Association of the CTLA-4 Gene 49 A/G Polymorphism With Type 1 Diabetes and Autoimmune Thyroid Disease in Japanese Children

Toshihide Ishihara, MD1, Yoshie Shimura, MD1, Koji Kobayashi, MD1, Kisho Kobayashi, MD1, Shin Amemiya, MD1, Mie Mochizuki, MD1, Yoshiko Nakagomi, MD1, Kazumichi Onigata, MD2, Shinya Tamai, MD3, Akira Kasuga, MD4, Shinpei Nanazawa, MD1

OBJECTIVE — To clarify the role of the T-lymphocyte–associated-4 (CTLA-4) polymorphism in the susceptibility to child-onset type 1 diabetes with regard to its clinical characteristics and complications with autoimmune thyroid disease (AITD) in the Japanese population.

RESEARCH DESIGN AND METHODS — The CTLA-4 49 A/G polymorphism was detected by the PCR-restriction fragment–length polymorphism (RFLP) method in 97 type 1 diabetic subjects and 20 patients with Graves’ disease, a cohort which included 4 patients who also had type 1 diabetes.

RESULTS — The genotypes and allele frequencies of this polymorphism did not differ between the type 1 diabetic subjects and the control subjects. The G allele frequency was 63.9% in the type 1 diabetic subjects. The G allele frequency in the subgroup of patients with a high titer of autoantibodies to the GAD antibody (Ab) was 72.9% (P = 0.0499 vs. control subjects), in the subgroup of patients without HLA DRB1*0405, it was 72.6% (P = 0.0271 vs. control subjects); and in the subgroup of patients with a residual β-cell function, it was 78.6% (P = 0.0391 vs. control subjects). The G allele frequency in the patients with Graves’ disease was also significantly higher at 78.1% (P = 0.0405 vs. control subjects). Furthermore, the frequency in our diabetic subjects complicated with Graves’ disease was even higher (87.5%).

CONCLUSIONS — We have demonstrated that a distinct association exists between the G allele of CTLA-4 and high values of GAD Ab, residual β-cell function, and the absence of HLA-DRB1*0405.

From the 1Department of Pediatrics, Faculty of Medicine, University of Yamanashi, Yamanashi, Japan; the 2Department of Pediatrics, Gunma University School of Medicine, Gunma, Japan; the 3Department of Pediatrics, Yamato Mutual Hospital, Kanagawa, Japan; and the 4Department of Internal Medicine, Tokyo Denryoku Hospital, Tokyo, Japan.

Address correspondence and reprint requests to Shin Amemiya, MD, Department of Pediatrics, Faculty of Medicine, University of Yamanashi, Shimokato 1110, Tamahochi, Nakakoma-gun, Yamanashi, 409-3898, Japan. E-mail: shina@res.yamanashi-med.ac.jp.

Received for publication 10 June 2002 and accepted in revised form 7 December 2002.

Abbreviations: Ab, antibody; AITD, autoimmune thyroid disease; CTLA-4, T-lymphocyte–associated-4; RFLP, restriction fragment–length polymorphism; TRAb, thyroid stimulating hormone receptor autoantibody; TSH, thyroid stimulating hormones.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.
Table 1—CTLA-4 exon 1 polymorphism in type 1 diabetic patients, AITD patients, and control subjects

<table>
<thead>
<tr>
<th>Genotype (%)</th>
<th>Allele (%)</th>
<th>P value: G allele frequency vs. control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>AA</td>
</tr>
<tr>
<td>Type 1 diabetes</td>
<td>97</td>
<td>17.5</td>
</tr>
<tr>
<td>with anti-pancreatic Ab+</td>
<td>74</td>
<td>20.3</td>
</tr>
<tr>
<td>with high (&gt;10 units/ml) GAD Ab</td>
<td>35</td>
<td>11.4</td>
</tr>
<tr>
<td>with TPO Ab+</td>
<td>32</td>
<td>12.5</td>
</tr>
<tr>
<td>with residual β-cell function</td>
<td>14</td>
<td>7.1</td>
</tr>
<tr>
<td>Type 1 diabetes with AITD</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>with Graves’ disease</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>AITD</td>
<td>27</td>
<td>7.4</td>
</tr>
<tr>
<td>with Graves’ disease</td>
<td>16</td>
<td>6.3</td>
</tr>
<tr>
<td>Control subjects</td>
<td>60</td>
<td>20.0</td>
</tr>
</tbody>
</table>

P determined by the χ² test using *Yates’ correction and †Fisher’s exact probability tests.

[TRAb] positive hyperthyroidism (also known as Graves’ disease). Informed consent was obtained from each patient, and the study was approved by the Medical Ethics Committee of the University of Yamanashi, Japan.

