Genetic association between the *IL2RA* and mode of onset of type 1 diabetes in the Japanese population

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Abbreviated title: *IL2RA* and type 1 diabetes in Japanese population

Key terms: IL2RA, Genetics, Japanese, Type 1 diabetes, Mode of onset, HLA, CTLA4

Word count: main text 2,226, abstract 230, 24 references, 4 tables, no figure

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This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

Disclosure statement: All authors have nothing to disclose.

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Precis: Genetic association of the *IL2RA* with Japanese type 1 diabetes is different according to the mode of disease onset.

Abstract

Context/Objective: The interleukin-2 receptor α (IL2RA), also known as CD25, is expressed on the regulatory T cells which play an important role in the control of immune responses and the maintenance of immune homeostasis. Our objective was to determine whether variants in the *IL2RA* gene are associated with type 1 diabetes in the Japanese population.

Design/Patients: We genotyped the four single-nucleotide polymorphisms (SNPs) (rs706778, rs3118470, ss52580101 and rs11594656) of the *IL2RA* in 885 patients with type 1 diabetes and 606 control subjects of Japanese origin. The allele and genotype frequencies were examined in the patient groups stratified by their mode of onset in a case-control study.

Results: We found evidence of association with acute-onset, but not slow-onset and fulminant, type 1 diabetes for two of the 4 SNPs genotyped (rs706778 and rs3118470). The rs706778 A allele and the rs3118470 G allele were associated with an increased disease risk (Odds ratio [OR] for rs706778 AA genotype 1.54, $P = 4.2 \times 10^{-4}$ and OR for rs3118470 GG genotype 1.50, $P = 0.0019$, respectively). Furthermore, the A-G haplotype was associated with increased type 1 diabetes risk in acute-onset form (OR 1.30, $P = 0.002$).

Conclusions: The present data confirmed the type 1 diabetes association with *IL2RA* and provide evidence that the different contributions of the *IL2RA* in the susceptibility to acute-onset and other forms of type 1 diabetes in the Japanese population.

Introduction

Type 1 diabetes is known as a multigenic organ-specific autoimmune disease characterized by T-cell–dependent destruction of the insulin-producing pancreatic β-cells, resulting in absolute dependence on insulin for survival and maintenance of health (1). Clinical features of Japanese type 1 diabetes are heterogeneous and there are at least three subtypes of type 1 diabetes in Japan, acute-onset 'classical', slow-onset, and fulminant type 1 diabetes (2). However, the underlying pathogenesis of β-cell destruction, including the genetic or environmental factors for each subtype of type 1 diabetes is largely unknown.

Recent first-stage results of genome-wide association (GWA) studies identified several loci other than the HLA on chromosome 6p21, the insulin gene (*INS-VNTR*) on 11p15, the *CTLA4* locus on 2q33, and the *PTPN22* on 1p13 include *IL2RA* locus on 10p15, *IFIH1* on 2q24 and, most recently, *CLEC16A* (*KIAA0350*) on 16p13, and *PTPN2* on 18p11 (3-5). The *IL2RA* encodes the α-chain of the IL-2 receptor (IL-2R) complex (also known as CD25) which is central to immune regulation as an important modulator of self-tolerance and immunity (6) and the *IL2RA* association with type 1 diabetes was originally identified by Vella and coworkers (7). IL2RA expression on $CD4^+$ $CD25^+$ regulatory T cells is essential for their function in suppressing T cell immune responses to autoantigens, alloantigens, tumor antigens, and pathogen-derived antigens. As direct evidence of the importance of $CD4^+$ $CD25^+$ regulatory T cells in human type 1 diabetes (8, 9), depletion of regulatory T cells by anti-CD25 antibody hastened development of diabetes in non-obese diabetic mice, an animal model of spontaneous type 1 diabetes (10). Furthermore, it has been evidenced that the type 1 diabetes-associated polymorphisms in the *IL2RA* region regulate the circulating concentration of soluble form of IL2RA (11). These findings implicate that the genetic associations of *IL2RA* polymorphisms to the rate of β-cell destruction in type 1 diabetes. In this study, we demonstrated the differences in the contribution of *IL2RA* polymorphisms to susceptibility to acute-onset, slow-onset, and fulminant type 1 diabetes in the Japanese population.

