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Association of Cytotoxic T Lymphocyte Antigen-4 Gene Polymorphisms and HLA Class II Alleles with the Development of Type 1 Diabetes in Korean Children and Adolescents

We studied the association of cytotoxic T lymphocyte antigen-4 gene (*CTLA4*) polymorphisms with the development of type 1 diabetes (T1D) in Korean children and adolescents. A total of 176 Korean subjects (92 females and 84 males) with childhood-onset T1D were studied. The A/G polymorphism at position 49 in *CTLA4* exon 1 and the C/T polymorphism at position -318 in the *CTLA4* promoter were analyzed by PCR-RFLP methods. The genotype and allele frequencies of the *CTLA4* polymorphisms in the T1D patients were not different from those in the controls. These polymorphisms were not associated with the clinical characteristics or the development of autoimmune thyroid disease in the T1D patients. The frequency of the A allele was significantly higher in the patients that did not have two out of the three susceptible HLA-DRB1 alleles, which were DRB1*0301, *0405 and *09012, compared to the controls (P<0.05). These results suggest that *CTLA4* polymorphisms do not directly confer any susceptibility to T1D. However, a *CTLA4*-mediated susceptibility effect on the development of T1D might be significant in children and adolescents that do not have susceptible HLA class II alleles.

Key Words : *Diabetes Mellitus, Type 1; Genes; Cytotoxic T-lymphocyte Antigen 4; HLA*

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INTRODUCTION

Type 1 diabetes (T1D) is an organ-specific autoimmune disease that is characterized by infiltration of lymphocytes into the pancreatic islets and pancreas-specific autoantibodies in the serum (1). A variety of genetically predisposed factors and contributing factors have been known to influence the pathogenesis of T1D. Some evidence has suggested that the susceptible genes to T1D are associated with the amplification of the immune response and the rate of progression of the disease; the role of these genes appear to be more important during childhood than during adult life (2).

The association of HLA alleles and the development of T1D has been shown by studies on diverse ethnic groups. However, a genetic predisposition solely conferred by the HLA is not sufficient to explain the mechanism that leads to the development of T1D. Therefore, it has been hypothesized that several genes are involved in the development of T1D (3).

The cytotoxic T lymphocyte antigen-4 gene (*CTLA4*) and the gene encoding CD28 have been mapped to chromosome 2q33. CTLA-4 is a glycoprotein receptor expressed on activated T cells and CD28 is involved in the regulation process of the activation of T cells by antigen-presenting cells and subsequent cellular immunity (4). *CTLA4* has been considered to be a permissive candidate gene involved in the etiology of autoimmune diseases; this is because CTLA-4 plays a role in the regulation of the activation of T cells and as well as T cell and B cell interactions (5).

According to prior studies on the association of the development of various autoimmune diseases and the *CTLA4* gene, the 49 A/G polymorphism in *CTLA4* exon 1 has been reported to be involved in the development of Graves' disease (6), Hashimoto thyroiditis (7), a portion of Addison's disease and rheumatoid arthritis (8). A significant association of T1D with the *CTLA4* polymorphisms was reported by Nistico et al. (9) for the first time; this association has been additionally reported by other studies in a variety of ethnic groups (10, 11). However, in studies conducted in Japan (12) and other countries (13, 14), the association of the *CTLA4* polymorphisms with the development of T1D has not been confirmed.

The primary purpose of this study was to investigate whether the *CTLA4* gene was associated with the development of T1D in Korean children and adolescents. Furthermore, we studied whether interactions between the *CTLA4* gene and susceptible HLA class II alleles had a role in the pathogenesis of T1D.

MATERIALS AND METHODS

This study included 176 patients (92 females, 84 males) with T1D, who were diagnosed during childhood and adolescence (mean age, 7.5±4.0 yr) from 1992 to 2002 at diabetes clinic of Seoul National University Children's Hospital. In addition, 90 healthy individuals were recruited as a control group. The study was explained to all patients, and their written consent was obtained. The diagnosis of T1D was based on the blood glucose level according to the World Health Organization diagnostic guidelines, clinical symptoms, absolute insulin-dependency, and pancreas-specific autoantibodies.

We reviewed the clinical characteristics, such as diabetic ketoacidosis at the initial presentation, age of onset, family history of T1D, pubertal status at the onset of diabetes, and presence of concomitant autoimmune thyroid disease to determine whether there was an association between the *CTLA4* polymorphisms and the clinical characteristics. Among the study subjects, there were 31 patients (17.6%) with autoimmune thyroid disease that were diagnosed by thyroid function tests, anti-thyroid autoantibodies, and/or TSH receptor stimulating antibodies.

