 seconds) and final extension at 72 C for 7 minutes. This produced an amplicon of 252 bp, which was visualized on a 1.5% ethidium bromide-stained agarose gel. Ten microliters of amplified DNA were digested with 1 U of restriction enzyme *BsmF*I (New England Biolabs, Hitchin, UK) in 1X NEBuffer 4 in a total volume of 15 µl at 65 C for 10-14 hours, followed by 3% agarose gel electrophoresis. Additionally, selected PCR products were analyzed by DNA sequencing to confirm the RFLP results.

Statistical analysis

Allele and genotype frequencies were determined by direct counting and then divided by the number of chromosomes to produce the allele frequency, or by the number of subjects to produce the genotype frequency. The goodness of fit to 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 appro-priate. A *P* value of <0.05 was considered significant. Odds ratios (OR) with 95% confidence interval (CI) were calculated using the statistical program Epi Info version 6 (Centers for Disease Control and Prevention [CDC], 1994).

Results

Polymorphism at -589C/T in the promoter region of the IL-4 gene were identified using the PCR-RFLP method. If a C were present at position -589, the enzyme would cut the 252 bp PCR product into two fragments, 192 and 60 bp. No digestion would occur if an T were present. A representative result of the RFLP pattern is shown in Fig. 1.

Genotype and allele frequencies for the -589 at the promoter of IL-4 gene in healthy controls and GD patients are summarized in Table 1. The Hardy-Weinberg analysis of the genotype data indicated that the frequencies were in the expected equilibrium and were, thus, randomly distributed. There were no significant differences in the genotype and allele frequencies of -589C/T polymorphism at the promoter of IL-4 gene between patients with GD and healthy controls.

Besides association study of cytokine gene polymorphisms with GD, the present study also provides the basic knowledge of the frequency of cytokine gene polymorphisms in a Thai population. The distributions of the cytokine gene polymorphisms between a Thai population and other populations in a previous report were compared (Table 2). The analysis showed significant differences in allele and genotype frequencies between a Thai population with Caucasians (χ^2 =65, p<0.05 and χ^2 =98.7, p<0.05, respectively), and Brazilians (χ^2 =19.2, p=0.00001 and χ^2 =39.6, p<0.05, respectively). However, no statistical differences were observed when comparing the Thai population with Japanese and Kuwaiti Arabs.

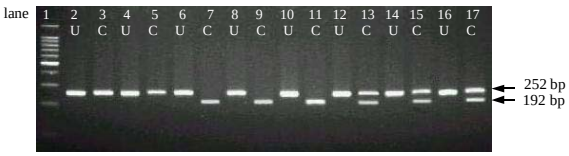

Fig 1. The representative of the PCR-RFLP pattern of IL-4 gene
Lane 1 is 100 bp molecular marker
Lane 2-5 (2-3 sample N2 and 4-5 sample N5) show results from homozygous of -589T
Lane 6-11 (6-7sample N4, 8-9sample N7 and 10-11 sample N33) show results from homozygous of -589C
Lane 12-17 (12-13 sample N3, 14-15 sample N6 and 16-17 sample N9) show results from heterozygous of -589C/T
Please note that under this electrophoresis condition, the 60 bp product is not visible
U = not add restriction enzyme, C = add restriction enzyme

Table 1. Genotype and allele frequencies for the -589 at the promoter of IL-4 gene in healthy controls and GD patients

	Healthy controls ***n*** **= 137**	**GD patients** ***n*** **= 137**
Genotype frequencies		
C/C	17 (12.4%)	15 (10.9%)
C/T	50 (36.5%)	55 (40.2%)
T/T	70 (51.1%)	67 (48.9%)
Allele frequencies		
C	84 (30.7%)	85 (31.0%)
T	190 (69.3%)	189 (69.0%)

Table 2. Comparison between allele frequencies of IL-4 gene polymorphism in the different population

Cytokine Position	**Control**						
	Author	Scarel-Caminaga	Hunt *et al.*	Heward *et al.*	Uboldi *et al.*	Noguchi *et al.*	Hijazi&Haider	Present study
	Year	*et al.* 2002	2000	2001	2003	1998	2000	2004
	Ethnic group	Brazilian	Caucasian	Caucasian	Caucasian	Japanese	Kuwaiti Arab	Thai[abcd]
	N	114	101	285	140	215	47	137
	Genotype							
IL-4 -589 (promoter)	**C/C**	43(37.7%)	75(74%)	222(77.9%)	111(79%)	17(7.9%)	3(6.4%)	17(12.4%)
	C/T	57(50.0%)	23(23%)	58(20.3%)	28(20%)	97(45.1%)	17(36.2%)	50(36.5%)
	T/T	14(12.3%)	3(3%)	5(1.8%)	1(1%)	101(46.8%)	27(57.4%)	70(51.1%)
	Allele							
	C	143(62.7%)	173(86%)	502(88%)	125(89%)	129(30%)	23(24.5%)	84(30.7%)
	T	85(37.3%)	29(14%)	68(12%)	15(11%)	301(70%)	71(75.5%)	190(69.3%)

