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Association between CTLA-4 +49 A/G Polymorphism and Type 1B Diabetes in Japanese Population

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+49 A/G polymorphism of CTLA-4 gene has been suggested to be associated with type 1 diabetes in some populations. However, a functional significance of the +49 A/G polymorphism is unknown, because it is believed the polymorphism does not affect the function of the CTLA-4 molecule. In this study, we examined the +49 A/G polymorphism of the CTLA-4 gene in 30 Japanese type 1 diabetic patients (14 type 1B and 16 type 1A) and 40 non-diabetic subjects in a case-control study, and stratified patients according to genotype of the polymorphism. The distribution of genotype frequencies differed between type 1 diabetic patients and controls ($p < 0.01$). When the subjects were subdivided into type 1A and type 1B subgroups, a significant difference in G allele frequency was found only between type 1B patients and controls, whereas G allele frequency tended to be higher in type 1A diabetic patients than controls. Type 1B patients displayed more severe metabolic decompensation (higher plasma glucose concentration, lower urinary C-peptide levels, higher insulin requirement, and higher serum amylase levels), and were found to be more prone to diabetic ketoacidosis than type 1A patients. After stratification by genotype, differences in urinary C-peptide and serum amylase levels between type 1A and type 1B patients were found to be due to differences in the GG genotype subgroup, whereas in the AG subgroup those differences disappeared. In conclusion, the +49 A/G polymorphism of CTLA-4 gene was associated with the occurrence of type 1B diabetes in a Japanese population, and type 1B diabetics with a GG genotype were associated with more severe cell dysfunction than their type 1A counterparts.

Key Words: CTLA-4; polymorphism; type 1 diabetes; type 1B diabetes; autoantibody.

Introduction

CTLA-4 is considered the most likely candidate gene for IDDM12 and other autoimmune disorders because of its important role in the T-cell proliferative response. However, the etiology of IDDM12 has not been identified *(1)*. The A/G variation at position +49 (+49 A/G) in the first exon of the gene, which was been reported as a +49*G allele associated with IDDM12 in several populations *(1,2)*, is an unlikely candidate itself because the threonine to alanine substitution in the leader peptide is not expected to affect the function of CTLA-4 molecule *(3)*. Furthermore, the association between +49 A/G polymorphism and islet autoantibodies remains divergent *(4–6)*. The above facts have prompted us to examine the role of +49 A/G polymorphism of CTLA-4 gene in type 1B diabetes, defined as diabetes not associated with immunological evidence of β-cell autoimmunity but for which insulin therapy is required for survival. It has been reported that approx 10% of Japanese patients with type 1 diabetes may have type 1B without ex-pression of any autoantibodies [antibodies to glutamic acid decarboxylase (GADAs), islet cells (ICA), and insulinoma-associated protein 2 (IA-2)] *(7)*. Although it is believed autoimmunity is not involved in β-cell dysfunction in type 1B diabetes, the pathogenesis is poorly understood.

In this study, we examined +49 A/G polymorphism of CTLA-4 gene in 30 type 1 diabetic patients to elucidate the genetic difference between type 1A and type 1B diabetes.

Results

Clinical Characteristics of Diabetic Patients at Diagnosis

Details of the clinical data regarding type 1A (antibody-positive) and 1B (antibody-negative) diabetic patients are shown in Table 1. Type 1B patients, compared with type 1A, significantly showed a higher mean fasting plasma glucose concentration, similar HbA_{1c} levels, and a lower mean value for urinary C-peptide. A lower mean arterial pH value was observed in type 1B diabetics, suggesting they were more prone to diabetic ketoacidosis than type 1A diabetics. Type 1B patients also had higher serum amylase levels than type 1As. Type 1Bs showed higher insulin requirements during follow-up 3 mo after the diagnosis. There were no differences in other clinical parameters (onset-age, BMI, dura-

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Table 1
Clinical Characteristics of Diabetic Patients at Diagnosis (All Patients)

	Type 1A (Autoantibody-positive)	Type 1B (Autoantibody-negative)	*p* Value
N	16	14	
Male:Female	8:8	6:8	
Age of onset (yr)	36.14 ± 5.06	26.25 ± 4.194	0.217
DKA onset (no.)	0	3	
Duration of hyperglycemic symptoms before diagnosis (days)	71.33 ± 22.42	33.67 ± 14.21	0.2386
BMI (kg/m^2)	20.44 ± 0.73	21.42 ± 0.73	0.456
FBS (mg/dL)	262.62 ± 13.79	466.40 ± 77.79	0.011
HbA_{1c} (%)	10.35 ± 0.68	10.24 ± 1.20	0.6639
Urinary C-peptide (μg/d)	17.50 ± 2.61	7.83 ± 1.32	0.002
Arterial pH	7.391 ± 0.008	7.234 ± 0.054	0.005
Serum amylase (IU/L)	102.67 ± 13.15	153.89 ± 20.47	0.0393
Insulin dose (U/kg of body weight)	0.256 ± 0.05	0.529 ± 0.074	0.0025

