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# Proinflammatory Cytokine Gene Polymorphisms Among Hashimoto's Thyroiditis Patients

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Proinflammatory cytokines are involved in the pathogenesis of Hashimoto's thyroiditis (HT). To test whether certain specific proinflammatory cytokine gene polymorphisms could be genetic markers for an individual's susceptibility to HT, we investigated single-site polymorphisms of certain proinflammatory cytokine genes of interest for 107 HT sufferers and 163 controls, subsequent to preparing the necessary experimental genomic DNA from peripheral blood, using a polymerase chain reaction (PCR)-based restriction analysis. The polymorphisms we detected were as follows: 1) C/T and E1/E2 polymorphisms for the interleukin (IL)-1β gene at promoter (−511) and exon 5, respectively; 2) a variable number of tandem repeats (VNTRs) for the IL-1 receptor antagonist (IL-1Ra) gene at intron 2; 3) a C/G polymorphism for the IL-6 gene at promoter (−572); and 4) an A/G polymorphism for the tumor necrosis factor (TNF)-α gene at promoter (−308). The data demonstrated an increased ratio of CG genotype and decreased ratios of CC and GG genotypes (chi-squared test; $P = 0.025$) for the IL-6 gene promoter for HT patients when compared with normal controls. The odds ratio (OR) for the CG genotype, as compared to the GG genotype, for HT patients was shown to be 4.065 (95% confidence interval (CI): 1.268–13.032). Comparison of the genotype analysis for the remaining gene polymorphisms and the allelic analysis for all of the screened gene polymorphisms, however, all revealed no statistically significant difference between the two study groups as regards frequency of genotype. In conclusion, we suggest that an IL-6 gene promoter (−572) C/G polymorphism could represent a potential "candidate" genetic marker to predict an individual's susceptibility to HT. J. Clin. Lab. Anal. 20:260–265, 2006. 

**Key words:** Hashimoto's thyroiditis; polymorphism; proinflammatory cytokine

## INTRODUCTION

Hashimoto' thyroiditis (HT) is a common organ-specific autoimmune disease, characterized by the presence of a diffuse thyroid enlargement (goiter) and by the presence of antimicrosomal antibody (AMiA) and antithyroglobulin antibody (ATA) against, respectively, thyroid peroxidase (TPO) and thyroglobulin (Tg) (1). Clinically, those individuals who are most likely to be susceptible to HT are, typically, either euthyroid or hypothyroid. Histologically, the thyroid glands of patients suffering from HT typically reveal diffuse lymphocyte infiltration and germinal-center formation, and also the obliteration of thyroid follicles by widespread apoptosis and fibrosis (2).

Intrathyroidal lymphocyte infiltration, the pathognomonic feature of HT, typically comprises T cells as well as B cells, implying a contribution to HT pathogenesis

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by both cell-mediated and humoral immunity processes (3). The immune reactions associated with HT begin as the stimulation of thyrocytes by a specific cytokine, interferon-γ (IFN-γ), with subsequent expression of both human leukocyte antigen (HLA) class-I and class-II molecules on the cellular surface of involved thyrocytes (4). The HLA class I-restricted pathway is involved in the activation of cytotoxic T cells and result in either direct damage to, or Fas-Fas ligand (FasL)-induced apoptosis of, thyrocytes (5). On the other hand, HLA class II-restricted reactions, when they are involved, activate helper T cells, stimulate B-cell proliferation and differentiation, and enhance the resultant production of the corresponding antibody (Ab). Both AMiA and ATA are complement-fixing and are thus directly cytotoxic to thyrocytes (6).

Cytokines are a large group of nonenzymatic protein hormones that participate in the induction and effector phases of all inflammatory and immune responses (7). Proinflammatory cytokines, a subgroup of the cytokine group, contain interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α. The IL-1 family consists of IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra). IL-1β features a similar structure and biological activity to IL-1α and is the predominant species of the human IL-1 class, whereas IL-1Ra antagonizes the activities of IL-1α and IL-1β (8). In addition to being responsible for inducing responses to acute illness, proinflammatory cytokines are also able to mediate multiple immunological reactions, such as T-cell activation, B-cell stimulation, and HLA-molecule and Fas expression, and are thus clearly involved in the pathogenesis of HT (5,9,10).

