Cytokine Gene Polymorphisms in Autoimmune Thyroid Disease


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ABSTRACT

Susceptibility to the autoimmune thyroid diseases, Graves’ disease (GD) and autoimmune hypothyroidism (AIH), depends on a complex interaction between environmental and genetic factors. The human leukocyte antigen and cytotoxic T lymphocyte-associated-4 regions appear to influence susceptibility to disease, but the effect is not major, and the other genes remain unknown. Cytokines are crucial in the regulation of immune and inflammatory responses and therefore are potential candidate genes for autoimmune thyroid disease. In a case-control study, using a unified method of genotyping, we have examined 15 polymorphisms in 9 cytokine genes in 215 patients with autoimmune thyroid disease (GD, 138; AIH, 77) and 101 normal controls.

Polymorphisms in the genes for interleukin-1α (IL-1α), IL-1β, IL-1 receptor antagonist, IL-1 receptor 1, IL-4, IL-4 receptor, IL-6, IL-10, and transforming growth factor-β were investigated. Genotyping was performed using the PCR and sequence-specific primers. Analysis showed a reduced frequency of the variant t allele in the IL-4 promoter polymorphism (position –590) in patients with GD and in the entire patient group (GD and AIH) compared with the control group [corrected \( P (\text{Pc}) = 0.00004 \) and \( P < 0.00001 \) for GD and all patients, respectively]. This was reflected in a reduction in the heterozygote genotype in the patient groups compared to the controls [\( \text{Pc} = 0.06 \), odds ratio, 0.4 (95% confidence interval, 0.2–0.7); all patients, 11%; \( \text{Pc} = 0.008 \); odds ratio, 0.4 (95% confidence interval, 0.2–0.7); control subjects, 23%]. There were no significant differences between the study groups for the other polymorphisms examined, and subgroup analysis revealed no association with clinical parameters of disease.

These results suggest that an IL-4 variant or a closely linked gene has a modest protective effect against the development of autoimmune thyroid disease, particularly GD. This variation in the IL-4 gene may provide further clues to the pathogenesis of autoimmune thyroid disease and other organ-specific autoimmune diseases. Furthermore, these results suggest that subtle variation in immunoregulatory genes may be associated with autoimmune disease states. (J Clin Endocrinol Metab 85: 2000)

AUTOIMMUNE THYROID disease, comprising predominantly Graves’ disease (GD) and autoimmune hypothyroidism (AIH), is common, affecting approximately 2% of women and 0.2% of men. The etiology of the disease appears to involve a complex interplay of multiple genetic and environmental influences. Evidence for the role of genetic factors is shown by the increased incidence of the disease within families and by twin studies. For well-defined Graves’ disease, the concordance rate in monozygotic twins is reported to be 22% and 0% for dizygotic twins (1). The human leukocyte antigen (HLA) class II genes contribute to susceptibility, and studies also suggest a role for the cytotoxic T lymphocyte-associated-4 (CTLA-4) gene (2). However, each of these candidate genes is likely to contribute no more than 5% to the overall genetic susceptibility (3). Other potential susceptibility loci have recently been identified by linkage analysis on chromosomes X (4), 14q31 (5), and 20q11 (6), but await confirmation. Candidate gene studies have proven very effective in detecting susceptibility genes for other diseases (7, 8) as well as genes important for disease progression (9) and is the strategy employed in this study.

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. Intrathyroidal inflammatory cells and thyroid follicular cells have been shown to produce a variety of cytokines, including interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-14, tumor necrosis factor-α, and interferon-γ (IFNγ) (10, 11). The cytokine network is complex, with cytokines having both diverse and overlapping functions, including effects that are promoted or inhibited by other cytokines. Cytokine secretion profiles can be considered as either pro- or antiinflammatory or, alternatively, on the basis of the animal model as either T helper cell type 1 (Th1) responses promoting cell-mediated immunity (IL-2 and IFNγ) or Th helper cell type 2 (Th2) responses promoting humoral immunity (IL-4, IL-5, IL-6 IL-10, and IL-13) (12). Despite attempts to classify autoimmune thyroid disease as a classical Th1- or Th2-mediated disease, no clear conclusions can be drawn, and a mixed Th1/Th2 response is seen in both GD and AIH (10). 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 (10). In thyroid-associated ophthalmpathy, cytokines (IL-1α, tumor
necrosis factor-α, and IFNγ) are pathogenic, promoting inflammation and fibroblast proliferation, leading to the accumulation of glycosaminoglycans (13). Polymorphisms in genes encoding these crucial immunomodulatory molecules may result in an altered level of expression and hence must be considered important candidate genes for autoimmune disease susceptibility and severity. As cytokines interact functionally, this study examines the contributions of several candidate genes with potential immunoregulatory roles in autoimmune thyroid disease. The candidate genes were selected for investigation if their gene product was likely to be important in the regulation of the cellular or humoral immune response and if a single nucleotide polymorphism within the gene was amenable to genotyping.