Table 2
Association of CTLA-4 +49 Polymorphism in Type 1 Diabetic Patients and Controls

	Type 1 diabetic patients (*n* = 29)	Controls (*n* = 40)
Genotype frequencies[a]		
GG	19(66%)	10(25%)
AG	10(34%)	27(68%)
AA	0(0%)	3(7%)
Allele frequencies[b]		
G	48(83%)	47(59%)
A	10(17%)	33(41%)
Phenotype frequencies		
G positive	29(100%)	37(93%)
A positive	10(34%)	30(75%)

[a] $p = 0.0023$.
[b] $p = 0.0026$.
Odds ratio for G allele = 3.37, 95%CI = 7.54–1.49.

Table 3
CTLA-4 +49 Polymorphism in Type 1 Diabetic Patients Analyzed with Respect to Type 1A and Type 1B Subtypes

	Type 1A diabetic patients (*n* = 16)	Type 1B diabetic patients (*n* = 14)	Controls (*n* = 40)
Genotype frequencies[a]			
GG	9(56%)	12(86%)	10(25%)
AG	7(44%)	2(14%)	27(68%)
AA	0(0%)	0(0%)	3(7%)
Allele frequencies[b]			
G	25(78%)	26(93%)	47(59%)
A	7(22%)	2(7%)	33(41%)

[a] $p = 0.0004$ (for Type 1B diabetic patients vs.controls).
[b] $p = 0.0009$ (for Type 1B diabetic patients vs.controls).
Odds ratio for G allele in Type 1B diabetic patients = 9.1, 95%CI = 40.85–2.01.

tion of hyperglycemic symptoms). Two type 1B patients with abrupt-onset (symptom duration of 2 and 5 d, respectively) high plasma glucose levels (710 and 860 mg/dL, respectively) but low HbA_{1c} (6.1 and 8.3%, respectively) were considered fulminant type 1B diabetics *(9)*.

Genotype and Allele Frequencies of the +49 A/G Polymorphism in Patients and Controls (Table 2)

Genotype frequencies differed significantly between type 1 diabetics and controls, with the GG genotype occurring more frequently in the former than the latter (66% vs 25%, $p = 0.0023$); the same tendency was found in allele frequency, with 83% G allele in diabetics vs 59% in controls ($p = 0.0026$). There was no difference in distribution of the polymorphism between male and female type 1 patients (data not shown).

CTLA-4 +49 A/G Polymorphism in Type 1A (Autoantibody-Positive) and Type 1B (Autoantibody-Negative) Diabetics (Table 3)

GG genotype in type 1B diabetics occurred more frequently than in controls (86% vs 25%, $p = 0.0004$). Similarly, the G allele in type 1Bs was found more frequently than in controls (93% vs 59%, $p = 0.0009$). The frequency of GG genotype and G allele tended to be higher in type 1A patients than in controls ($p = 0.0623$ and $p = 0.0532$, respectively), but was not as pronounced as that between type 1Bs and controls.

CTLA-4 +49 A/G Polymorphism and Clinical Characteristics

We compared clinical features of type 1A and type 1B diabetics stratified by genotype (Table 4). We found significantly

Table 4
Characteristics of Type 1A and Type 1B Diabetic Patients Stratified by Genotype of +49 A/G Polymorphism