The genetic mechanism(s) underlying HT would appear to be complex and, to the best of our knowledge, most studies that have attempted to deal with such a topic have focused principally on the role of the HLA region. HLA-DR5 has been reported to be associated with a 3.4-fold increased risk of HT for Caucasians, whereas high frequencies of HLA-DRB4*0101 and HLA-A2 have been demonstrated to be not uncommon amongst Japanese HT sufferers (11,12). In addition, HT has also been associated with particular alleles of the cytotoxic-T-lymphocyte associated protein 4 gene (*CTLA-4*) (13). To the best of our knowledge, however, it would appear that, currently, the research investigating the relationship between proinflammatory cytokine genes and HT is somewhat incomplete and limited, there being only one literature-based study that reported on the association between a specific allelic variant of the TNF-α gene (TNF 1) and HT (14). We have recently observed that a single-site polymorphism at promoter (−511 C/T) of the IL-1β gene is associated with an individual's relative susceptibility to succumbing to an analog of HT, namely Graves' disease (15). To further test whether certain proinflammatory cytokine genes could also be genetic markers for the prediction of HT development, we screened single-site polymorphisms of the IL-1β gene at promoter (−511) and exon 5, the IL-1Ra gene at intron 2, the IL-6 gene at promoter (−572), and the TNF-α gene at promoter (−308), using polymerase chain reaction (PCR)-based restriction analysis, to compare HT patients and normal controls from a selected Taiwanese study population.

## MATERIALS AND METHODS

### Patient Selection

A total of 107 unrelated Chinese HT patients (97 women) aged between 17 and 69 years (mean = 36.0 ± 12.5) were enrolled in this study, which ran from July 2004 to January 2005 inclusively. All study patients were of the Han race and resided in central Taiwan, and none of them revealed any current or previous history of hyperthyroidism and/or thyroid-associated ophthalmopathy. None of the female patients were pregnant at the time of the study, and none had delivered within the entire 1-year period prior to study enrolment. The presence of a palpable goiter and a high serum AMiA titer (1:100 or greater) were used as the definitive criteria to specifically define HT. Interestingly, a rather high serum ATA titer (1:100 or greater) was also noted for 87 (81%) of study-incorporated individuals. Thyroid ultrasonographic examination revealed a hypoechogenic pattern for all patients. The control group consisted of 163 (145 women) ethnically and residentially matched healthy volunteers over the age of 40 years who featured neither goiter nor any evidence of thyroid dysfunction. None of these controls exhibited any positive antithyroid autoantibodies in their sera. Further, these control-group individuals also exhibited no previous personal or family history of thyroid disease or any autoimmune disease. This study was approved by the institutional ethics committee of our institution, and informed consent was requested of, and obtained from, each study subject.

### Polymerase Chain Reaction

Experimental genomic DNA was prepared from peripheral blood using the genomic DNA isolation reagent kit (Genomaker Inc.; Taipei, Taiwan). A conventional PCR was used to identify the genotypes of the IL-1β, IL-1Ra, IL-6, and TNF-α genes. The PCR for the various polymorphisms was performed to a total volume of 50 μL, it contained genomic DNA (2–6 pmol of each primer), 1 × Taq polymerase buffer (1.5 mM $MgCl_2$), and 0.25 units of AmpliTaq DNA polymerase (PerkinElmer; Foster City, CA). The appropriate

primers for the polymorphisms of the IL-1β gene promoter (−511) and exon 5 and the IL-1Ra gene intron 2, the IL-6 gene promoter (−572), and the TNF-α gene promoter (−308) are listed in Table 1 and are as referred to in the studies of Cantagrel et al. (16), Chung et al. (17), and Galbraith and Pandey (18), respectively. PCR amplification was performed using a programmable thermal cycler GeneAmp PCR System 2400 (Perkin-Elmer), the cycling conditions for which are presented in Table 1.