IL-1α and IL-1β are pleiotropic cytokines with primarily proinflammatory effects, including stimulation of IL-2 and IL-6. Both IL-1α and IL-1β as well as the naturally occurring IL-1 receptor antagonist (IL-1RA) act via the IL-1 receptor 1. Hence, disruption of the balance among IL-1α, IL-1β, IL-1RA, and their receptors may result in disease. Polymorphisms of IL-1α (position −889) and IL-1β (exon 5, +3962) (14) have been associated with juvenile rheumatoid arthritis (15, 16) and insulin-dependent diabetes mellitus (14, 17), respectively. Variation in the IL-1RA gene (exon 2) has been associated with a variety of autoimmune disorders (18–21), including Graves’ disease (22), although this finding has not been replicated (23).

IL-4 mediates the humoral immune response, and polymorphisms in IL-4 (position −590) and its receptor gene (nucleotide 1902) have been associated with atopy (24, 25). IL-6 is a key inflammatory cytokine, and elevated systemic levels are seen in many conditions, including autoimmune thyroid disease (10). Promotor region variation of the IL-6 gene has been associated with juvenile rheumatoid arthritis (26). IL-10 enhances B cell proliferation and an IL-10 polymorphism (position −1082) has been associated with systemic lupus erythematosus (27) and rheumatoid arthritis (28). Transforming growth factor-β (TGFβ) is another cytokine with important modulatory functions, including an inhibitory role in B cell maturation.

To investigate whether variability in these immunoregulatory genes may influence disease susceptibility or severity, polymorphisms were assessed in a case control association study using a unified method of genotyping.

**Subjects and Methods**

**Subjects**

The patient cohort comprised 215 Caucasian patients with autoimmune thyroid disease [135 GD (111 women) and 77 AIH (67 women)] recruited from the endocrinology clinics in Oxford and Sheffield. GD was defined by the presence of hyperthyroidism and diffuse goiter, supported by the presence of either thyroid antibodies (peroxidase and/or thyroglobulin) or thyroid eye disease. AIH was diagnosed by the presence of primary hypothyroidism and positive thyroid antibodies with or without goiter. Information was also obtained on age at diagnosis, size of goiter, presence of other autoimmune disease, and, for GD, severity of ophthalmopathy and relapse rates. The study was approved by the respective local ethics committees, and all subjects gave written informed consent.

The control population comprised 101 Caucasian cadaveric renal allograft donors. The representative nature of this control population of the general Caucasian population has previously been shown in HLA genotyping reports (29).

**Genotyping methodology**

All genotyping was performed using PCR-sequence specific primers (PCR-SSP). DNA was extracted from 10 mL ethylenediamine tetraacetate blood using the Puregene kit (Gentra Systems, Minneapolis, MN). PCR primers were designed with allele specificity determined by the terminal 3′-nucleotide. For detection of two or more closely related polymorphisms within the same gene, forward and reverse allele-specific primers were used (PCR-haplotyping) (30), thus minimizing the number of PCR reactions and formally identifying the cis/trans orientation of the alleles. Primers and concentrations used for IL-1α (−889/ c), IL-1β (+3962t/c), IL-4 (−590c/t), IL-6 (+3247a/g), IL-10 (−1082a/g, −819c/t, −592c/a), and TGFβ (−880g/a, −509c/t, aa10L/P, aa263T/l) have been previously described (31). Primers for IL-1α (−511 c/t), IL-1RA, IL-1 receptor 1, and IL-4 receptor are listed in Table 1. To confirm adequate DNA amplification, all reaction mixes also contained control primers. Further details of the PCR-SSP methodology, including PCR amplification and gel electrophoresis, have been published previously (32). An example is shown in Fig. 1.

**Statistical analysis**

Allele frequencies were compared between the patient groups and the control group using the binomial distribution probability. Genotype frequencies were also compared between the entire patient group and the control group and between the subgroups of GD and AIH compared to the control group using the genotype relative risk method of Lathrop (33). P values were corrected for the number of independent comparisons of haplotype or allele variants that were made (n = 12) and a corrected P (Pc) value of less than 0.05 was considered significant.