	AG				GG				
	All	Type 1A	Type1B	*p*	All	Type 1A	Type 1B	*p*	*p**
N	9	7	2		21	9	12		
Male:Female	5:4	3:4	2:0		9:12	5:4	4:8		
Age of onset (yr)	32.78 ± 5.99	35.17 ± 8.87	28.00 ± 4.51	0.7963	30.88 ± 4.57	37.5 ± 8.69	24.88 ± 6.19	0.1556	0.7341
DKA onset (no.)	1	0	1		2	0	2		
Duration of hyperglycemic symptoms before diagnosis (d)	31.71 ± 8.36	31.75 ± 10.87	31.67 ± 15.90	0.8597	55.36 ± 16.06	90.83 ± 30.51	34.25 ± 19.44	0.2008	0.6277
BMI (kg/m^2)	21.19 ± 1.16	20.60 ± 1.66	22.37 ± 1.13	0.4386	20.47 ± 0.62	20.71 ± 0.78	20.5 ± 0.99	0.848	0.7528
FBS (mg/dL)	413.71 ± 94.33	271.00 ± 8.07	613.67 ± 165.12	0.0253	325.47 ± 39.70	257.38 ± 22.28	390.13 ± 62.62	0.0929	0.6468
HbA1c (%)	12.19 ± 0.82	12.35 ± 0.46	11.93 ± 2.65	0.4386	8.90 ± 0.61	8.67 ± 0.75	9.22 ± 1.12	0.8075	0.0069
Urinary C-peptide (μg/d)	12.48 ± 3.70	15.16 ± 3.80	7.03 ± 1.29	0.0518	13.86 ± 2.35	18.53 ± 3.26	8.42 ± 1.68	0.0066	0.5107
Arterial PH	7.31 ± 0.06	7.38 ± 0.01	7.21 ± 0.09	0.0339	7.33 ± 0.04	7.40 ± 0.01	7.26 ± 0.06	0.0216	0.4159
Serum amylase (IU/L)	140.50 ± 17.01	125.66 ± 6.23	165.33 ± 45.57	0.4561	107.08 ± 19.12	86.29 ± 20.43	148.17 ± 23.89	0.0321	0.1579
Insulin dose (U/kg of body weight)	0.36 ± 0.09	0.22 ± 0.02	0.54 ± 0.14	0.0339	0.39 ± 0.07	0.16 ± 0.08	0.52 ± 0.09	0.0104	0.9368

*p = AG vs GG (all).

greater HbA_{1c} in the AG group (12.19 ± 0.83%) than in the GG group (8.90 ± 0.61%; $p = 0.0069$). The data also revealed that the difference in glucose concentration observed in all patients (Table 1) was attributable to a more pronounced difference in the AG group. Similarly, differences for urinary C-peptide and serum amylase levels were more pronounced in the GG group (Table 4).

Discussion

CTLA-4 functions as an important regulator of T-cell activation, and plays an important role in maintaining peripheral T-cell tolerance and thus protects against autoimmunity *(10)*. It is usually considered a candidate gene for IDDM12. Association of the +49 A/G polymorphism of CTLA-4 gene with type 1 diabetes has been observed in some populations. However, the functional significance of this polymorphism is unknown, as it was believed that it did not affect the function of the CTLA-4 leader peptide *(3)*. Furthermore, a lack of association between +49 A/G polymorphism and islet autoantibodies has been reported *(6)*. Thus, we hypothesized that the mechanism of association between +49 A/G polymorphism and type 1 diabetes does not involve autoimmunity. We therefore presumed that this polymorphism associates with non-antibody-associated type 1B diabetes. In the present study, we found a significant association between the polymorphism and type 1B diabetes in a Japanese population (Table 3). Type 1A diabetics showed a higher frequency of GG genotype and G allele than controls; although our sample size was small, the possibility cannot be denied of an association between the CTLA-4 +49 A/G polymorphism and type 1A diabetes. However, the difference between type 1A patients and controls was not as clear as that seen between type 1B patients and controls, suggesting that this polymorphism is more prevalent in type 1B than type 1A diabetes. The type 1B patients in our study manifested significantly higher plasma glucose concentration, lower urinary C-peptide levels, and higher insulin requirement compared with type 1A patients. We also found type 1Bs were more prone to diabetic ketoacidosis and significantly higher serum amylase levels than type 1As. Of the 14 type 1B diabetics, only 2 patients were consistent with fulminant type 1B diabetes, characterized by the absence of GAD antibodies, abrupt-onset, short duration of hyperglycemic symptoms (approx 1 wk), high serum amylase levels, and low HbA_{1c} values (approx 8%) *(9)*. This is at variance with the subtype described by several previous studies characterized by DKA-onset and subsequent non-insulin dependency *(11–13)*.

Some studies reported type 1B diabetes was possibly secondary to viral infection *(14,15)*, but only two type 1B diabetics in our study appeared to have symptoms of viral infections before the onset of disease [neither of them had any anti-virus antibody: Coxsackie virus A-9; Coxsackie virus B-3, 4, 5; influenza A (H1N1, H3N2); mumps virus; cytomegalovirus]. Consistent with a previous study *(16)*, our data revealed a relationship between positive GADAs and preserved β-cell function, because type 1A diabetics (who were all GADAs-positive in our study) had higher urinary C-peptide levels than autoantibody-negative type 1B diabetics (Table 1).

