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Differences in the Contribution of CTLA4 Gene to Susceptibility to Fulminant and Type 1A Diabetes in Japanese Patients

Running Title: *CTLA4 Polymorphism in Fulminant Type 1 Diabetes*

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Abstract

OBJECTIVE: We examined the contribution of the *CTLA4* gene in the susceptibility to fulminant type 1 diabetes (T1D), and compared them with “classic” type 1A diabetes (T1AD).

RESEARCH DESIGN AND METHODS: We genotyped the +49G>A and CT60 G>A of the *CTLA4* in fulminant T1D (n=55), “classic” T1AD (n=91), and healthy control subjects (n=369). We also assessed serum levels of soluble form of CTLA4.

RESULTS: The +49GG and CT60GG genotypes were associated with T1AD (P<0.001). In contrast, the CT60AA genotype, but not +49G>A, was associated with fulminant T1D (P<0.05), especially in patients carrying HLA-DR4 (P<0.001). Serum levels of sCTLA4 were significantly decreased in patients with fulminant T1D (P<0.05).

CONCLUSIONS: These results suggest that *CTLA4* CT60 affects the genetic susceptibility to fulminant T1D. Furthermore, the contribution of the *CTLA4* to the disease susceptibility is distinct between fulminant T1D and “classic” T1AD.

Fulminant type 1 diabetes (T1D) is a subtype of T1D characterized by extremely rapid onset that can be classified in type 1B diabetes (1). Although frequent flu-like symptoms just before the onset suggest the contribution of virus infection in the etiology of fulminant T1D, both environmental and genetic factors are largely unknown. Susceptibility to “classic” type 1A diabetes (T1AD) is determined by multiple genes within the HLA region and non-HLA genes including *INS-VNTR*, *CTLA4*, and *PTPN22* (2). Among them *CTLA4* is associated with autoimmunity, cancer, allergy, and infectious disease. In the *CTLA4* region, a number of variants, like the +49G>A and CT60, have shown T1D association (3). Although the association between class II HLA and fulminant T1D has been reported (4), the contribution of the non-HLA genes to the susceptibility to fulminant T1D has not been investigated. In this study, we examined the genetic contribution of *CTLA4* gene to fulminant T1D compared with “classic” T1AD.

Research Design and Methods

We examined 55 patients with fulminant T1D (49% female, median age-at-onset 35.0 years), 91 patients with “classic” T1AD (57% female, median age-at-onset 17.0 years) and 369 healthy controls. Diagnostic criteria for fulminant T1D were described elsewhere (1). The criterion for the recruitment of T1AD were 1) presence of diabetic ketosis at onset, 2) the duration of hyperglycemic symptoms before starting insulin therapy was < 3 months, 3) positive for at least one of the anti-islet autoantibodies. This study was approved by the appropriate ethical committees and informed consent was obtained from all subjects.

Genotyping of two SNPs in the *CTLA4*, +49G>A (rs231775) and CT60 (rs3087243), were performed as reported previously (5). Serum concentration of sCTLA4 were measured by ELISA using human sCTLA4 kit (MedSystems Diagnostics, Vienna, Austria), according to the manufacturer's protocol. Sera from T1D patients were obtained at disease onset and stored at -20 C until use.

The significance of differences in the distribution of genotypes between cases and controls was determined by a Chi-squared test or Fisher's exact probability test. Comparisons of the sCTLA4 levels were made by ANOVA with phenotypic group alone and ANOVA with phenotypic group and *CTLA4* genotype. $P < 0.05$ was considered to be statistically significant.

Results

The +49G>A was associated with "classic" T1AD, but not with fulminant T1D (Table 1). In contrast, the contribution of CT60 to disease is distinct from that of +49G>A. The frequency of CT60AA genotype in fulminant T1D was significantly higher than in controls ($P=0.021$) or T1AD ($P=0.031$). The CT60GG was associated with T1AD ($P=0.008$). Because of the strong association of the HLA-*DR4* in both patient groups (1), the effect of *CTLA4* on T1D susceptibility relative to HLA-*DR4* was also examined. Among *DR4*-positive individuals, the frequency of CT60AA genotype was significantly increased in patients with fulminant T1D ($P=0.005$). However, stratification of patients by the presence or absence of HLA-*DR4* did not affect the association between +49GG genotype and T1AD (Table 1).

It has been reported that CT60 G allele might associated with the lower production of sCTLA4 mRNA (3). We therefore determined serum sCTLA4 levels. The mean sCTLA4 level for fulminant T1D (0.56 ± 0.24 ng/ml; mean \pm SD, $n=36$) was significantly lower than those in T1AD (0.94 ± 0.87 ng/ml, $n=45$) and controls (0.89 ± 0.76 ng/ml, $n=23$) ($P=0.043$). A mixed model ANOVA using phenotypic group (fulminant T1D, T1AD, and controls) and CT60 genotypes (GG and GA+AA) as factorial fixed effects revealed no differences in sCTLA4 levels between CT60 genotypes ($P=0.76$) or phenotype/genotype interactions ($P=0.40$).