**Results**

Fifteen polymorphisms in nine genes were analyzed in both the patients and control subjects. The three polymorphisms in the IL-10 gene and the two in the TGFβ gene (−800 and −509) were haplotyped (see Subjects and Methods). All control allele frequencies were in Hardy-Weinberg equilibrium.

The major finding was a striking reduction in the frequency of the IL-4 (−590) variant t allele in the patients with GD and the overall patient group (GD and AIH) compared to the control group (GD: Pexact = 0.00003; Pc = 0.0004; GD and AIH: Pexact < 0.00001; Pc < 0.0001; Table 2). For the patients with AIH, a similar trend was seen, but this was not significant when corrected for the number of comparisons made (Pexact = 0.006; Pc = 0.07). Genotype analysis showed that the reduction in t allele frequency resulted in fewer IL-4 heterozygote genotypes in the patients with GD compared to controls [P = 0.005; Pc = 0.06; odds ratio (OR), 0.4; 95% confidence interval, 0.2–0.7] which was also seen when all patients were compared with controls (P = 0.0007; Pc = 0.008; OR, 0.4; 95% confidence interval, 0.2–0.7; Table 3). Again, a similar tendency was seen for the patients with AIH (P = 0.01; Pc = 0.11), but this was not significant when corrected for the number of observations. Allele and genotype frequencies for the other gene polymorphisms did not differ significantly between the patient and control groups (data not shown, but available from the authors). Further subgroup analysis was performed, and no significant associations of polymorphisms (including the IL-4 −590 polymorphism) were detected with age of onset of disease, size of goiter, recurrent GD, titer of thyroid peroxidase or thyroxin.
roglobulin antibodies, thyroid eye disease, or presence of other autoimmune disease (data not shown).

Discussion

This study provides the first evidence for a genetic association between autoimmune thyroid disease and the IL-4 cytokine gene. This association is due to a decreased prevalence of the variant t allele in the patients with GD, resulting in fewer c/t heterozygote genotypes, compared to controls. In addition, in the AIH group a similar trend was seen, although these results were not significant when multiple comparisons were taken into account.

IL-4 is a key cytokine in immune regulation. It is produced by T cells, mast cells, and eosinophils and causes proliferation of IgE- and IgG-secreting B cells. It also stimulates the expression of HLA class II antigens via STAT6 (signal transducer and activator of transcription-6) (34) and opposes the Th1 cell inflammatory response. Indeed, IL-4 is considered the pivotal cytokine polarizing the immune response toward a Th2 cell response, and although the initial trigger for IL-4 production remains unknown, genetic variation is likely to play a role (35). The IL-4 polymorphism investigated in this study is a c to t base change in the promotor region of IL-4 at position −590 (36). This promotor polymorphism appears to be functional, with increased transcriptional activity attributed to the variant allele (24). Association of this polymorphism (24, 37, 38) and an IL-4 receptor gain of function polymorphism (25) with atopy provides evidence that IL-4 is an important mediator of allergic disease.

