Association of CTLA-4 Gene A/G Polymorphism in Japanese Type 1 Diabetic Patients With Younger Age of Onset and Autoimmune Thyroid Disease

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OBJECTIVE — We studied the association between type 1 diabetes with autoimmune thyroid disease (AITD) and A/G allele polymorphism in exon 1 of the CTLA-4 gene in a Japanese population.

RESEARCH DESIGN AND METHODS — We studied 74 Japanese type 1 diabetic patients with or without AITD and 107 normal subjects to identify the association between CTLA-4 polymorphism and type 1 diabetes using polymerase chain reaction–restriction fragment length polymorphism analysis.

RESULTS — The frequency of the CTLA-4 G allele differed significantly between the type 1 diabetic patients (61%) and the normal control subjects (48%) ($P = 0.016$). The difference in the CTLA-4 G allele became greater between patients with a younger age of onset of type 1 diabetes (age at onset <30 years) and the normal control subjects (64% and 48%, respectively). However, the frequency of the CTLA-4 G allele did not differ between type 1 diabetic patients with younger and older age of onset (64% vs. 57%). The G allele frequencies in the patients with younger-onset type 1 diabetes and AITD increased more than in the control patients ($P = 0.025$). These differences reflected a significant increase in the frequency of G/G genotype—that is, 54% in those with younger-onset type 1 diabetes and AITD, 39% in those without AITD, and 28% in control subjects.

CONCLUSIONS — An association was detected between the CTLA-4 gene polymorphism and younger-onset type 1 diabetes with AITD. The G variant was suggested to be genetically linked to AITD-associated type 1 diabetes of younger onset in this Japanese population. The defect in these patients presumably lies in a T-cell–mediated autoimmune mechanism.

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Type 1 diabetes, as well as such autoimmune thyroid diseases (AITDs) as Graves' and Hashimoto disease, is said to be a T-cell–mediated autoimmune disease (1). The development of type 1 diabetes is controlled by multiple susceptibility genes, including HLA class II region (IDDM1) and the insulin promoter region (IDDM2) (1–3). The CTLA-4 gene, which has been mapped to the *IDDM 12* locus (2q33), is a good candidate gene for this disease (3–8).

The activation of T-cells requires a costimulatory signal mediated by CD28/B7 interaction (9,10). The CTLA-4 gene product, a T-cell surface molecule that binds to the B7 molecule on the antigen-presenting cell, delivers a negative signal to the T-cell and mediates apoptosis (11,12). The expression of CTLA-4 on the T-cells may affect the course of an ongoing immune process (13,14). It is known that CTLA-4Ig, a fusion protein that combines the extracellular binding domain of CTLA-4, prolongs the survival of rabbit xenografts in NOD mice (15).

Yanagawa et al. (16) were the first to report an association between Graves' disease and the G allele at position 49 in the CTLA-4 gene among Caucasian patients. A linkage to type 1 diabetes of the A/G mutation in exon 1 of CTLA-4 gene, leading to a Thr/Ala substitution in the leader peptide, has been demonstrated in different populations (6–8,17). Because type 1 diabetes sometimes occurs in association with AITD (18,19), we investigated whether or not the CTLA-4 variation was associated with type 1 diabetes with AITD in patients with type 1 diabetes with onset at a younger age (<30 years) (6,7,18). Because the patients with type 1 diabetes previously reported included middle-aged individuals, we recruited patients with type 1 diabetes with onset at a younger age to exclude the autoimmune process that may occur in older patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS

We analyzed the CTLA-4 gene Thr[17]Ala polymorphism. We recruited for study 51 Japanese patients with type 1 diabetes (18 men and 33 women; mean age at type 1 diabetes onset was 10.9 ± 7.7 years). Another 23 type 1 diabetic patients developed diabetes after the age of 30 years (11 men and 12 women, mean age 45.8 ± 12.8 years). The diagnosis of type 1 diabetes was based on the patient's clinical features and laboratory data, including the presence of anti-GAD antibody (GAD65) and a urinary C-pep-

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Abbreviations: AITD, autoimmune thyroid disease; PCR, polymerase chain reaction; TG, thyroglobulin; TPO, thyroid peroxidase.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical and laboratory findings in type 1 diabetic patients and normal control subjects*

	Type 1 diabetic patients			Normal control subjects
	Total	Onset <30 years	Onset ≥30 years	
n	74	51	23	107
Sex (M/F)	29/45	18/33	11/12	48/59
Onset age (years)	21.8 ± 18.8	10.9 ± 7.7	45.8 ± 12.8	—
Range of age (years)	0.0–71.5	0.0–26.4	30.1–71.5	—
GAD65				
Antibody positive (%)	93.6*	94.6*	92.9*	—
Antibody titer (U/ml)	36.3 ± 60.4†	30.8 ± 44.5†	36.7 ± 43.0†	—
IA-2				
Antibody positive (%)	75 (21/28)*	85 (17/20)*	50 (4/8)*	—
Antibody titer (U/ml)	8.4 ± 14.6†	9.6 ± 16.3†	4.4 ± 7.3†	—