Subjects and Methods

Subjects

We examined 885 patients with type 1 diabetes (56% female) and 606 healthy control subjects of Japanese origin. Patients with type 1 diabetes include 627 patients with acute-onset (57% female), 222 with slow-onset (56% female) and 25 with fulminant type 1 diabetes (28% female). The remaining 11 patients were unclassified type 1 diabetes. The criterion for the recruitment of acute-onset type 1 diabetes 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. Diagnosis of slow-onset type 1 diabetes were done by the following criteria: 1) originally diagnosed as type 2 diabetic and no sign of ketosis at diabetes onset; 2) proven anti-islet autoantibody positivity; and 3) insulin treatment started ≥12 months after the diagnosis. Diagnostic criteria for fulminant type 1 diabetes were described elsewhere (2). The median age-at-onset and serum C-peptide concentration of acute-onset, slow-onset and fulminant type 1 diabetes was 21.0, 40.0, and 44.0 years and 0.14, 0.45, and 0.005 ng/ml, respectively. The associations of clinical and genetic parameters such as age-at-onset, gender, co-occurrence of autoimmune thyroid disease (AITD), glutamic acid decarboxylase 65 (GAD65) autoantibody titer, *CTLA4* and class II HLA with *IL2RA* risk haplotype were also examined. AITD was defined as Graves' disease, Hashimoto's thyroiditis which were diagnosed based on the finding of palpable goiter or the presence of chronic thyroiditis with ultrasonography examination in the absence of goiter, abnormal levels of thyroid hormones, and positivity for autoantibodies to

thyroid peroxidase, thyroglobulin and/or thyrotropin receptor. The healthy control subjects had normal glucose tolerance and no family history of type 1 diabetes or other autoimmune diseases. This study was approved by the appropriate ethical committees and informed consent was obtained from all subjects.

Genotyping

Two SNPs in intron 1 of the *IL2RA*, rs706778 A>G and rs3118470 A>G, and two SNPs in the 5' regions of the *IL2RA* and *RBM17*, ss52580101 (rs41295061) C>A and rs11594656 T>A, were genotyped. Genotyping of the rs706778 A>G was performed by PCR-restriction fragment length polymorphism (RFLP) method (1.5 mM $MgCl_2$, T_{anneal} 58°C) with forward primer (rs706778F2); 5'-TCCCCTTTCTCTTCTCTCAG-3' and reverse primer (rs706778R3); 5'-GAGCCCTGAGGGACTAGTAAATTTCC ACCA-3' followed by digestion with *Bsr* I (New England Biolabs, Ipswich, MA). The rs3118470 A>G was genotyped by PCR-RFLP (1.0 mM $MgCl_2$, T_{anneal} 58°C) with forward primer (rs3118470F2); 5'-CTCTTTCTGACCACATCCCA-3' and reverse primer (rs3118470R3); 5'-AGCCCCAAGGGATCAGTAAATTTCCA CCA-3' followed by digestion with *Bsr* I. The ss52580101 C>A was genotyped by PCR-RFLP (2.0 mM $MgCl_2$, T_{anneal} 62°C) with forward primer (rs41295061F); 5'-CACCCTCATCCATAAAGACC-3' and reverse primer (rs41295061R); 5'-ATTCATCCCACACCCACCAG-3' followed by digestion with *Mwo* I. Finally, the rs11594656 T>A was genotyped by PCR-RFLP (2.0 mM $MgCl_2$, T_{anneal} 58°C) with forward primer (rs11594656F); 5'-CTCCCCAGTCATTCACCAAA-3' and reverse primer (rs11594656R); 5'-TCTTTTGGCTTTTCTCACTATGATGCC GTC-3' followed by digestion with *BsmA* I. The digested products were resolved on a 3.5% agarose gel and stained with ethidium bromide. The number of cases and controls we were able to genotype were 877 and 602 for rs706778, 872 and 592 for rs3118470, 881 and 606 for ss52580101, and 882 and 606 for rs11594656, respectively. No deviations from Hardy-Weinberg equilibrium were observed for all genotypes in control subjects. The HLA-*DR* and +6230G>A (CT60, rs3087243) in the *CTLA4* were also genotyped as reported previously (12). The data on HLA-*DR* and *CTLA4* +6230G>A polymorphism were available in 587 and 798 patients with type 1 diabetes, respectively.

Anti-islet autoantibody assays

Autoantibodies to glutamic acid decarboxylase 65 (GAD65), insulinoma associated antigen-2 (IA-2), insulin, and zinc transporter-8 (ZnT8) were measured by radioimmunoassay and islet cell antibodies (ICA) were determined by immunoenzymatic staining of human pancreas sections, as described previously (13, 14). Subjects positive for at least one of autoantibodies to GAD65, IA-2, ZnT8, insulin and/or ICA were defined as positive for anti-islet autoantibodies.

