YUKIO IKEDA, WAKAKO YOSHIDA, TORU NOGUCHI, KOICHI ASABA, TATSUYA NISHIOKA, TOSHIHIRO TAKAO AND KOZO HASHIMOTO

Department of Endocrinology, Metabolism and Nephrology, Kochi Medical School, Kochi University, Nankoku, Kochi 783-8505, Japan

Abstract. Interleukin (IL)-12 is a key factor in cell-mediated immunity that drives the development of Th1 cells and stimulates T lymphocytes and natural killer cells to produce interferon (IFN)-γ. The IL-12B gene, which encodes the p40 subunit of IL-12, is located at chromosome 5q31-33 and a linkage finding for autoimmune thyroid disease (AITD) on 5q31-33 in a Japanese population has been reported. It is also reported that the A/C polymorphism in the 3' untranslated region (UTR) of the IL-12B gene (1188A/C) is associated with IL12B mRNA expression levels. We attempted to determine whether genetic polymorphisms of the IL-12B gene are associated with AITD. One hundred three patients with Hashimoto’s thyroiditis, 90 patients with Graves’ disease, and 123 healthy control subjects were recruited. We detected the 1188A/C polymorphism using a PCR-RFLP method and the A/T polymorphism in intron 4 of the IL-12B gene using a cycle sequencing method. These IL-12B gene polymorphisms showed strong linkage disequilibrium, and their genotype and allele frequencies in the patients did not differ from those in the control subjects. Our results suggest that IL-12B gene polymorphisms were unlikely to have an effect on the development of Hashimoto’s thyroiditis or Graves’ disease in Japanese patients.

Key words: Interleukin-12, Interleukin-12B, Autoimmune thyroid disease, Hashimoto’s thyroiditis, Graves’ disease

GENETIC susceptibility to an autoimmune thyroid disease (AITD) such as Graves’ disease and Hashimoto’s thyroiditis has been reported to depend on the human leukocyte antigen (HLA) class II gene [1]; however, the association is not as strong as that of type 1 diabetes mellitus [2, 3]. Further, the concordance rate for AITD in HLA class II identical siblings has been found to be only 7%, suggesting that the HLA region is not intimately involved in susceptibility to AITD. Other non-HLA genetic factors have been presented as candidates for influencing the autoimmunity of AITD, such as the cytotoxic T lymphocyte antigen 4 (CTLA-4) gene. Binding of B-7 on antigen-presenting cells to CD28 on T lymphocytes activates an antigen specific proliferation of T lymphocytes and immune response. CTLA-4 on T lymphocytes regulates this pathway by binding competitively with B7 and down-regulating the T cell receptor, and is considered to be the most likely candidate for IDDM 12 [3, 4]. In addition, there is evidence supporting an association between CTLA-4 and Graves’ disease [5–10], as well as Hashimoto’s thyroiditis [8, 11]. Other candidate genes, such as T cell receptor, TSH receptor and immunoglobulin G marker genes [12, 13], have been investigated; however, no firm conclusions have been reached [14, 15].

Inflammatory cytokines have also been investigated as candidates, as they participate in the induction and effector phases of immune and inflammatory response. Intra-thyroidal inflammatory cells and thyroid follicular cells have been shown to produce a variety of cytokines, thus, these cytokines might play a critical role in the development of AITD. Hunt et al. [16] investigated gene polymorphisms associated with many kinds
of cytokines and found that an interleukin (IL)-4 variant or a closely linked gene had a modest protective effect against the development of AITD, particularly Graves’ disease, although this association remains controversial [17].

IL-12 is a proinflammatory cytokine that drives the development of Th1 cells, which are the main mediators of cell-mediated immunity and, since it also stimulates T lymphocytes and natural killer cells to produce interferon (INF)-γ, the cytokine is known to be a key factor in cell-mediated immunity. IL-12 is composed of 2 subunits, p35 and p40, and the IL-12B gene encodes the p40 subunit of IL-12. Huang et al. [18] identified several polymorphisms in IL-12B genes, including repeat elements in introns 2 and 4, and single nucleotide polymorphic sites in intron 4 and the 3’ untranslated region (UTR). Further, IL-12B has been proposed to be a candidate for involvement in type 1 diabetes mellitus susceptibility [19], though a negligible effect was recently reported [20, 21]. In addition, a whole genome analysis of Japanese AITD [22] found a suggestive linkage with 5q31-q33, where the IL-12B gene is located. In the present study, we investigated the association of IL-12 B gene polymorphisms with AITD in a Japanese population as well as the CTLA-4 gene polymorphism.