**Methods.** Genomic DNA was extracted from peripheral mononuclear cells from each subject using a Smitest Ex-R&D DNA extraction kit (Sumitomo Metal, Ibaragi, Japan). HLA-DRB1 typing was performed by the PCR-restriction fragment—length polymorphism (RFLP) method using the Smitest HLA-DRB1 genotyping kit (Sumitomo Metal). Since the HLADR1B1 genotype varies to a large extent, a large number of control subjects should be prepared for any statistical comparison with a relatively small number of type 1 diabetic patients, as in the case of many other autoimmune diseases. Therefore, we used data for the HLADR1B1 genotype frequencies in the Japanese general population provided by the HLA workshop (25), as shown by Sugihara et al. (26).

The A/G allele polymorphism in the CTLA-4 gene exon 1 position 49 (Thr49Ala) on chromosome 2q33 was defined by the PCR-RFLP method using the specific primers 5'-GCT CTA CCT GAA GAC CT-3' and 5'-AGT CTC ACT CAC CTT TGC AG-3', as described by Donner et al. (10). PCR was performed using 0.2 μg genomic DNA, 1 unit AmpliTaq DNA polymerase (Perkin-Elmer, NJ), 20 pmol of each primer, and 8 mmol dNTPs under the following conditions: initial denaturation for 4 min at 94°C, followed by 30 cycles of annealing for 45 s at 58°C, extension for 45 s at 72°C, and denaturation for 45 s at 94°C, with a final extension for 4 min at 72°C. The restriction enzyme Bbv1 (England Bio-Rad, Boston) digested the sequence if an A allele was present at position 49, resulting in 88/74-bp fragments. A sequence with an A allele at position 49 was not digested and resulted in a 162-bp fragment, as determined by 3% acrylamide gel electrophoresis.

The serum levels of antipancreatic β-cell autoantibodies including GAD Ab and IA-2 Ab were measured as previously described (27–29). Any serum sample with >0.020 units/ml of GAD Ab index, 1.30 units/ml of GAD Ab, or 1.30 units/ml of IA-2 Ab was considered positive. Sera from patients with type 1 diabetes were collected on their first visit to our hospitals. Patients were divided into three groups according to their GAD Ab values: patients with negative GAD Ab values, patients with low GAD Ab values (<10.0 units/ml), and patients with high GAD Ab values (≥10.0 units/ml)). The classification of GAD Ab levels was determined by the classification of the Japanese multicenter trial in prevention of β-cell destruction in type 1 diabetes (30).

The abrupt onset of type 1 diabetes was defined as the presence of clinical symptoms at the time of diagnosis, as we also had type 1 diabetic patients who had no clinical symptoms at the time of diagnosis whose diabetes was detected mostly by urine glucose screening at school (29).
Table 2—Distribution of CTLA-4 exon 1 polymorphism in Japanese type 1 diabetic patients

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>AA</td>
</tr>
<tr>
<td>*0405</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>47</td>
</tr>
<tr>
<td>(−)</td>
<td>42</td>
</tr>
<tr>
<td>*0901</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>41</td>
</tr>
<tr>
<td>(−)</td>
<td>48</td>
</tr>
<tr>
<td>Control subjects</td>
<td>60</td>
</tr>
</tbody>
</table>

Data represent percentage of patients in genotypes or chromosomes in alleles. P is determined by χ² test.

was 63.9% in the type 1 diabetic subjects. However, this increased significantly to 72.9% in the subgroup of patients with a high titer of GAD Ab (P = 0.0499) and to 78.6% in the subgroup with a residual β-cell function 3 years after onset (P = 0.0391), as compared with the control subjects.

HLA DRB1 alleles and CTLA-4 gene polymorphism

Analysis of the distribution of HLA DRB1 alleles revealed that the susceptibility alleles, including DRB1*0405, *0802, and *0901, occurred with significantly higher frequencies in the type 1 diabetic patients than in the control subjects (25,26). With respect to HLA, these results are consistent with those of a previous report on Japanese patients with child-onset type 1 diabetes (26,31).

As shown in Table 2, the frequency of the G allele was significantly higher in the patients strictly without DRB1*0405 (P = 0.0486 vs. DRB1*0405 positive patients, P = 0.0271 vs. control subjects).

CTLA-4 gene polymorphism andAITD and residual β-cell functions

The G allele frequency in the type 1 diabetic subjects with a residual β-cell function 3 years after onset was higher than that in the control subjects (Table 1). Furthermore, the genotype’s frequency in the subgroup, which has a residual β-cell function 3 years after onset, shows statistical significance (AA 5.7, AG 13.8, GG 32.8%, P = 0.0365 vs. control subjects).

CTLA-4 gene polymorphism andAITD

Of the 97 diabetic patients, 4 were affected by AITD (2 had Graves’ disease, 2 had Hashimoto disease). This incidence rate (4.1%) was obviously higher in comparison with the incidence rate (0.008–0.01%) among young Japanese <19 years old and the incidence rate (0.14–0.26%) among the Japanese control subjects (32,33).

The G allele frequency among the Graves’ disease patients was significantly higher than the frequency in control subjects (P = 0.0405, Table 1). However, the frequency of the G allele in our diabetic patients complicated with Graves’ disease was much higher (87.5%), although the value did not reach statistical significance due to the small number of patients. These four patients characteristically had adolescent-onset diabetes and consistently high GAD Ab titers.