Table 1. PCR-SSP primer specificities and sequences

<table>
<thead>
<tr>
<th>Gene polymorphism</th>
<th>Allele position</th>
<th>Genebank accession no</th>
<th>Forward primer (5' to 3')</th>
<th>Reverse primer (5' to 3')</th>
<th>Annealing position</th>
<th>Conc. (μmol/L)</th>
<th>Annealing position</th>
<th>Conc. (μmol/L)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β [X04500]</td>
<td>2511</td>
<td>c</td>
<td>CTCATCTggCATTgATCTgg</td>
<td>ggTgCTgTTCTCTgCCTCg</td>
<td>1226–1245</td>
<td>3.4</td>
<td>1441–1423</td>
<td>1.36</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t</td>
<td>CTCATCTggCATTgATCTgg</td>
<td>ggTgCTgTTCTCTgCCTCg</td>
<td>1226–1245</td>
<td>3.4</td>
<td>1441–1423</td>
<td>1.36</td>
<td>215</td>
</tr>
<tr>
<td>IL-1 RA [X64532]</td>
<td>11100</td>
<td>c</td>
<td>CCTTCATCCgCTCAgACAgT</td>
<td>TgACgCCTTCTgAGGGTC</td>
<td>11081–11100</td>
<td>0.68</td>
<td>11378–11361</td>
<td>1.7</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t</td>
<td>CCTTCATCCgCTCAgACAgT</td>
<td>TgACgCCTTCTgAGGGTC</td>
<td>11081–11100</td>
<td>0.68</td>
<td>11378–11361</td>
<td>1.7</td>
<td>297</td>
</tr>
<tr>
<td>IL-1 receptor 1 [U14179]</td>
<td>970</td>
<td>c</td>
<td>CCAgCCTggATTTgTCCgg</td>
<td>CAgTggTCgAgTCTgCAg</td>
<td>700–718</td>
<td>3.4</td>
<td>988–970</td>
<td>3.4</td>
<td>288</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t</td>
<td>CCAgCCTggATTTgTCCgg</td>
<td>CAgTggTCgAgTCTgCAg</td>
<td>700–718</td>
<td>3.4</td>
<td>988–970</td>
<td>3.4</td>
<td>288</td>
</tr>
<tr>
<td>IL-4 receptor [NM000418]</td>
<td>11902</td>
<td>a</td>
<td>CAgTCCTCTggCCAgAgAg</td>
<td>CACCgCATgTACAAACTCCT</td>
<td>1221–1239</td>
<td>5.1</td>
<td>1921–1202</td>
<td>3.4</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g</td>
<td>CAgTCCTCTggCCAgAgAg</td>
<td>CACCgCATgTACAAACTCCT</td>
<td>1221–1239</td>
<td>5.1</td>
<td>1921–1202</td>
<td>3.4</td>
<td>700</td>
</tr>
</tbody>
</table>

Polymorphisms are listed as nucleotide substitutions in lower case letters. Annealing positions refer to the numbering of the reference sequence. Primer mixes included control primers that amplify a nonpolymorphic region of HLA-DRB1.

FIG. 1. Example of PCR-SSP genotyping. PCR products have been electrophoresed through 1% agarose and photographed after UV illumination. One sample is represented. Each lane has a control amplicon with or without an allele- or haplotype-specific amplicon. In lanes 13–16 the control band is uppermost (250 bp), with the allele-specific band below. In all other lanes the control band is lowermost (796 bp), with the allele or haplotype specific bands above. Polymorphisms represented are: lanes 1 and 2, IL-1a t/c; lanes 3 and 4, IL-1β (+3962) t/c; lanes 5 and 6, IL-1β (−511) t/c; lanes 7 and 8, IL-1RA t/c; lanes 9 and 10, IL-1 receptor 1 c/t; lanes 11 and 12, IL-4 t/c; lanes 13 and 14, IL-6 a/g; lanes 15 and 16, IL-4 receptor a/g; lanes 17–19, IL-10 haplotypes acc/ata/gcc; lanes 20–23, TGFβ haplotype (−880 to −509) gc/gt/ac/at; lanes 24 and 25, TGFβ (aa263) L/P; lanes 26 and 27, TGFβ (c10) T/I. Lanes 1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 19, 20, 24, and 26 are positive.
TABLE 2. Allele frequencies for IL-4 polymorphism

<table>
<thead>
<tr>
<th>Allele frequencies for IL-4 polymorphism</th>
<th>Graves’ disease (n = 138; 276 chromosomes)</th>
<th>Autoimmune hypothyroidism (n = 77; 154 chromosomes)</th>
<th>All patients (n = 215; 430 chromosomes)</th>
<th>Controls (n = 101; 202 chromosomes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 -590 c allele</td>
<td>259 (0.94)</td>
<td>143 (0.93)</td>
<td>402 (0.93)</td>
<td>173 (0.86)</td>
</tr>
<tr>
<td>IL-4 -590 t allele</td>
<td>17 (0.06)</td>
<td>11 (0.07)p</td>
<td>28 (0.07)</td>
<td>29 (0.14)</td>
</tr>
</tbody>
</table>

\[ P_{\text{exact}}^a = 0.006 \text{ vs. } \text{controls}; P_{\text{c}} = 0.07. \]
\[ P_{\text{exact}}^b = 0.00003 \text{ vs. } \text{controls}; P_{\text{c}} = 0.0004. \]
\[ P_{\text{exact}}^c < 0.00001 \text{ vs. } \text{controls}; P_{\text{c}} < 0.00001. \]

