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Trinucleotide repeats of programmed cell death-1 gene are associated with susceptibility to type 1 diabetes mellitus

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Abstract

Multiple genes are involved in conferring susceptibility to autoimmune type 1 diabetes mellitus. The immunoreceptor programmed cell death-1 (PDCD-1), an inhibitory costimulatory molecule regulating peripheral tolerance, is reported to play an important role in the development of type 1 diabetes mellitus, making the human PDCD-1 gene, *PDCD1*, a candidate for disease susceptibility. The aim of this study was to clarify the contribution of *PDCD1* to genetic susceptibility to type 1 diabetes mellitus in humans. To screen for sequence variants, we sequenced all 5 exons and exon-intron junctions of *PDCD1* in Japanese subjects, 16 with type 1 diabetes mellitus and 16 without the disease. Some of the sequence variations identified were genotyped in larger samples (n = 275) with and without type 1 diabetes mellitus by polymerase chain reaction restriction fragment length polymorphism method or a fluorescence-based method. The distributions of polymorphisms were compared between patients with type 1 diabetes mellitus and healthy controls by contingency table analysis and Pearson χ^2 test. In this study, we found 16 sequence variants, including a TGC repeating variant in the 3′ untranslated region. We found this variant to be associated with the development of type 1 diabetes mellitus. These data suggest the contribution of *PDCD1* and its gene product to the development of type 1 diabetes mellitus.

1. Introduction

Type 1 diabetes mellitus is caused by autoimmune destruction of insulin-producing beta cells of the pancreas. Whereas the development of type 1 diabetes mellitus is influenced by both environmental and genetic factors, genetic susceptibility to type 1 diabetes mellitus is determined by multiple genes [1]. In humans, the strongest component, termed *IDDM1*, is linked to the human leukocyte antigen (HLA) region [2]. In addition to *IDDM1* in the HLA region, a number of studies have demonstrated that other genes not linked to HLA are also important for disease susceptibility. Most of these non-HLA genes have yet to be identified, except for a few genes, such as genes for insulin (*INS*) [3], cytotoxic T lymphocyte antigen 4 (*CTLA4*) [4], and protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*) [5]. Elucidation of susceptibility genes for type 1 diabetes mellitus is not only useful for identifying individuals at high risk for type 1 diabetes mellitus, but would also facilitate our understanding of the disease pathogenesis, leading to the development of novel strategies for more effective methods of prevention and cure of the disease.

Programmed cell death-1 (PDCD-1) is involved in immune responses as an inhibitory regulator and plays a role in the regulation of peripheral tolerance [6]. In vivo, blockade of the interaction between PDCD-1 and its ligand, PD-L1 [7], or disruption of PDCD1 [8] has recently been reported to accelerate type 1 diabetes mellitus in nonobese diabetic (NOD) mice, indicating a role of PDCD-1 in the development of type 1 diabetes mellitus in NOD mice. PDCD-1 is one of the costimulatory receptor members that belong to the B7-CD28 superfamily. CTLA4 is also a member of this family with some sequence homology to PDCD-1. Recently, Ueda et al [4] demonstrated that a polymorphism of *CTLA4* is involved in susceptibility to type 1 diabetes mellitus and is responsible for *IDDM12* in

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Table 1
Clinical characteristic of patients and controls

	Patients with T1D	Controls
No. of subjects	154	153
Age (y), mean ± SD		42.0 ± 10.9
Sex (female/male)	95/59	44/109
Age of onset (y), mean ± SD	16.1 ± 12.4	
Therapy		
Multiple (≥4) insulin injections	147	
Continuous subcutaneous insulin infusion	7	

T1D indicates type 1 diabetes mellitus.

humans. Given the similarity between CTLA4 and PDCD-1 in molecular structure as well as their function as inhibitory costimulatory molecules, together with the data in the NOD mouse showing direct involvement of PDCD-1 in the development of type 1 diabetes mellitus, *PDCD1* is a strong candidate gene for type 1 diabetes mellitus susceptibility.

To clarify the contribution of *PDCD1* to genetic susceptibility to type 1 diabetes mellitus, we searched for sequence variations of *PDCD1* by determining the nucleotide sequences of all exons and exon-intron junctions of the gene and studied the association of the sequence variations identified with type 1 diabetes mellitus in humans.

2. Materials and methods

For the screening of sequence variants, we studied Japanese subjects, 16 with type 1 diabetes mellitus and 16 without the disease. The diagnosis of type 1 diabetes mellitus was defined by both clinical features and laboratory data. All the patients were ketosis prone, lacked endogenous insulin secretion as judged by urinary C-peptide level of less than 3.3 nmol/d, which was a more stringent criterion than that previously proposed [9], and had needed intensive insulin treatment since diagnosis (Table 1). Control subjects consisted of medical staff in our department. They had normal glucose tolerance and no family history of type 1 diabetes mellitus or other autoimmune diseases. All patients and controls subjects were of Japanese origin and resided in the Osaka area (Western Japan). Informed consent was obtained from all subjects, and the human genome study was approved by Osaka University (approval number 66). Genomic DNA was extracted from peripheral blood leukocytes. Ten pairs of primers were designed by using Primer3 Input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to cover all 5 exons and exon-intron junctions in the published human *PDCD1* sequence (Ensemble Human Geneview) (Table 2). Polymerase chain reaction (PCR) amplification was performed in a TAKARA Ex Taq, TAKARA LA Taq, or TAKARA PrimeSTAR HS system (TAKARA BIO, Otsu, Japan), in a TaKaRa PCR Thermal Cycler. Sequencing was performed by using an ABI PRISM BigDye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

Selected sequence variants were genotyped in larger samples (n = 275) with and without type 1 diabetes mellitus by PCR restriction fragment length polymorphism method or a fluorescence-based method as reported previously [10].