The percentage of positive TPO Ab patients was 33.0% (32/97) of the total type 1 diabetic patients (Table 1), a value clearly much higher than the percentage of positive TPO Ab subjects (2.0–5.2%) in the Japanese control subjects.

CONCLUSIONS — The binding signal of the CTLA-4 molecule to the T-cell receptor molecule delivers a negative signal for T-cell activation (4). However, the function of CTLA-4 according to its genotype remains unclear.

Regarding the Th1/Th2 imbalance, the acute-onset and the early deterioration of β-cell function in type 1 diabetes are caused when Th1 is predominant, whereas high GAD Ab values depend on Th2 predominance. Our results demonstrate that the G allele of CTLA-4 gene had close association of residual β-cell function and high titer of GAD Ab in juvenile-onset type 1 diabetes.

Similar to Abe et al. (17), we report that there is a high frequency of GG genotypes among GAD Ab–positive patients with adult-onset type 1 diabetes. On the other hand, Awata et al. (9) reported that the frequency of the G allele did not differ among type 1 diabetic patients, including adult-onset patients. The subgroup of type 1 diabetic patients who required insulin treatment initiation within 1 month of onset had a significantly higher frequency of the G allele. All the patients in our study required insulin treatment initiation within 1 month of onset.

Takara et al. (16) reported the association between the G allele of this polymorphism in Japanese younger-onset type 1 diabetic patients (onset at <30 years) and AITD. In their report, the G allele frequency (66%) in younger-onset type 1 diabetic patients with AITD was higher than those without AITD (61%). The G allele frequency among our child-onset diabetic patients with AITD (71.4%) was higher than in that study, which suggests that child-onset type 1 diabetic patients with AITD may experience a stronger immune response, including CTLA-4, than adult-onset patients with AITD.

In an in vitro study, Guo et al. (34) reported that a shift from Th1 to Th2 responses increased production of the thyroid antibody. Therefore, the onset of Graves’ disease is most likely affected by a cytokine imbalance of Th2 predominance caused by TRAb. The association of the G allele of CTLA-4 with Graves’ disease among Japanese people has been previously demonstrated (7,9). Furthermore, in the present study, the frequency of the G allele was high among type 1 diabetic patients with AITD, in particular among those with Graves’ disease. In addition, measurement of GAD Ab values among AITD patients has revealed that their positive rate of GAD Ab is higher than that of patients without diabetes (21). Furthermore, among type 1 diabetic patients, the frequency of GAD Ab is higher in patients with AITD than in those without AITD, and GAD Ab remains positive longer in patients with AITD (22). The association of the immune reaction with the G allele and Th2 is supported by the above findings. Due to the relatively few cases, the present study is unable to prove a meaningful association between the CTLA-4 G gene and type 1 diabetic patients, but a strong association was shown between this gene and patients with AITD, particularly among those with Graves’ disease.

Our series of patients consisted of
classic child-onset type 1 diabetic patients according to HLA distribution. Among a Caucasian population, previous analyses of the correlation between the polymorphism of CTLA-4 and HLA-DRB1 have revealed that the frequency of the G allele is significantly higher in patients with DR4 than in the control subjects (11,13). Saïah et al. (11) explained that the activation of the pathway depends on DR genotypes (T-cells through T-cell receptor DR-antigen complex molecule followed by CTLA-4). In the patients without DR3, the G allele of CTLA-4 has a stronger diabetogenic effect than DR3-positive subjects. In the present study, the G allele was more prevalent in the type 1 diabetic patients, who do not have DRB1*0405. HLA-DRB1*0405 and DRB1*0901 alleles are susceptible for type 1 diabetes in the Japanese population (26,31). It may be possible to speculate in the cases without DRB1*0405, T-cell activation is less sensitive to the CTLA-4-mediated pathway after the initiation of T-cell receptor and DR molecule-antigen complex. Therefore, the CTLA-4-mediated diabetogenic effect is significant in the DRB1*0405-negative population but not significant in the DRB1*0405-positive type 1 diabetic patients.

The present study has demonstrated that there are some associations between the G allele of CTLA-4 and autoantibody production, between high values of GAD Ab and complications such asAITD, and between the A allele of CTLA-4 and early exhaustion of β-cell function and HLA-DRB1*0405 Japanese child-onset type 1 diabetes. These findings suggest that CTLA-4 gene polymorphism may directly influence Japanese type 1 diabetic patients, such as a G allele subgroup with increased production of autoantibodies or with Graves’ disease, by means of predominant Th2 in the immunoregulatory balance.

Acknowledgements—This study was supported by a Scientific Research Grant from the Ministry of Education, Science, Sports and Culture, Japan (C-11670750 to S.A., 1999–2000).

This study was partly presented to the 6th Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society and European Society for Pediatric Endocrinology, 6–10 July 2001, Montreal, Canada.

References