Data are *n*, %, or means ± SD. *Within 1 year of onset of type 1 diabetes; †mean ± SD in patients with positive antibody.

tide level of <20 µg/day (20). Blood was sampled at intervals of 6–12 months for the measurement of the thyroid autoantibodies (anti–thyroid peroxidase [TPO] and anti-thyroglobulin [TG] antibodies), GAD65 antibody, and IA-2 antibody. Antibodies were determined by radioimmunoassay (20,21). Cutoff levels of GAD65 antibody, IA-2 antibody, and thyroid autoantibody were 1.2, 0.5, and 0.4 U/ml, respectively. Thyroid autoantibodies were measured >3 times in all patients with diabetes during the follow-up period. When the thyroid autoantibodies were detected >2 times in same patient, autoantibodies were considered to be positive. The diagnosis of AITD was based on the finding of palpable goiter or the presence of chronic thyroiditis with ultrasonography examination in the absence of goiter, and the presence of thyroid autoantibodies. Only 1 of the 74 patients had hypothyroidism. Another had hyperthyroidism of Graves' disease. A total of 107 normal healthy subjects (48 men and 59 women) were used as control subjects. Informed consent for participation was obtained from all. To facilitate analysis, the patients with type 1 diabetes were divided into 4 groups according to the age of disease onset and the presence of AITD.

Genomic DNA was prepared from peripheral white blood cells. The CTLA-4 genotypes were determined in duplicate by polymerase chain reaction (PCR) using the methods described by Donner et al. (5). The presence of G alleles was determined in each subject by PCR amplification of CTLA-4 followed by digestion with *Bbv*I, which acts on the G variant but not on the A variant. PCR products were detected by electrophoresis in agarose gel.

Statistical analysis of the differences between groups was determined by analysis of variance, χ^2 test with Yates' correction, or Fisher's exact probability test. Either 2×2 or 3×2 contingency tables were used with analysis by allele or genotype, respectively. A level of $P < 0.05$ was considered statistically significant.

RESULTS

The clinical and laboratory findings in the patients with type 1 diabetes and control subjects are shown in Tables 1 and 2. Slightly more patients with younger-onset type 1 diabetes with AITD than those without AITD were positive for IA-2 antibodies. No difference in the frequency of GAD65 antibody was obtained between type 1 diabetic patients with younger and older age of onset. When thyroid autoantibodies were measured at intervals of 6–12 months, 55% of patients with younger-onset type 1 diabetes had AITD (Table 2) and 52% of patients with older-onset diabetes had AITD (data not shown). More parents of younger-onset type 1 diabetes patients with AITD were positive for thyroid autoantibody than were parents of those without AITD ($P = 0.037$, Table 2).

There was a significant difference in the CTLA-4 G allele frequency between the patients with type 1 diabetes (61%) and the normal control subjects (48%) ($P = 0.016$) (Table 3). However, there was no difference in the frequency of the CTLA-4 G allele between patients with younger- and older-onset type 1 diabetes (64 vs. 57%). The G allele frequency of CLTA-4 gene was 66% in patients with younger-onset type 1 diabetes with AITD, 61% in those without AITD, and 48% in normal control subjects (Table 3). Younger-onset type 1 diabetes with AITD increased the G allele frequency over that of control subjects ($P = 0.025$). However, there was no difference in relation to G allele frequency between patients with younger-onset type 1 diabetes without AITD and control subjects. Only the G/G frequencies of the CTLA-4 gene in younger-onset type 1 diabetes with AITD became greater than in control patients (54 vs. 28%; $P = 0.038$). But there was no difference the G/G frequencies between younger-onset patients without AITD and normal control subjects (39 vs. 28%, respectively).

CONCLUSIONS

The identification of genes involved in the development of type 1 diabetes presents a major challenge (1–3). The genes of the immune system are attractive candidate genes, and nearly all

Table 2—*Clinical and laboratory findings in younger-onset type 1 diabetic patients with or without AITD*

	Type 1 diabetes (onset <30 years)	
	With AITD	Without AITD
n	28	23
Sex (M/F)	8/20	10/13
Age of onset (years)	11.0 ± 7.5	10.8 ± 8.0
Range of age (years)	0.0–26.4	1.0–25.1
GAD65		
Antibody positive (%)	90.0*	100*
Antibody titer (U/ml)	27.3 ± 41.6†	35.0 ± 48.6†
IA-2		
Antibody positive (%)	92.3 (12/13)*	71.4 (5/7)*
Antibody titer (U/ml)	6.0 ± 9.3†	16.4 ± 24.3†
Thyroid antibody positivity in parents (%)	58.3 (7/12)‡	12.5 (1/8)

Data are *n*, %, or means ± SD. *Within 1 year of onset of type 1 diabetes; †mean ± SD in patients with positive antibody; ‡$P = 0.037$ (Fisher's exact probability test).