For the IL-1Ra gene intron 2, a 10 μL aliquot of each of the various PCR products was loaded onto a separate well of a 3% agarose gel containing ethidium bromide. Following this, the gel was then subjected to electrophoresis, each allele being recognized according to its particular size. The 86-bp variable number tandem repeats (VNTRs) of the IL-1Ra gene were classified as being one of a total of five different variants, namely "I" for the 410-bp PCR product, "II" for the 240-bp option, "III" for the 500-bp species, "IV" for the 325-bp variant, and the "V" allele for the 595-bp analog. By contrast, however, the remaining polymorphisms of interest, the IL-1β gene promoter (−511) and exon 5, the IL-6 gene promoter (−572), and the TNF-α gene promoter (−308), were able to be discerned individually by digestion with *Ava*I, *Taq*I, *Bsr*BI, and *Nco*I, respectively, prior to electrophoresis being conducted. Hence, subsequent to the PCR amplification for these four site polymorphisms, the PCR products were firstly incorporated, separately, with aliquots of the above-mentioned enzymes and then with reaction buffer according to the manufacturer's instructions, with both steps of the reaction necessitating incubation of the reaction mix for three hours at 37°C. Following this, an aliquot (10 μL) of each PCR product was loaded onto a separate lane of a 3% agarose gel containing ethidium bromide and then subjected to electrophoresis. The resultant genotypes thus elicited were classified into one of the following categories: 1) an excisable allele homozygote (CC for the IL-1β gene promoter, E1E1 for the IL-1β gene exon 5, GG for the IL-6 gene, and AA for the TNF-α gene); 2) a nonexcisable allele homozygote (TT, E2E2, CC, and GG for the IL-1β gene promoter, IL-1β gene exon 5, IL-6 gene, and TNF-α gene, respectively); and 3) a heterozygote (CT, E1E2, GC, and AG).

The laboratory work for both patients and controls was uniformly performed by a single trained professional technician contemporaneously and the work was subsequently repeated in a similar fashion by another trained technician at a different time, and all gels were inspected by investigators who were blinded to the clinical phenotypes of the individuals being studied.

## Statistical Analysis

The genotype and allelic frequencies of the polymorphisms for the HT and control groups were statistically compared using the chi-square test performed with the Statistical Package for Social Sciences (SPSS) version 8.01 software (SPSS for Windows; SPSS Inc.; Chicago, IL) following the examination of allelic frequencies incorporating the application of Hardy-Weinberg equilibrium (HWE) proportions for genotype frequencies. When the inherent assumption of the chi-squared test was violated (i.e., a single cell featured an expected count of $<1$, or, greater than 20% of the cells revealed an expected count of $<5$), Fisher's exact

**TABLE 1. Primer sequences (5′ to 3′ orientation) and conditions for polymerase chain reaction (PCR) in polymorphisms of the proinflammatory cytokine genes**