Statistical analysis

A χ^2 test was used for statistical analysis unless otherwise indicated. Odds ratio and its 95% confidence interval (95% CI) was also calculated. Haplotype frequencies were estimated based on the HPlus v2.5 software program (http://qge.fhcrc.org/hplus/) (15). Linkage disequilibrium, evaluated by D' coefficients, was calculated to evaluate linkage disequilibrium by Haploview v4.0 software (http://www.broad.mit.edu/mpg/haploview). Deviations from Hardy-Weinberg equilibrium were tested by comparison of observed and expected genotype frequencies. Patient-only logistic regression analysis was performed to test for the association of the *IL2RA* risk haplotype with age at onset, gender, co-occurrence of AITD, log-transformed GAD65 autoantibody titer, class II HLA and *CTLA-4* CT60 genotype as variables. StatView Ver.5.0 (SAS Institute, Cary, NC) were used for these tests. A *P* value less than 0.05 was considered statistically significant.

Results

IL2RA variants and type 1 diabetes

First, we compared *IL2RA* genotype and allele frequencies between the overall type 1 diabetes and control subjects. Among the four SNPs genotyped in this study, two SNPs (rs706778 and rs3118470) had statistically significant type 1 diabetes association. As shown in Table 1, the AA genotype at rs706778 SNP and the GG genotype at rs3118470 SNP were significantly associated with type 1 diabetes (OR 1.37, 95%CI: 1.09-1.71, P = 0.0062 for rs706778 and OR 1.28, 95%CI: 1.00-1.63, P = 0.048 for rs3118470).

Association of IL2RA variants with mode of diabetes onset and class II HLA

Then we divided these patients into three groups (acute-onset, slow-onset, and fulminant) according to the mode of diabetes onset and examined the genetic contribution of the two significant *IL2RA* SNPs in each group (Table 2). We found a significant genetic association between the *IL2RA* and acute-onset type 1 diabetes (OR 1.54, 95%CI: 1.21-1.96, $P = 4.2 \times 10^{-4}$ for rs706778 and OR 1.50, 95%CI: 1.16-1.94, P = 0.0019 for rs3118470), whereas no significant difference was observed between slow-onset type 1 diabetes, fulminant type 1 diabetes and control subjects (Table 2). Furthermore, the stratification of acute-onset patients by the presence or absence of high diabetes risk class II HLA (*DR*4/4, 4/X, 9/9, but not *DQB1**0601/*0602) showed that the disease association of *IL2RA* polymorphisms was observed only in patients carrying high diabetes risk HLA (OR 1.52, 95%CI: 1.13-2.04, $P = 5.5 \times 10^{-4}$ for rs706778 and OR 1.48, 95%CI: 1.08-2.02, P = 0.015 for rs3118470). Therefore, the association of *IL2RA* with Japanese type 1 diabetes originates in the association with acute-onset patients, especially with patients carrying high risk class II HLA. However, neither the ss52580101 nor rs11594656 were associated with mode of diabetes onset and class II HLA (data not shown).

IL2RA haplotype analysis

Two significant *IL2RA* SNPs are in high linkage disequilibrium (D' = 0.953, r^2 = 0.69) and rs706778A;rs3118470G haplotype (frequency 52.3%) was associated with increased risk in acute-onset type 1 diabetes (OR 1.30, 95%CI 1.10-1.53, P = 0.002) (Table 3).

Association with clinical and genetic parameters of type 1 diabetes

We evaluated the relationship of the disease associated *IL2RA* haplotype with clinical and genetic parameters in acute-onset patients by logistic regression analysis (Table 4). There were no associations of *IL2RA* risk haplotype with age-at-onset, gender, GAD65 autoantibody titer or presence of established genetic risk factors for Japanese type 1 diabetes such as the carriage of the *CTLA4* CT60GG genotype and high risk HLA-*DR* genotype. However, the *IL2RA* risk haplotype was associated with the co-occurrence of thyroid autoimmunity (OR 1.66, 95%CI 1.00-2.75, P = 0.048). In patients with slow-onset type 1 diabetes, any clinical and genetic parameters were not associated with *IL2RA* risk haplotype (data not shown).

