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

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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.

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Type 1 diabetes is a multifactorial disease to which the HLA region provides a major genetic contribution. Several non-HLA loci have been detected as putative susceptibility genes by genome-wide scanning (1–3). Chromosome 23q31 contains two genes encoding cytotoxic T-lymphocyte–associated-4 (CTLA-4) and CD28. The CTLA-4 gene encodes a T-cell surface molecule that binds to the B7 molecule in antigen-presenting cells (4). This binding signal delivers a negative signal to a T-cell and mediates the inactivation of T-cell or B-cell functions (4–6). CTLA-4 is widely believed to play a role in some autoimmune diseases. The association of the A/G allele polymorphism at position 49 in exon 1 (49 A/G) of the CTLA-4 gene with autoimmune thyroid disease (AITD) has been investigated in Japanese as well as other ethnic populations (7–11). The association between this polymorphism and type 1 diabetes has been confirmed among Caucasian patients and other ethnic groups (10–14), but not in Japanese people (9,15–17). Among type 1 diabetic patients, the percentage of patients with an antithyroid autoantibody is high (17,18), as is the percentage of patients who also have AITD (16,18–20). In addition, among AITD patients, the positive ratio of patients with autoantibodies to the GAD antibody (Ab) is high, in addition with the high titers (21,22).

Therefore, we aimed to clarify the role of this polymorphism in the susceptibility to child-onset type 1 diabetes in Japanese patients, with the emphasis on its clinical characteristics, including the early deterioration of β-cell function and complications associated with AITD.

RESEARCH DESIGN AND METHODS

A total of 97 subjects with child-onset type 1 diabetes who were <16 years old at the onset of diabetes (37 males and 60 females) were examined along with 60 nondiabetic age-matched control subjects. Type 1 diabetes was diagnosed according to the classification of the Japan Diabetes Society and the American Diabetes Association (23,24). We also studied a group of 20 AITD patients (4 diabetic patients and 16 similarly-aged nondiabetic patients with the thyroid stimulating hormone receptor autoantibody

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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*

		Genotype (%)			Allele (%)		*P* value: G allele frequency *vs.* control subjects
	n	AA	AG	GG	A	G	
Type 1 diabetes	97	17.5	37.1	45.4	36.1	63.9	NS*
with anti-pancreatic Ab+	74	20.3	33.8	45.9	37.2	62.8	NS*
with high (>10 units/ml) GAD Ab	35	11.4	31.4	51.2	27.1	72.9	0.0499*
with TPO Ab+	32	12.5	37.5	50	31.3	68.8	NS*
with residual β-cell function	14	7.1	5.6	64.3	21.4	78.6	0.0391†
Type 1 diabetes with AITD	7	0	57.1	42.9	28.6	71.4	NS†
with Graves' disease	4	0	25	75	12.5	87.5	NS†
AITD	27	7.4	40.7	51.9	27.8	72.2	NS*
with Graves' disease	16	6.3	31.3	62.5	21.9	78.1	0.0405†
Control subjects	60	20.0	45.0	35.0	42.5	57.5	

P determined by the χ^2 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 HLADRB1 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 HLADRB1 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 CTT 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 *Bbv*I (England Bio-Rad, Boston) digested the sequence if a G 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 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) according to the classification of a 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). Residual β-cell function was determined by evaluation of the serum or urine C-peptide level at 3 years after onset. If the serum C-peptide level was <0.13 nmol/l (0.4 ng/ml) or the urine level <3.3 μmol/day (10 μg/day) or 3.2 nmol/μmol creatinine (5 μg/g creatinine), islet β-cell function was considered lost. Two subjects were excluded, as they had not yet had a 3-year history of diabetes at the time of this study.

Among the type 1 diabetic patients, the measurement of antithyroid peroxidase (TPO) Ab was performed by radioimunoassay (Cosmic, Japan), with the limit of detection being 0.3 units/ml.

The diagnosis of AITD was based on the criteria below. The diagnosis of Graves' disease was based on hyperthyroidism, high levels of the thyroid hormones, low levels of thyroid stimulating hormones (TSH), and the presence of TRAb or the high uptake of I^{131}. The diagnosis of Hashimoto disease was based on the presence of the positive antithyroglobulin antibody (TgAb) in addition to positive TPO Ab. In the present study, all the patients with Hashimoto disease had hypothyroidism and required thyroid hormone supplements.

Statistical analysis. Statistical analysis was done by the χ^2 test using Yates' correction or Fisher's exact probability test in the cases indicated. Statistical significance was established at the $P < 0.05$ level.

RESULTS

Frequencies in the 49 A/G polymorphism of CTLA-4

The genotypes and allele frequencies of the 49 A/G polymorphism of CTLA-4 did not differ between the type 1 diabetic subjects and the control subjects (Table 1).

The frequency of antipancreatic β-cell autoantibodies did not differ significantly between each genotype. However, among the 35 type 1 diabetic patients with high GAD Ab values, the frequency of the G allele was significantly higher than among the control subjects ($P = 0.0499$, Table 1).

Characteristics of type 1 diabetic patients with AA, AG, and GG genotypes in CTLA-4 polymorphism

The distribution of sex, age, and onset-type did not differ significantly among patients with AA, AG, and GG genotypes. As shown in Table 1, the G allele frequency

Table 2—*Distribution of CTLA-4 exon 1 polymorphism in Japanese type 1 diabetic patients*

		Genotype			Allele	
	n	AA	AG	GG	A	G
*0405						
(+)	47	19.1	44.7	36.2	41.5	58.5
(−)	42	14.3	26.2	59.5	27.4	72.6*†
*0901						
(+)	41	12.2	51.2	36.6	37.8	62.2
(−)	48	20.8	22.9	56.3	32.3	67.7
Control subjects	60	20.0	45.0	35.0	42.5	57.5

Data represent percentage of patients in genotypes or chromosomes in alleles. *P* is determined by χ^2 test. *$P = 0.0486$ vs. *0405(+); †$P = 0.0271$ vs. control subjects.

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. DRBI*0405 positive patients, $P = 0.0271$ vs. control subjects).

CTLA-4 gene polymorphism and AITD 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 and AITD

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 adolescence-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 in 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). Saiah 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 diabetologenic 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 as AITD, 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.

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