TABLE 3. Genotype frequencies for IL-4 polymorphism

<table>
<thead>
<tr>
<th>Genotype frequencies for IL-4 polymorphism</th>
<th>Graves’ disease (n = 138)</th>
<th>Autoimmune hypothyroidism (n = 77)</th>
<th>All patients (n = 215)</th>
<th>Controls (n = 101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4 -590 c/c</td>
<td>122 (88)</td>
<td>68 (88)</td>
<td>190 (88)</td>
<td>75 (74)</td>
</tr>
<tr>
<td>IL-4 -590 c/t</td>
<td>15 (12)p</td>
<td>7 (9)p</td>
<td>22 (11)p</td>
<td>23 (23)</td>
</tr>
<tr>
<td>IL-4 -590 t/t</td>
<td>1 (&lt;1)</td>
<td>2 (3)</td>
<td>3 (1)</td>
<td>3 (3)</td>
</tr>
</tbody>
</table>

Percentages are in parentheses.

\[ a P = 0.005 \text{ vs. } \text{controls}; OR 0.4 (0.2–0.7); P_{\text{c}} = 0.06. \]
\[ b P = 0.01 \text{ vs. } \text{controls}; OR 0.3 (0.1–0.8); P_{\text{c}} = 0.11. \]
\[ c P = 0.007 \text{ vs. } \text{controls}; OR 0.4 (0.2–0.7); P_{\text{c}} = 0.008. \]

The mechanism by which IL-4 is involved in the development of autoimmune thyroid disease, particularly GD, requires explanation. T cell activation is believed to be the key event in the initiation of autoimmune thyroid disease (39), and thus, cytokines are likely to be intimately involved in the process. The patients with autoimmune thyroid disease in our study exhibited a lower prevalence of the variant t allele than the controls, which may reflect a overall lower activity of IL-4. This would favor an inflammatory immune response mediated by Th1 cells. In humans, Th1 cytokines stimulate the production of an IgG1 isotype response (40), which is the predominant pathogenic TSH receptor autoantibody seen in GD (and sometimes in AIH) (41, 42). IgG1 isotypes are also prevalent among thyroglobulin and peroxidase antibodies (43, 44). Hence, in autoimmune thyroid disease, lower IL-4 activity may result in a propensity to immune response toward cell-mediated immunity. Supporting this mechanism, IL-4 has been shown to inhibit organ-specific autoimmune disease in animals (45, 46).

For GD, the reduced frequency of the variant t allele was highly significant and associated with small confidence intervals (OR, 0.4; 95% confidence interval, 0.2–0.7). Although not directly comparable, these results are in keeping with previously identified candidate genes for GD, such as HLA DR3 and CTLA-4 (relative risks of 2–5 and 2–3, respectively) (2). It is of interest that a similar trend for an association of the IL-4 polymorphism was seen in the group with AIH compared to the control group. There is some evidence suggesting that the two disorders are related and may represent two ends of a spectrum of autoimmune thyroid disease phenotype. Thus, the two diseases commonly cluster within the same family (47), monozygotic twins are described where one has GD and the other has Hashimoto’s thyroiditis (48), and clinically an individual may fluctuate between GD and AIH. Aspects of the pathogenesis of the two disorders are also related; both are associated with antibodies to thyroid tissue and exhibit some similarities in immune function (10).

The lack of a significant association after Bonferroni correction for the group with AIH may be because fewer individuals with AIH were genotyped than those with GD (type 1 error).

Although the above explanation supports a pathophysiological mechanism for the association of this IL-4 variant with autoimmune thyroid disease, it is also possible that this association is not due to the IL-4 gene, but to another gene in linkage disequilibrium. The genes for IL-3, IL-5, IL-13, and granulocyte-macrophage colony-stimulating factor are all in close proximity. Of these, IL-13 deserves further attention because of the major role it plays in the cytokine network (34).

The other cytokine gene polymorphisms evaluated in this study did not reveal any significant associations with autoimmune thyroid disease. Although these negative results exclude any major genetic effect, identifying genes exerting very minor effects or influencing clinical phenotype requires extremely large study cohorts, particularly when several candidate genes are evaluated, and adjustments for multiple comparisons are necessary.

This study has demonstrated a significant association of IL-4 genotype with susceptibility to autoimmune thyroid disease. This suggests that variation in amplification and regulation of immune responses within the thyroid determine clinical disease. However, variation in IL-4 accounts for only part of the abnormal immunoregulatory response seen in autoimmune thyroid disease, and as in other complex diseases, there are likely to be many polymorphic genes, each exerting only a minor effect. This finding is not only of interest to autoimmune thyroid disease, but may also be of relevance to other organ-specific autoimmune diseases. Further examination of polymorphisms in cytokine genes should enhance our understanding of the cytokine network and provide clues to the pathogenesis of particular diseases.

Acknowledgments

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References