| Set | Position | Primer and PCR conditions | PCR product (bp) | Restriction site |
|---|---|---|---|---|
| IL-1β promoter | −511 C/T | U 5′-TGGCATTGATCTGGTTCATC-3′<br>D 5′-GTTTAGGAATCTGGACCAGA-3′<br>95°C × 30 sec, 55°C × 30 sec and 72°C × 30 sec | 190+114 or 304 | *Ava*I |
| IL-1β exon 5 | Exon 5 | U 5′-GTTGTCATCAGACTTTGACC-3′<br>D 5′-TTCAGTTCATATGGACCAGA-3′<br>95°C × 30 sec, 55°C × 30 sec and 72°C × 30 sec | 135+114 or 249 | *Taq*I |
| IL-1Ra | Intron 2 | U 5′-CTCAGCAACACTCCTAT-3′<br>D 5′-TCCTGGTCTGCAGGTAA-3′<br>95°C × 30 sec, 58° × 30 sec and 72°C × 30 sec | I: 410<br>II: 240<br>IV: 325 | VNTRs |
| IL-6 promoter | −572 G/C | U 5′-GCAAAGTCCTCACTGGGAGGA-3′<br>D 5′-TCTGACTCCATCGCAGCCC-3′<br>95°C × 30 sec, 60°C × 30 sec and 72°C × 45 sec | 201+95 or 296 | *Bsr*BI |
| TNF-α promoter | −308 A/G | U 5′-AGGCAATAGGTTTTGAGGGCCAT-3′<br>D 5′-ACACTCCCCATCCTCCCGGCT-3′<br>95°C × 30 sec, 60°C × 30 sec and 72°C × 45 sec | 97+20 or 117 | *Nco*I |

U, upstream primers; D, downstream primers; VNTRs, variable number tandem repeats.

test was applied. Results were considered to be statistical significance when the probability of a finding of intergroup difference occurring simply by chance was less than 5% ($P<0.05$). Odds ratios (OR) with 95% confidence intervals (CI) were calculated for disease susceptibility associated with specific genotypes and alleles.

## RESULTS

From the initial examination for genotype frequencies, the polymorphisms of screened genes were all found to be in HWE for both control and patient groups.

For the genotype analysis, HT patients featured a greater ratio of the CG heterozygous genotype (41.1%) and lesser ratios for the CC and the GG homozygous genotypes (55.2% and 3.7%, respectively) at the IL-6 gene promoter than was the case for normal controls (CG: 28.2%, CC: 61.4% and GG: 10.4%; chi-squared test, $P = 0.025$; Table 2). The OR for the risk of the appearance of the CG genotype, compared with the GG genotype, for the HT patient group was 4.065 (95% CI: 1.268–13.032). As regards the genotype distributions for the remaining genes, e.g., the IL-1β gene promoter (chi-squared test, $P = 0.524$), IL-1β gene exon 5 (Fisher's exact test, $P = 0.395$), IL-1Ra gene intron 2 (Fisher's exact test, $P = 0.644$), and the TNF-α gene promoter (Fisher's exact test, $P = 0.670$), there appeared to be no significant difference between the corresponding value for both study groups (Table 2).

With respect to allelic analysis, unlike the results of genotype analysis, we noted no significant difference in frequencies for the C allele (75.7% and 75.5%, respectively) and the G allele (24.3% and 24.5%, respectively) for the IL-6 gene promoter when comparing the patient and control groups (chi-squared test, $P = 0.949$; Table 3). Also, no statistically significant difference as regards the allelic analysis for all of the remaining genes was noted when comparing HT and control groups (Table 3).

**TABLE 2. Genotype frequencies of interleukin (IL)-1β gene promoter and exon 5, IL-1Ra gene intron 2, IL-6 gene promoter and tumor necrosis factor (TNF)-α gene promoter between healthy control subjects and Hashimoto's thyroiditis (HT) patients**

| Genotype | Hashimoto's thyroditis patients $N = 107$ (%) | Controls $N = 163$ (%) | $P$ |
|---|---|---|---|
| IL-1β promoter | | | 0.524[a] |
| -511*T/-511*T | 36 (33.7) | 51 (31.3) | |
| -511*C/-511*T | 44 (41.1) | 78 (47.9) | |
| -511*C/-511*C | 27 (25.2) | 34 (20.8) | |
| IL-1β exon 5 | | | 0.395[b] |
| E1/E1 | 105 (98.1) | 155 (95.1) | |
| E1/E2 | 2 (1.9) | 7 (4.3) | |
| E2/E2 | 0 (0.0) | 1 (0.6) | |
| IL-1Ra | | | 0.644[b] |
| I/I (410/410) | 97 (90.7) | 146 (89.6) | |
| I/II (410/240) | 8 (7.5) | 15 (9.2) | |
| II/II (240/240) | 0 (0.0) | 1 (0.6) | |
| I/IV (410/325) | 1 (0.9) | 0 (0.0) | |
| IV/IV (325/325) | 1 (0.9) | 1 (0.6) | |
| IL-6 promoter[c] | | | 0.025[a] |
| -572*G/-572*G | 4 (3.7) | 17 (10.4) | |
| -572*G/-572*C | 44 (41.1) | 46 (28.2) | |
| -572*C/-572*C | 59 (55.2) | 100 (61.4) | |
| TNF-α promoter | | | 0.670[b] |
| -308*A/-308*A | 0 (0.0) | 1 (0.6) | |
| -308*A/-308*G | 16 (15.0) | 27 (17.6) | |
| -308*G/-308*G | 91 (85.0) | 135 (82.8) | |