Conclusions

The CTLA4, which delivers inhibitory signals to T-cell activation, is expressed on the surface of activated T-cells and regulatory T cells, and the lack of CTLA4 results in uncontrolled T

cell-mediated lymphoproliferative disease (6). Furthermore, CTLA4 also has a significant biological role in attenuating T cell responses in the context of an inflammatory environment such as infection with a pathogen (7). We showed that *CTLA4* is associated with an increase risk of fulminant T1D, and its contribution is distinct from “classic” T1AD. As is reported previously (5, 8), a significant association between “classic” T1AD and +49GG and CT60GG genotype was also found in the present study. However, the CT60AA genotype has a contribution to the susceptibility to fulminant T1D. Moreover, it is implicated that HLA-*DR4* has influence on the association of fulminant T1D with the CT60AA genotype.

In this study, we also revealed that the serum sCTLA4 level in fulminant T1D was significantly lower than those in T1AD and controls. Although it remains unknown how sCTLA4 regulate the T-cell activation, recombinant sCTLA4 inhibits T-cell proliferation *in vitro*. Furthermore, sCTLA4 is constitutively expressed on nonstimulated T cells and its expression is downregulated after T cell activation (9). Therefore, the decreased levels of sCTLA4 might indicate a lower potential of T cell inhibition in fulminant T1D, cause of which might be functional defects leading to reduced production of sCTLA4 or ongoing activation of the immune system eventually leading to decreased levels of sCTLA4.

In conclusion, the present study implicates that *CTLA4* confers susceptibility to fulminant T1D. Furthermore, the different contributions of *CTLA4* to susceptibility to fulminant and “classic” T1AD indicate that the underlying immune process primed β -cell injury might be distinct between these subtypes of T1D.

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Table 1 *CTLA4* polymorphisms in patients with fulminant type 1 diabetes, type 1 A diabetes, and healthy controls

	Fulminant	Type 1A	Control	Pvalue			OR (95% CI)		
				F vs. CTRL	1A vs. CTRL	F vs. 1A	F vs. CTRL	1A vs. CTRL	F vs. 1A
<i>+49 G>A</i>	n=55	n=91	n=369						
AA	13 (23.6)	7 (7.7)	61 (16.5)						
AG	23 (41.9)	36 (39.6)	186 (50.4)						
GG	19 (34.5)	48 (53.7)	122 (33.1)	NS	0.0013	0.012			
GG	19 (34.5)	48 (53.7)	122 (33.1)	NS	0.0005	0.033		2.26 (1.41-3.60)	0.47 (0.24-0.94)
AA+AG	36 (65.5)	43 (46.3)	247 (66.9)						
<i>CT60 G>A</i>	n=55	n=91	n=369						
AA	8 (14.5)	4 (4.4)	22 (6.0)						
AG	26 (47.3)	28 (30.8)	165 (44.7)						
GG	21 (38.2)	59 (64.8)	182 (49.3)	0.043	0.029	0.0036			
GG	21 (38.2)	59 (64.8)	182 (49.3)	NS	0.008	0.0017		1.89 (1.18-3.05)	0.33 (0.17-0.67)
AA+AG	34 (61.8)	32 (35.2)	187 (50.7)						
AA	8 (14.5)	4 (4.4)	22 (6.0)	0.021	NS	0.031	2.68 (1.13-6.37)		3.70 (1.06-12.9)
AG+GG	47 (85.5)	87 (95.6)	347 (94.0)						
<i>DR4 (+)</i>	n=42	n=52	n=121						
+49GG	14 (33.3)	27 (51.9)	38 (31.4)	NS	0.011	NS		2.35 (1.21-4.59)	
CT60AA	8 (19.0)	2 (3.8)	6 (5.0)	0.005	NS	0.018	4.51 (1.46-13.9)		5.88 (1.18-29.4)
<i>DR4 (-)</i>	n=13	n=36	n=183						
+49GG	5 (38.5)	19 (52.8)	61 (33.3)	NS	0.026	NS		2.24 (1.08-4.61)	
CT60AA	0 (0)	3 (8.3)	16 (8.7)	NS	NS	NS			

Data are the number of subjects (percentage). F, Fulminant; 1A, Type 1A; CTRL, Controls; OR, Odds ratio; NS, Not significant. The interaction between *CTLA4* polymorphisms and HLA-*DR4* was assessed by a Chi-square test with a 2x2 contingency table (+49 GG vs. AG+AA or CT60 AA vs. AG+GG) in *DR4*-positive

or -negative patients and the corresponding controls.