Discussion

We demonstrated 1) the type 1 diabetes association of the SNPs in the *IL2RA* and 2) the differences in the contribution of *IL2RA* to susceptibility of type 1 diabetes depending on the mode of diabetes onset in the Japanese population. Furthermore, it was suggested that the class II HLA have influence on the association of type 1 diabetes with the *IL2RA*. The association of *IL2RA* variants with acute-onset, but not with slow-onset and fulminant, type 1 diabetes suggests that *IL2RA* might relate to the rate of autoimmune β-cell destruction. The different contributions of type 1 diabetes susceptibility gene in the mode of diabetes onset have been also documented in class II *HLA*, *CTLA4*, *PTPN22*, and *NeuroD* in the Japanese population (16-19). Therefore, the underlying immune process primed β-cell injury might be distinct between subtypes of type 1 diabetes (acute, slow, and

fulminant).

The IL2R-signaling complex is composed of three distinct subunits, the α-chain (CD25), β-chain (CD122), and γ-chain (CD132) each of which have unique roles in facilitating IL2-dependent signal transduction (6). Among these IL2Rα acts to confer high-affinity binding of IL2 to the IL2R complex highly expressed by activated T-cells and $CD4^+$ $CD25^+$ regulatory T cells. The *IL2RA* gene has also been associated with Graves' disease (20), rheumatoid arthritis (4) and multiple sclerosis (21) which imply that this locus may have a general effect on predisposition to autoimmunity. Since the involvement of IL2R complex on the regulation of immune function is not limited to the specific-organs (6), the current observation showing the much stronger association between *IL2RA* polymorphisms and type 1 diabetes with AITD is consistent with this hypothesis.

Two SNPs associated with Japanese type 1 diabetes in this study (rs706778 and rs3118470) are located in intron 1 and have been reported the type 1 diabetes association in two independent studies in subjects of British and Canadian descent (11, 22). Furthermore, recent WGA study reported that other SNPs close to these two SNPs (< 300bp distance), rs12722489 and rs2104286, had a significant association with multiple sclerosis (21). Taken together, these results implicate that causal variants for multiple autoimmune diseases localize in the *IL2RA* intron 1. Of note, the two most associated markers with European patients (11), ss52580101 (rs41295061) and rs11594656, were less polymorphic in Japanese population compared to white populations and were not associated with Japanese type 1 diabetes, suggesting that there is an allelic heterogeneity between Japanese and white populations.

To date, at least six positive regulatory regions have been identified in the regulation of *IL2RA* expression in the promoter region and intron 1 (6). Although it is unknown how rs706778 and rs3118470 SNPs in intron 1 act to influence the expression of *IL2RA*, searching the TFSEARCH transcription factor binding site data base with the DNA sequence surrounding the SNPs sites identified the binding site for the Cut-like repressor protein (CR3HD) on the antisense strand of both SNPs. The polymorphic nucleotide in rs706778 A>G and rs3118470 A>G is located at the critical region of the core motif of the CR3HD binding consensus sequence (cAT**T**GATgga; core motif is shown in uppercase, the position at polymorphic site is in bold) (23). Furthermore, it is reported that single nucleotide substitution in the Cut-like protein binding site of the TGF-β type II receptor gene (*TβR-II*) promoter increase the CR3HD binding affinity which induce the reduction of *TβR-II* transcription (24). Thus, it is possible that these SNPs may be involved in regulating the *IL2RA* transcription or altering the splicing of the *IL2RA* transcript, which could affect the regulatory T cell functions. Further studies of expression of *IL2RA* transcripts in different blood cell subsets, together with the transfection experiment with CR3HD transcription factor are required to characterize the functional role of these SNPs. In conclusion, the present study indicated that *IL2RA* is associated with Japanese type 1 diabetes and its contribution to susceptibility to type 1 diabetes is different according to the mode of diabetes onset.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan. We thank Miyuki Moritani and Masaki Miwa for their excellent technical assistance.

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Table 1 *IL2RA* polymorphisms in patients with type 1 diabetes and healthy control subjects

		Genotypes					Alleles		
	n	AA	Aa	aa	OR * (95%CI)	*P* value *	A allele	OR (95%CI)	*P* value
rs706778 A>G									
Controls	602	170 (28.2)	309 (51.3)	123 (20.4)			649 (53.9)		
Type 1 diabetes	877	307 (35.0)	421 (48.0)	149 (17.0)	1.37 (1.09-1.71)	0.0062	1035 (59.0)	1.23 (1.06-1.43)	0.0059
rs3118470 A>G									
Controls	592	159 (26.9)	298 (50.3)	135 (22.8)			616 (52.0)		
Type 1 diabetes	872	206 (23.6)	427 (49.0)	239 (27.4)	1.28 (1.00-1.63)	0.048	839 (48.1)	0.85 (0.74-0.99)	0.037
ss52580101 C>A									
Controls	606	605 (99.8)	1 (0.2)	0 (0.0)			1211 (99.9)		
Type 1 diabetes	881	881 (100)	0 (0.0)	0 (0.0)	NA		1762 (100)	NA	
rs11594656 T>A									
Controls	606	570 (94.1)	35 (5.8)	1 (0.2)			1175 (96.9)		
Type 1 diabetes	882	836 (94.8)	43 (4.9)	2 (0.3)	1.15 (0.73-1.80)	NS	1715 (97.3)	1.10 (0.71-1.70)	NS