[a]Chi-squared test.
[b]Fisher's exact test.
[c]OR for G/C = 4.065 (95% CI = 1.268–13.032), for C/C = 2.508 (95% CI = 0.805–7.807).

## DISCUSSION

Proinflammatory cytokines participate in a number of different steps of thyroid autoimmunity and are thus involved in the pathogenesis of HT (5,9,10). Having noted this, however, to the best of our knowledge, from

**TABLE 3. Allelic frequencies of interleukin (IL)-1β ge ne promoter and exon 5, IL-1Ra gene intron 2, IL-6 gene promoter, and tumor necrosis factor (TNF)-α gene promoter between healthy control subjects and Hashimoto's thyroiditis (HT) patients**

| Allelic variant | Hashimoto's thyroiditis patients $N = 214$ (%) | Controls $N = 326$ (%) | $P$ |
|---|---|---|---|
| IL-1β promoter | | | 0.818[a] |
| Allele T | 116 (54.2) | 180 (55.2) | |
| Allele C | 98 (45.8) | 146 (44.8) | |
| IL-1β exon 5 | | | 0.142[b] |
| E1 | 212 (99.1) | 317 (97.2) | |
| E2 | 2 (0.9) | 9 (2.8) | |
| IL-1Ra | | | 0.477[a] |
| I (410 bp) | 203 (94.9) | 307 (94.2) | |
| II (240 bp) | 8 (3.7) | 17 (5.2) | |
| IV (325 bp) | 3 (1.4) | 2 (0.6) | |
| IL-6 promoter | | | 0.949[a] |
| Allele G | 52 (24.3) | 80 (24.5) | |
| Allele C | 162 (75.7) | 246 (75.5) | |
| TNF-α promoter | | | 0.559[a] |
| Allele A | 16 (7.5) | 29 (8.9) | |
| Allele G | 198 (92.5) | 297 (91.1) | |

[a]Chi-squared test.
[b]Fisher's exact test.

a thorough review of the literature, there would appear to have been a relative paucity of relevant literature/published studies investigating the relationship between the proinflammatory cytokine genes and an individual's susceptibility to HT. Our study observed an association between the presence of a proinflammatory cytokine gene polymorphism and HT, the data demonstrating that there appeared to be an increased frequency of the CG heterozygous genotype for the IL-6 gene promoter (−572) among HT patients when compared with normal controls. We therefore suggest that the above-mentioned IL-6 gene promoter (−572) C/G polymorphism might represent a candidate genetic marker to predict the development of HT for an individual.