Genomic DNA was extracted from peripheral mononuclear cells using the Wizard DNA purification kit (Promega, Maison, WI, U.S.A.), and quantified by spectrophotometer. HLA-DRB1 alleles were analyzed by grouping of DRB1 using a Dynal RELI SSO HLA-DRB1 typing kit (Dynal Biotech Inc., Lake Success, NY, U.S.A.) and primers described previously (15). Each HLA-DRB1 allele was determined by the single strand conformation polymorphism (SSCP) method. HLA-DQB1 alleles were analyzed by applying PCR-RFLP and PCR-SSCP methods as described previously (16). The subjects were classified according to the known susceptible DRB1 alleles (17).

For the analysis of the A/G polymorphism at position 49 in the *CTLA4* exon 1, the PCR-RFLP method was used as described previously (7). The primers used were 5′-GCTCTACTTCCTGAAGACCT-3′ (forward) and 5′-AGTCTCACTCACCTTTGCAG-3′ (reverse). Amplified samples were digested with the specific restriction enzyme *Bbv*I (New England BioLabs, Beverly, MA, U.S.A.) at 37°C for 2 hr, electrophoresed on a 3% agarose gel for 30 min, stained with ethidium bromide, and evaluated. A 162 bp band was determined to be the A allele and the 88 bp and 74 bp bands were determined to be the G allele.

The C/T polymorphism at position -318 in the *CTLA4* promoter was analyzed by PCR-RFLP methods using known primers 5′-AAATGAATTGGACTGGATGGT-3′ (forward) and 5′-TTACGAGAAAGGAAGCCGTG-3′ (reverse) as described previously (18). The amplified DNA products were treated with the *Mse*I restriction enzyme (New England BioLabs) at 37°C for 2 hr. The formation of 132/115 bp fragments was determined to be the T allele and the detection of the complete 247 bp fragment was identified as the C allele.

The measured values are presented as mean±standard deviation. The comparisons of the HLA genotypes of the patients and the controls were performed using the two-tailed Fisher's exact test. The corrected *P* value (P_c) was obtained by multiplying the number of all genotypes included. The odds ratio (OR) was calculated by the Woolf method, and the 95% confidence interval (CI) was applied. For the cases with 0 variables, a Haldane's modification method was used. A $P<0.05$ was considered statistically significant.

RESULTS

The genotype and allele frequencies of the *CTLA4* exon 1 polymorphism in T1D patients were not different from those in the control subjects (Table 1). The distribution of the *CTLA4* exon 1 polymorphsims in patients with T1D and autoimmune thyroid disease (n=31) was not different from that in the control subjects. There was no relationship of the *CTLA4* exon 1 polymorphism with the clinical characteristics of the patients, such as presence of ketoacidosis, age of disease onset, gender, pubertal status at initial presentation, and concomitant autoimmune thyroid disease.

We analyzed the distribution of the *CTLA4* exon 1 poly-

Table 1. Distribution of the polymorphism at position 49 in exon 1 of the *CTLA4* gene in patients with type 1 diabetes and controls

Patients	n	Genotypes			Alleles	
		A/A	A/G	G/G	A	G
Type 1 diabetes	176	24 (13.6)	58 (33.0)	94 (53.4)	106 (30.1)	246 (69.9)
With AITD	31	5 (16.1)	10 (32.3)	16 (51.6)	20 (32.3)	42 (67.7)
DKA at diagnosis	43	6 (14.0)	12 (27.9)	25 (58.1)	24 (27.9)	62 (72.1)
SMR at diagnosis						
Prepubertal	143	22 (15.3)	46 (32.2)	75 (52.4)	90 (31.5)	196 (68.5)
In puberty	33	2 (6.1)	12 (36.4)	19 (57.6)	16 (24.2)	50 (75.8)
Controls	90	13 (14.4)	31 (34.4)	46 (51.1)	57 (31.7)	123 (68.3)

Data are n (%).
AITD, autoimmune thyroid disease; DKA, diabetic ketoacidosis; SMR, sexual maturity rating.

morphism according to the presence or absence of susceptible HLA-DRB1 alleles (DRB1*0301, *0405 and *09012). In the patients without the HLA-DRB1*0301 allele, the frequency of the A allele was significantly higher than in the control subjects (43.6% vs. 31.7%; $P<0.05$).