Data are n (%) unless otherwise indicated. A, major allele; a, minor allele. * AA vs. Aa+aa for rs706778 and rs11595656, aa vs. AA+Aa for rs3118470

NS, not significant. NA, not applicable

Table 2 ***IL2RA*** **polymorphisms in patients with type 1 diabetes classified by mode of diabetes onset**

	Acute-onset	Slow-onset	Fulminant	Controls
rs706778 A>G				
n	623	219	24	602
Genotype frequencies				
AA	235 (37.7)	63 (28.8)	7 (29.2)	170 (28.2)
AG	281 (45.1)	122 (55.7)	12 (50.0)	309 (51.3)
GG	107 (17.2)	34 (15.5)	5 (20.8)	123 (20.4)
OR (95%CI) *	1.54 (1.21-1.96)	1.03 (0.73-1.44)	1.05 (0.43-2.57)	
P value *	0.00042	NS	NS	
Allele frequencies				
A	751 (60.3)	248 (56.6)	26 (54.2)	649 (53.9)
G	495 (39.7)	190 (43.4)	22 (45.8)	555 (46.1)
OR (95%CI)	1.30 (1.11-1.52)	1.12 (0.90-1.39)	1.01 (0.57-1.80)	
P value	0.0014	NS	NS	
rs3118470 A>G				
n	622	221	25	592
Genotype frequencies				
AA	145 (23.3)	51 (23.1)	8 (32.0)	159 (26.9)
AG	286 (46.0)	128 (57.9)	12 (48.0)	298 (50.3)
GG	191 (30.7)	42 (19.0)	5 (20.0)	135 (22.8)
OR (95%CI) *	1.50 (1.16-1.94)	0.79 (0.54-1.17)	0.85 (0.31-2.30)	
P value *	0.0019	NS	NS	
Allele frequencies				
A	576 (46.3)	230 (52.0)	28 (56.0)	616 (52.0)
G	668 (53.7)	212 (48.0)	22 (44.0)	568 (48.0)
OR (95%CI)	0.80 (0.68-0.93)	1.00 (0.80-1.24)	1.17 (0.66-2.08)	
P value	0.0048	NS	NS	

Data are n (%) unless otherwise indicated. Genotype and allele frequencies were compared between type 1 diabetes and control subjects. * AA vs. AG+GG for rs706778, GG vs. AG+AA for rs3118470. NS, not significant

Table 3 *IL2RA* haplotype frequencies in acute-onset patients with type 1 diabetes and healthy control subjects

Haplotypes (rs706778: rs3118470)	Acute-onset type 1 diabetes (n=613) (%)	Controls (n=588) (%)	OR (95%CI)	*P* value
G:A	38.2	45.1	1.00 (reference)	
A:G	52.3	46.8	1.30 (1.10-1.53)	0.002
A:A	8.0	7.2	1.27 (0.93-1.74)	NS
G:G	1.5	0.9	1.69 (0.76-3.75)	NS

Haplotypes were estimated by an EM algorithm using Hplus (version 2.5). NS, not significant

Table 4 Logistic regression analysis for the association of clinical and genetic parameters with *IL2RA* risk haplotype among acute-onset patients with type 1 diabetes

	rs706778: rs3118470 A:G haploype (+) vs A:G haploype (-)		
Variable	OR	95% CI	*P* value
Age at onset (year)	0.99	0.98-1.01	NS
Male	0.87	0.60-1.29	NS
With AITD	1.66	1.00-2.75	0.048
GAD65 autoantibody titer	1.14	0.74-1.75	NS
CTLA4 CT60GG present	1.01	0.69-1.50	NS
High-risk HLA-*DR* present	1.31	0.78-2.20	NS

AITD, autoimmune thyroid disease. CT60, *CTLA4* +6230G>A. High-risk HLA-*DR*, *DR*4/4, 4/X, 9/9, but not *DQB1**0601/*0602. NS, Not significant