IL-6 is mainly produced by mononuclear phagocytes, and following its release from monocytes, IL-6 exerts its inflammatory effect, resulting in the induction of fever and the synthesis of a number of acute-phase proteins in the liver. Furthermore, IL-6 is also an important growth and differentiation factor for T cells and B cells, and is able to modulate both cellular and humoral immunity (10). Intrathyroidal IL-6 production has been observed to play a critical role in the early stages of T-cell activation and the expression of certain autoantibodies, such as TPO (19,20). Any alteration in the synthesis of IL-6 may thus interfere with thyroid autoimmunity and the development of HT. The gene encoding IL-6 is located in the short arm of chromosome 7 (7p21) (21). In our study, we observed that the specific polymorphism arising at the IL-6 gene promoter was related to the tested individual's susceptibility to HT. We therefore propose that, by means of altering the transcriptional activity, the IL-6 gene promoter polymorphism may be able to influence the expression of the IL-6 gene, i.e., IL-6 production, and the resultant likelihood of occurrence of HT. In actuality, some previous studies have demonstrated the role of the IL-6 gene promoter polymorphism in the development of clinical diseases, such as multiple myeloma, carotid atherosclerosis and juvenile chronic arthritis (22–24). Following on from this, however, because we did not detect either serum or intrathyroidal IL-6 levels for individuals enrolled in this study, we are unable to provide any direct evidence suggesting that an IL-6 gene promoter polymorphism could contribute to the development of HT. Further study is clearly needed in order to elucidate the relationship between the above-mentioned IL-6 gene polymorphism and the occurrence of HT.

TNF-α is also produced mainly by monocytes, and exhibits cytotoxic as well as cytostatic effects upon thyrocytes, and further, TNF-α is also able to upregulate HLA class-I expression, and, acting synergistically with IFN-γ, enhance HLA class-II expression (10). The gene encoding TNF-α is located in the short arm of chromosome 6 (6p21.3), which also contains genes encoding HLA molecules (25). Owing to the biological effect of TNF-α upon the thyroid gland and also its specific gene location, TNF-α should, in principle, be able to influence an individual's susceptibility to HT, although it would appear, to the best of our knowledge, that literature-published studies investigating the relationship between TNF-α gene polymorphisms and HT are rare, and in this realm, to date, there would appear to have been only one relevant study published which claims to have obtained meaningful results (14). In their 2004 study, Bougacha-Elleuch et al. (14) assessed the polymorphism of the TNF-α gene promoter (−308 A/G) for Tunisian HT sufferers and also for normal matched individuals, these authors reporting an increased frequency of the G allelic variant for HT patients as compared to normal controls. Investigating at the same position for this gene, we observed no difference in distributions for genotype and allelic frequencies between HT patients and normal controls. Such seemingly disparate findings emerging from our study and that of Bougacha-Elleuch et al. (14) possibly reflect different mechanisms for the pathogenesis of HT for different racial populations.

From earlier research, IL-1-related genes have been observed to be associated with an individual's susceptibility to Graves' disease (15,26); however, to the best of our knowledge, to date, there would not appear to have been any study yet published, which has been able to specially define the relationship between IL-1-related gene polymorphisms and the occurrence of HT. Further, our data also reveal no association between polymorphisms of the IL-1-related genes and the incidence of HT. Because IL-1 exerts multiple effects upon a number of immunological reactions and the thyroid gland itself, it would be appear entirely possible to be able to define the correlation between an IL-1-related gene polymorphism and an individual's HT susceptibility subsequent to future comprehensive investigations in this realm.

The IL-6 gene polymorphism referred to above may demonstrate a potential functional effect upon an individual's susceptibility to HT. However, because of the relatively small sample size in the current study, it is not our stated intention here to suggest that the IL-6 gene polymorphism referred to above must be a direct cause of HT, but that there does exist some sort of an association between the IL-6 gene polymorphism and the presence of HT. In essence, such a suggestion implies that other factors, either individually or in concert, such as population stratification and statistical artifacts, may have contributed to produce the results chronicled in this study.

In conclusion, our study provides some preliminary findings linking certain proinflammatory cytokine gene

polymorphisms and an individual's HT susceptibility. The data suggest that a specific IL-6 gene promoter (−572 C/G) polymorphism might constitute a specific genetic marker for the prediction of the development of HT for an individual. Following on from this, however, because both the genetics of HT and the activities of cytokines are typically complex, and also because cytokine gene polymorphisms would appear to be abundant, further work should be undertaken in order to more comprehensively determine the specific details of the pathogenesis of HT.

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