**Genotyping of CTLA-4 and IL-12B gene polymorphisms**

Genomic DNA was extracted from whole blood using a commercial kit (SMI test; Sumitomo, Tokyo, Japan). The A/G polymorphism in exon 1 (49A/G) of the CTLA-4 gene was analyzed using a polymerase chain reaction (PCR) method and digestion of the amplified fragments with restriction enzymes (RFLP), as described by Vaidya et al. [5]. Primers used for amplification of the polymorphic region were 5’-CCACGGCTTCTTTCTCGTA-3’ (forward) and 5’-AGTCTCACTCACCTTTGCAG-3’ (reverse). The PCR product was digested with BbvI (New England Biolabs, Beverly, MA), and the digested products were separated on a 2% agarose gel and identified by ethidium bromide staining.

The A/T polymorphism in intron 4 of the IL-12B gene was determined using a cycle sequencing method. The DNA fragments were amplified by a PCR method using sense (5’-ATTAAGCATTGGGGTCTCAC-3’) and antisense (5’-GAACTAGGATCAAATTGTATAC-3’) primers. PCR fragments separated by electrophoresis on an agarose gel were recovered and purified using a commercially available kit (QIAquick PCR Gel Extraction Kit, QIAGEN). The sequences of the PCR fragments were detected using a commercial kit and analyzer (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and ABI PRISM™310 Genetic Analyzer; PE Applied Biosystems, Foster City, CA), with the sense primer used in the PCR, as the sequencing primer.

The A/C polymorphism in the 3’UTR (1188A/C) of the IL-12B gene was analyzed using a PCR-RFLP method previously described by Huang et al. [18]. Primers used for amplification of the polymorphic regions were 5’-TTTGGAGGAAAAGGTGGAAGA-3’ (forward) and 5’-AACATTCCACATCATCCCTGGC-3’ (reverse). PCR products were digested using TaqI (Toyobo, Osaka, Japan), and the digested products were separated on a 3% agarose gel and identified by ethidium bromide staining.

**Statistical analysis**

All data are presented as the mean ± SD. Genotype and allele frequencies were estimated using a chi-square
test. All statistical analyses were performed using StatView version 5.0 (SAS Institute Inc., Cary, NC) on a personal computer. Statistical significance was defined as \( P<0.05 \).

**Results**

**CTLA-4 gene 49A/G polymorphism**

The G allele frequencies of the 49A/G polymorphism were 60.2%, 67.0% and 70.0% in the controls, patients with Hashimoto’s thyroiditis, and those with Graves’ disease, respectively. The frequency was significantly higher in patients with Graves’ disease than in the control subjects \( (P = 0.036) \). The G allele frequency (68.4%) was also significantly higher in total patients with AITD including both Hashimoto’s thyroiditis and Graves’ disease than in the control subjects \( (P = 0.034) \), but was not significantly higher in patients with Hashimoto’s thyroiditis alone. The frequency of the GG genotype was significantly higher in patients with Graves’ disease than in the control subjects \( (54.4\% \text{ vs. } 35.8\%, \text{ respectively, } P = 0.028) \), and the difference was more pronounced between younger (<50 years old) patients with Graves’ disease and younger control subjects \( (30 \text{ of } 51, 58.8\% \text{ vs. } 15 \text{ of } 51, 29.4\%, \text{ respectively, } P = 0.003) \). The GG genotype frequency was also higher in total AITD patients \( (48.7\%) \) than in the control subjects \( (P = 0.028) \), although the frequency in patients with Hashimoto’s thyroiditis alone \( (43.7\%) \) did not differ statistically from that in the control subjects. These results did not change when patients with type 1 diabetes were excluded (data not shown).

**IL-12 B gene polymorphisms**

The A/T polymorphism in intron 4 and the 1188A/C polymorphism of the IL-12B gene showed a strong linkage disequilibrium (Table 1). Allele frequencies of the A/T polymorphism in intron 4 and 1188A/C were comparable between the 3 groups, with T allele frequencies of the A/T polymorphism in intron 4 at 47%, 46% and 50%, and C allele frequencies of the 1188A/C polymorphism at 53%, 54% and 51% in the controls, Hashimoto’s thyroiditis patients, and Graves’ disease patients, respectively. The genotype frequencies in patients with Hashimoto’s thyroiditis or Graves’ disease did not differ from those in the control subjects (Table 2), and the results did not change when compared with those in younger subjects or subjects without type 1 diabetes (data not shown).