We analyzed the distribution of the *CTLA4* exon 1 polymorphism in a subgroup of patients that did not have two out of the three susceptible DRB1 alleles. According to the analysis of the genotype frequency, the frequency of the A/A genotype in the patients without the DRB1*0301 and *09012 alleles was significantly higher compared to the control subjects (36.0% vs. 14.4%; $P<0.05$) (Table 2). According to the analysis of the allele frequency, the frequency of the A allele in the patients without the DRB1*0301 and *0405 alleles was significantly higher than in the control subjects (45.5% vs. 31.7%; $P<0.05$). In addition, the frequency of the A allele was significantly higher in the patients without the DRB1*0301 and *09012 alleles (52.0% vs. 31.7%; $P<0.01$), and the patients without the DRB1*0405 and *09012 alleles (54.2% vs. 31.7%; $P<0.05$), compared to the control subjects.

The genotype and allele frequencies of the *CTLA4* promoter polymorphism were not different in the comparison between the T1D patients and the control subjects (Table 3). The distribution of the *CTLA4* promoter polymorphism in patients with both T1D and autoimmune thyroid disease was not different from that in the control subjects. When the patients were divided into subgroups based on the presence of the susceptible HLA-DRB1 alleles, the distribution of the *CTLA4* promoter polymorphism was not different in the patients and the control subjects.

DISCUSSION

It has been reported that the intracellular transduction signals formed by the complex of T cell receptor molecules and CTLA-4 molecules suppress the activation of T cells (19). The CTLA-4 molecule encodes the T cell receptor that plays a role in the control of the proliferation of T cells, as well as mediating apoptosis of T cells. Therefore, it has been considered to be a strong candidate molecule involved in the development of T cell-mediated autoimmune disease. However, the functional relationship of the CTLA-4 protein with human disease has not been elucidated to date.

The distribution of the *CTLA4* exon 1 polymorphism among Asians and Caucasians shows a clear difference. The frequency of the G allele of *CTLA4* exon 1 has been reported to be 68% among Koreans (20), 57.5% (21) and 63% (22) among Japanese, and 34.2% (10) and 36% (7) among Caucasians. On the other hand, the frequency of the T allele in the *CTLA4* promoter region has been reported to be 7-14%

Table 2. Distribution of the polymorphism at position 49 in exon 1 of the *CTLA4* gene in type 1 diabetic patients according to susceptible HLA-DRB1 alleles

HLA	n	Genotypes			Alleles	
		A/A	A/G	G/G	A	G
DRB1*0301(-)/0405(-)	33	8 (24.2)	14 (42.4)	11 (33.3)	30 (45.5)†	36 (54.5)
DRB1*0301(-)/09012(-)	25	9 (36.0)*	8 (32.0)	8 (32.0)	26 (52.0)‡	24 (48.0)
DRB1*0405(-)/09012(-)	12	3 (25.0)	7 (58.3)	2 (16.7)	13 (54.2)§	11 (45.8)
Controls	90	13 (14.4)	31 (34.4)	46 (51.1)	57 (31.7)	123 (68.3)

Data are n (%).
*$P<0.05$, OR=3.33 (95% CI 1.21-9.11) vs. controls; †$P<0.05$, OR=1.80 (95% CI 1.01-3.21) vs. controls; ‡$P<0.01$, OR=2.34 (95% CI 1.24-4.42) vs. controls; §$P<0.05$, OR=2.55 (95% CI 1.08-6.02) vs. controls.

Table 3. Distribution of the C/T polymorphism at position -318 in the promoter of the *CTLA4* gene in patients with type 1 diabetes and controls

Patients	n	Genotypes			Alleles	
		C/C	C/T	T/T	C	T
Type 1 diabetes	176	140 (79.5)	34 (19.3)	2 (1.1)	314 (89.2)	38 (10.8)
With AITD	31	25 (80.6)	6 (19.4)	0	56 (90.3)	6 (9.7)
DKA at diagnosis	43	33 (76.7)	9 (20.9)	1 (2.3)	75 (87.2)	11 (12.8)
SMR at diagnosis						
Prepubertal	143	113 (79.0)	28 (19.6)	2 (1.4)	254 (88.8)	32 (11.2)
In puberty	33	27 (81.8)	6 (18.2)	0	60 (90.9)	6 (9.1)
Controls	90	71 (78.9)	17 (18.9)	2 (2.2)	159 (88.3)	21 (11.7)

Data are n (%).
AITD, autoimmune thyroid disease; DKA, diabetic ketoacidosis; SMR, sexual maturity rating.

among Asians and Caucasians (23), without a marked ethnic difference noted to date.