Table 3—*The frequency of the CTLA-4 A/G genotypes and alleles in type 1 diabetic patients and normal control subjects*

	Genotype				Allele	
	A/A	A/G	G/G	P (χ^2)	G allele frequency	P (χ^2)
Type 1 diabetes	16/74 (22)	25/74 (34)	33/74 (45)	0.062 (5.556)	91/148 (61)	0.016 (4.253)
Younger onset (onset age <30)	10/51 (20)	17/51 (33)	24/51 (47)	0.052 (5.920)	65/102 (64)	0.013 (6.135)
With AITD	6/28 (21)	7/28 (25)	15/28 (54)	0.038 (6.525)	37/56 (66)	0.025 (5.026)
Without AITD	4/23 (17)	10/23 (44)	9/23 (39)	0.338 (2.167)	28/46 (61)	0.160 (1.995)
Older onset (onset age ≥30)	6/23 (26)	8/23 (35)	9/23 (39)	0.577 (1.117)	26/46 (57)	0.384 (0.757)
Normal control subjects	34/107 (32)	43/107 (40)	30/107 (28)	—	103/214 (48)	—

Data are *n* (%). *P* values are vs. normal control subjects.

the accessible genes have been analyzed extensively (3). Among the candidate genes, CTLA-4 gene variation has been investigated (4–7,18). Recently, Marron et al. (8) suggested that *IDDM12* was either CTLA-4 or an unknown gene in close proximity. Three polymorphic regions within the CTLA-4 gene are known: A/G polymorphism at position 49 in exon 1, a microsatellite polymorphism in a 3′ untranslated region of exon 3, and a promoter polymorphism with cysteine/thymine substitution at position −318 (5,16). Among the 3 polymorphic regions within the CTLA-4 gene, the primary risk marker for type 1 diabetes remains unknown. Although Donner et al. (5) suggested that the microsatellite 102 bp polymorphism was the dominant variant in type 1 diabetes, the promoter polymorphism may be linked to exon 1 polymorphism and the predisposition to type 1 diabetes as seen with AITD (16,17). Whether CTLA-4 A/G polymorphism in exon 1 affects the concentration of CTLA-4 protein remains to be clarified.

Positive associations can sometimes be detected in only 1 population due to genetic heterogeneity. Thus, to ensure that the association of a gene with a disease is relevant, such studies have to be repeated in different populations. The present study detected an association between the CTLA-4 gene polymorphism and younger-onset type 1 diabetes with AITD in a Japanese population. We recruited younger-onset type 1 diabetic patients in the analysis of AITD. The higher prevalence of AITD among these patients resulted from the serial measurement of thyroid autoantibodies in 1 patient. Some patients had a transient increase of autoantibodies. The prevalence of autoantibodies was 28% within 1 year of disease onset, with a prevalence of 27% in younger diabetic and 33% in older diabetic patients (data not shown). We also reported that the prevalence of AITD was 22% at onset of disease and 28% at 3 years after onset (20). Recently, Imagawa et al. (22) reported that the prevalence of AITD was 40% in new-onset type 1 diabetics.

The higher frequency of the CTLA-4 G allele was obtained in the patients with type 1 diabetes of younger onset as compared with normal control subjects. Differences became more significant when analysis considered the presence of AITD. If we could analyze a larger number of patients, the apparent difference in G allele frequency would be obtained between type 1 diabetic patients with and without AITD. Any dysregulation of the T-cell activation process may affect the pathogenesis of such autoimmune disorders as type 1 diabetes or AITD. Although the CTLA-4 association may be weaker in type 1 diabetes than in an AITD such as Graves' disease (18,23), it may confer susceptibility to the destruction of β-cells by a different mechanism (20). The CTLA-4–linked susceptibility locus in type 1 diabetes may differ from that in Graves' disease. In that respect, it is of interest that the gene that codes for IA-2 (tyrosine phosphatase), a type 1 diabetes autoantigen, has been mapped to chromosome 2q35 in humans (24). This may be relevant to the increased prevalence of IA-2 antibody in younger-onset type 1 diabetic patients with AITD.

We conclude that the CTLA-4 Ala^{17} polymorphism in exon 1 is a marker for AITD-associated type 1 diabetes of younger onset in a Japanese population. Although the exon 1 Ala/Thr substitution of the CTLA-4 gene is not known to have functional relevance, this polymorphism may be linked to the (AT)n microsatellite that could affect RNA stability (16). Further studies are needed to analyze the association among the A/G polymorphism in exon 1, a microsatellite polymorphism in exon 3, and a promoter at position −318.

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