**Table 1.** Linkage disequilibrium between A/T polymorphism in intron 4 and A/C polymorphism in 3’ UTR of the IL-12B gene in all of the study subjects

<table>
<thead>
<tr>
<th>3’ Untranslated Region (1188)</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>1</td>
<td>85</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>157</td>
<td>1</td>
<td></td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>72</td>
<td>0</td>
<td>0</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as number of subjects (percentage in parentheses).

**Table 2.** Genotype distribution of the IL-12B polymorphisms

<table>
<thead>
<tr>
<th>A/T polymorphism in intron 4</th>
<th>AA</th>
<th>AT</th>
<th>TT</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects/Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>31</td>
<td>63</td>
<td>29</td>
<td>123</td>
<td>N.S.</td>
</tr>
<tr>
<td>Hashimoto’s thyroiditis</td>
<td>27</td>
<td>55</td>
<td>21</td>
<td>103</td>
<td>N.S.</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>28</td>
<td>40</td>
<td>22</td>
<td>90</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A/C polymorphism in 3’ UTR (1188A/C)</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects/Patients</td>
<td></td>
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<td>64</td>
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<td>N.S.</td>
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<td>103</td>
<td>N.S.</td>
</tr>
<tr>
<td>Graves’ disease</td>
<td>22</td>
<td>39</td>
<td>29</td>
<td>90</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Data are shown as number of subjects (percentage in parentheses). Genotype frequencies were estimated using a chi-square test. N.S.: not significant vs. control.
Discussion

The present results show that the G allele of the CTLA-4 49A/G polymorphism is likely to be found in patients with Graves’ disease, which confirm the results of previous reports [5–10]. The G allele frequencies in the controls and patients with Graves’ disease (60.2% and 70.0%, respectively) were similar to those of the previous study in a Japanese population (58% and 69%, respectively) [10]. In addition to Graves’ disease, an allelic association between the CTLA-4 49A/G polymorphism and Hashimoto’s thyroiditis has also been reported [8, 11]. However, in this study, we could not find a statistical difference in allele frequency or genotype distribution of the CTLA-4 49A/G polymorphism between patients with Hashimoto’s thyroiditis and control subjects. This may suggest that the association is less important in Hashimoto’s thyroiditis than in Graves’ disease in the Japanese population, although the subject number in this study was too small to arrive at any definitive conclusions. It has also been reported that the CTLA-4 49A/G polymorphism is unlikely to be the primary etiological polymorphism; rather, it may be acting as a marker, that is, as a linkage disequilibrium with the etiological polymorphism, which may lie outside the CTLA-4 gene in one of the neighboring genes [23]. Although HLA class II and CTLA-4 genes may be associated with susceptibility to AITD, the contribution of these genetic factors might be no more than 10%, and many other genes, each exerting a minor effect, might possibly be influencing the development of AITD [24].

Increased serum levels of IL-12 have been reported in patients with Graves’ disease as well as those with silent thyroiditis [25, 26]. It was also reported that IL-12 is involved in the development of animal experimental AITD [27]. Morahan et al. [19] reported that the 1188A/C polymorphism of the IL-12 B gene showed strong linkage disequilibrium with the type 1 diabetes susceptibility locus, and suggested that a variation in IL-12 p40 production may influence T-cell responses crucial for either mediating or protecting against this and other autoimmune diseases. However, Dahlman et al. [21] were unable to confirm that finding in a much larger group of type 1 diabetic patients or in those with Graves’ disease. In the present study, we also did not find a significant association between the IL-12 B gene polymorphism and AITD. Morahan et al. [28] reported an association between IL-12B promoter polymorphism and severity of asthma in Australian children, although Noguchi et al. [29] showed no association between the 1188A/C polymorphism and atopic asthma in a Japanese population. In our study population, we found 2 patients with Hashimoto’s disease and 3 patients with Graves’ disease were under treatment for asthma, and that 6 patients with Hashimoto’s disease and 6 patients with Graves’ disease had history of asthma. However, the exclusion of these patients from the study subjects did not change the results (data not shown). Although a definitive conclusion cannot be made until analysis of a much larger group of subjects with AITD, our results suggest that the A/C polymorphism in the 3’ UTR and the A/T polymorphism in intron 4 of the IL-12B gene, which are in linkage disequilibrium, are unlikely to exert an effect toward the development of Hashimoto’s thyroiditis or Graves’ disease in Japanese patients.

References