Our results showed that the distribution of the *CTLA4* exon 1 polymorphism and the the *CTLA4* promoter polymorphism in patients with T1D were not different from those in control subjects. Recent studies on the association of the *CTLA4* polymorphisms and susceptibility to T1D in various ethnic populations showed inconsistent results. According to the studies that included Japanese populations, the distribution of the *CTLA4* exon 1 polymorphisms in patients with T1D was not different from that in normal individuals (21, 22). By contrast, Steck et al. (24) observed that the frequency of the C/C genotype at position -318 in the *CTLA4* promoter was significantly lower in patients with T1D compared to controls, and they concluded that this polymorphism was associated with T1D.

Meanwhile, a clear association was detected between the *CTLA4* genotype and the degree of expression of the CTLA-4 protein (25). According to this study, in individuals with thymidine (-318T) in *CTLA4* promoter region, after the stimulation of cells, the expression of CTLA-4 on the cell surface and the expression of *CTLA4* mRNA in unstimulated cells were significantly increased. In a recent study, Ueda et al. (26) reported that a reduction in the level of a soluble isoform of CTLA-4 (sCTLA-4) mRNA, associated with the disease-susceptible haplotype of *CTLA4*, could lead to reduced blocking of CD80/CD86, causing increased activation through CD28, or to less stimulation of CD80/CD86. In order to verify the association of the *CTLA4* polymorphisms with the pathogenesis of T1D, distinctive difference in the distribution of *CTLA4* genotypes between patients and controls should be identified. However, this has not been consistently demonstrated to date.

The results of recent studies suggest that *CTLA4* polymorphisms are associated with the clinical characteristics of patients with T1D. Abe et al. (22) reported a patient group with initial diabetic ketoacidosis and positive for the ICA512 antibody had a different distribution of the *CTLA4* polymorphisms compared to control subjects. Other studies showed that the frequency of the G allele of *CTLA4* exon 1 was higher in patients with a high titer of GAD antibodies and high residual function of β-cells compared to the control subjects (21, 27). Such results may indicate that *CTLA4* polymorphisms are involved in the more potent immune response and specific clinical characteristics of patients with T1D. In addition, Takara et al. (28) reported that the frequency of the G allele of *CTLA4* exon 1 was significantly higher in patients with both T1D and autoimmune thyroid disease concomitantly, compared to the controls. However, the results of our study showed no association of *CTLA4* polymorphisms and the clinical characteristics of patients with T1D. Therefore, further study is needed to clarify the association of *CTLA4* polymorphisms with specific clinical characteristics of patients with T1D.

We analyzed the relationship between the *CTLA4* exon 1 polymorphism and HLA-DRB1 alleles to determine whether their interactions had a role in the pathogenesis of T1D. In the diabetic patients that did not have two out of the three susceptible DRB1 alleles, that is DRB1*0301, *0405 and *09012, the frequency of the A allele of *CTLA4* exon 1 was significantly higher than that in the control subjects. This finding suggests that the *CTLA4* polymorphism was associated with the development of T1D only in the patients that had no specific susceptible HLA-DRB1 alleles.

There have been several reports regarding the association of *CTLA4* polymorphisms with HLA-DRB1 allele in the pathogenesis of T1D. Djilali-Saiah et al. (29) reported that in diabetic patients with the DR4 haplotype, the frequency of the G allele of *CTLA4* exon 1 was significantly higher. Mochizuki et al. (21) reported that in children with T1D that did not have susceptible DRB1*0405 allele, there was a higher frequency of G allele of *CTLA4* exon 1 compared to normal children. On the other hand, Donner et al. (30) conducted a combined transmission analysis of the *CTLA4* polymorphisms and HLA DQA1-DQB1 and reported that in patients with T1D that had susceptible haplotypes, such as HLA DQA1*0301-DQB1*0302 or DQA1*0501-DQB1*0201, the protective 84-bp allele of the $(AT)_n$ repeat of *CTLA4* polymorphism was not protective against the development of T1D. Therefore, when patients do not have susceptible HLA class II alleles, the *CTLA4* polymorphism appears to play some role in the pathogenesis of T1D.

Mochizuki et al. (21) explained that the sensitivity of T-cell activation to the CTLA4 mediated pathway after the initiation of the T-cell receptor and DR molecule-antigen complex may be decreased in the absence of susceptible HLA-DRB1 alleles. However, because the distribution of both *CTLA4* and the HLA class II alleles vary in different ethnic populations, further study of the association of these two genes is needed.

In conclusion, the results of this study suggest that HLA-DRB1 and DQB1 alleles play a primary role in pathogenesis of T1D and that the *CTLA4* exon 1 polymorphism is a genetic factor that mediates the disease associated susceptibility in patients that do not have specific susceptible HLA class II alleles. This concept requires further study and confirmation among different ethnic groups.

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