[a] compared with Caucasian (genotype frequency reported by Heward *et al.*) χ^2 = 98.7 p < 0.05
[b] compared with Caucasian (allele frequency reported by Heward *et al.*) χ^2 = 65 p < 0.05
[c] compared with Brazilian (genotype frequency reported by Scarel-Caminoga *et al.*) χ^2 = 39.6 p < 0.05
[d] compared with Brazilian (allele frequency reported by Scarel-Caminoga *et al.*) χ^2 = 19.2 p < 0.05

Discussion

The result from the present study did not show an association between -589C/T promoter polymorphism of the IL-4 gene and GD in a Thai population. The present result was in keeping with the Heward et al report suggesting that the protective effect of -589C/T genotype described by from Hunt et al in UK subjects may be a false positive result. It is possible that IL-4 gene may hold minor effects in GD susceptibility, particularly when several genes are contributed to pathogenesis of this complex disease, which then requires extremely large study cohorts to see positive association. In addition, the screening of only 1 polymorphism within the gene is not the best way to access the positive role of the gene. It might be necessary to analyze other SNPs within IL-4 gene. In fact, a recent study has reported several novel polymorphisms within the IL-4 gene which remain to be investigated in a disease association study [41]. Furthermore, linkage disequilibrium within IL-4 gene was shown to be deviated from prediction determined by the distance between polymorphisms [41], suggesting the use of haplotype instead of single SNP as the genetic marker. Chromosome 5q31, a linked susceptible loci for GD, contains several candidate genes besides IL-4 such as IL-3, IL-5, IL-13 and granulocyte colony stimulating factor gene (GM-CFS) [6]. Therefore, it is possible that other genes on chromosome 5q31 might also influence the susceptibility to GD. Further study of the other genes will lead to better understanding of GD pathogenesis.

One interesting report has recently shown that IL-4 -589 allele frequencies were significantly more divergent than the neutral SNP frequencies (varies from 0.17 in southern Italy to 0.76 in Cameroon) and suggested that selection for immune function is the most likely explanation for it [42,43]. Thus, the distribution of IL-4 gene polymorphisms in a Thai population compared to other previously published results were also analyzed in the present study. In summary, the distribution of -589T allele, which was associated with high IL-4 production is low in Caucasians (11-14%), intermediate in South Americans (37%), and high in Asians (69-75%). The frequencies of IL-4 polymorphisms in a particular population were different and might be useful in identifying the disease susceptibility marker. The ethnic difference in the distribution pattern of IL-4 promoter polymorphism may explain the ethnic differences in susceptibility or severity of many diseases.

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ไม่พบความสัมพันธ์ระหว่างความหลากหลายของยีน interleukin 4 ที่ตำแหน่ง promoter (-589) กับโรคเกรฟในผู้ป่วยไทย

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ไซโตไคน์มีความสำคัญในการควบคุมปฏิกริยาตอบสนองทางภูมิคุ้มกันและขบวนการอักเสบทำให้ยีนไซโตไคน์ถูกนำมาศึกษาหาความสัมพันธ์กับการเกิดโรค autoimmune thyroid โดยความหลากหลายในยีนไซโตไคน์อาจมีผลต่อกระบวนการ transcription ทำให้การสร้างไซโตไคน์เปลี่ยนแปลงไป และมีหลายการศึกษาที่พบความสัมพันธ์ระหว่างยีน interleukin 4 (IL-4) กับโรคภูมิต้านเนื้อเยื่อของตัวเอง ดังนั้นงานวิจัยนี้ได้ทำการศึกษาความหลากหลายในยีน IL-4 ที่ตำแหน่ง promoter (-589)ระหว่างผู้ป่วยโรคเกรฟเปรียบเทียบกับคนปกติและศึกษาความสัมพันธ์กับการเกิดโรคเกรฟในคนไทย โดยใช้การศึกษาแบบ population-based รวบรวมผู้ป่วย 137 คน และคนปกติ 137 คนซึ่งมีเชื้อสายและถิ่นกำเนิดเดียวกัน โดยใช้วิธี PCR-RFLP หารูปแบบความหลากหลายของยีน IL-4 ที่ตำแหน่ง -589 ในส่วน promoter จากการศึกษาของ Hunt และคณะพบความสัมพันธ์แบบ protective กับรูปแบบ -589T ในกลุ่มประชากร Caucasian แต่ไม่พบความสัมพันธ์นี้ในประชากรไทยโดยความถี่ของรูปแบบ -589T ใกล้เคียงกันระหว่างผู้ป่วยและคนปกติ (69% กับ 69.3%) ดังนั้นความหลากหลายของยีน IL-4 ที่ตำแหน่ง -589 ไม่สามารถใช้เป็นเครื่องหมายสำหรับยีนที่กำหนดความเสี่ยงในการเกิดโรคเกรฟในคนไทย