Adjusted values of clinical features according to the CLTA-4 +49 A/G genotype revealed GG was associated with lower HbA_{1c} values, and GG/type 1B with more β-cell destruction (Table 4). However, the mechanism by which +49 A/G polymorphism affects β-cells, independent of autoimmunity in the pancreas, is unknown. Although GG/type 1B accounted entirely for the difference of serum amylase levels observed in all patients (Table 1), whether or not it is involved in pancreatic exocrine dysfunction as suggested in a previous study by Imagwa et al. *(9)* remain to be clarified, because we could not obtain the histopathological data of the patients' pancreas within 5 mo after the initial diagnosis of diabetes. The role of this polymorphism on development of type 1 diabetes should thus be further evaluated.

In conclusion, the +49 A/G polymorphism was found to be associated with type 1B diabetes in a Japanese population. Compared with type 1A diabetics, type 1B diabetics in our population were prone to diabetic ketoacidosis, had more severe β-cell dysfunction and required greater doses of insulin therapy. The mechanism by which the GG genotype was associated with more severe β-cell dysfunction remains to be elucidated.

Materials and Methods

Subjects

We studied 30 Japanese type 1 diabetic patients (14 males/16 females) at Kanazawa University Hospital over a 10-yr period. Subjects had a mean age at onset of 25.3 ± 3.0 yr and required insulin continuously after the diagnosis of the type 1 diabetes. The diagnosis of type 1 diabetes was based on the 1997 Committee of the American Diabetes Association criteria *(8)*. Fourteen patients were classified as having type 1B (idiopathic) diabetes due to lack of evidence of autoantibodies (GADAs, ICA, IA-2). Of these 14, 2 were a subtype of abrupt onset with the characteristics described by Imagawa et al. *(9)*. The other 16 patients with at least one GAD antibody, ICA, or IA-2 were classified as type 1A diabetics. All of them had GADAs, 4 had both GADAs and ICA, and 2 were positive for both ICA and IA-2. Forty non-diabetic age- and sex-matched subjects (20 males and 20 females, age 21–60, BMI: 21.7 ± 0.8) with a normal glucose tolerance (as assessed by a 75-g oral glucose tolerance test according to the 1985 World Health Organization criteria), and an absence of family history of diabetes mellitus and autoimmune disease served as control subjects. The study was performed under informed written consent from all subjects and was approved by the Ethics Committee of Kanazawa University.

PCR–Restriction Fragment Length Polymorphism

Genomic DNA was extracted from peripheral blood leukocytes using standard procedures. An A → G substitution at nucleotide 49 in exon 1 of the CTLA-4 gene was defined as CTLA-4 +49A/G polymorphism. This SNP was determined by the PCR–restriction fragment length polymorphism (RFLP). The sequences of the primers were 5'-AAG GCTCAGCTGAACCTGGT-3' (forward primer) and 5'-CTGCTGAAACAAATGAAACCC-3' (reverse primer) as described previously *(3)*. The forward primer was designed with a single base mismatch for the last nucleotide (underline), generating a BstEII (New England Biolabs Inc.) site for the detection of the +49 A/G SNP. PCR products were incubated at 60°C overnight using 6 U of BstEII per reaction. Digested products were electrophoresed on 10–20% gradient acrylamide gel (PAGEL, ATTO, Tokyo, Japan). Digested A allele yields a fragment of 130 bp and the G allele yields an intact 152 bp fragment.

Autoantibody Measurement

Serum antibodies were measured within 3 mo after the initial diagnosis of diabetes. GAD antibody (GADA) was determined at sampling by means of a commercially available radioimmunoassay (RIA) kit (Cosmic, Tokyo, Japan). Sera were considered to be GADA-positive when the level was >1.5 units/mL (the detection limit of the assay was determined to be 1.3 units/mL). Islet-cell antibodies were measured by an indirect immunofluorescence method (with an abnormal value defined as <5 Juvenile Diabetes Foundation units), IA-2 antibodies with an immunoprecipitation assay kit (with an abnormal value defined as <0.75 units per milliliter).

Statistical Analysis

All data are shown as mean ± SEM. χ^2-test for 3× or 2 × 2 tables was used to compare differences. StatView 5.0 for Macintosh (Abacus Concepts, Berkeley, CA) was used for these analyses. A p value of less than 0.05 was considered statistically significant.

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