Hardy-Weinberg equilibrium was tested by using a χ^2 goodness-of-fit test. The distributions of polymorphisms were compared between patients with type 1 diabetes mellitus and healthy control subjects by contingency table analysis and Pearson χ^2 test. The correlation between age of onset and genotype status of *PDCD1* was estimated. Values of P less than .05 were considered significant.

3. Results

A total of approximately 4000 base pairs (bp) of *PDCD1*, including all 5 exons and exon-intron junctions, were sequenced in 32 subjects (16 subjects with type 1 diabetes mellitus and 16 control subjects). In total, 16 sequence variants were identified: 13 single nucleotide polymorphisms (SNPs) including one nonsynonymous SNP in the coding region, 2 insertion/deletion polymorphisms, and 1 microsatellite polymorphism (Table 3). Of these, 7 variants were not previously reported in the "Ensemble Human Gene SNP View" (http://www.ensembl.org/Homo_sapiens/genesnpview?db=core;gene=ENSG00000188389). SNPs identified were as follows: C157T, C268G, C5355T, C5384G, T5707C, and G5745C in intron 1; G6437A in intron 2; C7209T and A7499G in intron 4; C7625T and

Table 2
Sequences of PCR primers

Region	Forward primer	Reverse primer	Product size (bp)
Segment 1	GGCCAGGATGGTTCTTAGGT	CCACACAGCTCAGGGTAAGG	249
Segment 2	CCCTTACCCTGAGCTGTGTG	AAGCCAAGGTTAGTCCCACA	297
Segment 3	GGATATGGAAAGAGGCCACA	GCCAGGGACTGAGAGTGAAA	596
Segment 4	TCAGGAGGTCCTTGTCTTGG	ACGAAGCTCTCCGATGTGTT	802
Segment 5	CTTCCGTGTCACACAACTGC	GGTGGGGTCCTGGCTATAAT	951
Segment 6	GCCTGCAGGACTCACATTCT	AGGGTCTGCAGAACACTGGT	788
Segment 7	TCCCTGAGCAGACGGAGTAT	TAAAGGTGGAGGGGTTTCCT	764
Segment 8	TCATGTCTCAATGCCCACAG	ACCGTAGGATGTCCCTCTCC	609
Segment 9	AGGAAACCCCTCCACCTTTA	TTTCAGGAATGGGTTCCAAG	328
Segment 10	ACATCCTACGGTCCCAAGGT	CTGTGTGTTTCTGGGACAGC	569

Table 3
Sequence variations of *PDCD1* and their allele frequencies in subjects in initial sequencing study

nt	Allele	n	T1D (%)	n	Control (%)	nt	Allele	n	T1D (%)	n	Control (%)
157	T	20	62.5	14	50.0	7209	C	15	68.2	20	66.7
	C	12	37.5	14	50.0		T	7	31.8	10	33.3
268	C	20	62.5	14	50.0	7499	G	17	70.8	20	66.7
	G	12	37.5	14	50.0		A	7	29.2	10	33.3
834	D	11	36.7	16	53.3	7625	C	12	50.0	16	53.3
	I	19	63.3	14	46.7		T	12	50.0	14	46.7
5355	T	28	87.5	27	96.4	7785	C	20	71.4	22	68.8
	C	4	12.5	1	3.6		T	8	28.6	10	31.3
5384	C	31	96.9	27	96.4	8185	TGC10	30	93.8	29	90.6
	G	1	3.1	1	3.6		TGCnon10	2	6.3	3	9.4
5638	A	31	96.9	27	96.4	8737	A	26	81.3	25	78.1
	–	1	3.1	1	3.6		G	6	18.8	7	21.9
5707	T	31	96.9	27	96.4	9010	G	29	96.7	30	100.0
	C	1	3.1	1	3.6		A	1	3.3	0	0.0
5745	G	31	96.9	27	96.4						
	C	1	3.1	1	3.6						
6437	G	13	40.6	18	56.3						
	A	19	59.4	14	43.8						

T1D indicates type 1 diabetes mellitus.

T7785C in exon 5; and G8737A and G9010A in the 3′ untranslated region (UTR). C7625T in exon 5 was a nonsynonymous polymorphism leading to an amino acid substitution from alanine to valine. Two insertion/deletion polymorphisms were a 23-bp insertion/deletion polymorphism at nucleotide (nt) position 834 [834I/D] and an insertion/deletion of A at nt 5638 [A5638(−)]. The microsatellite polymorphism was trinucleotide (TGC) repeats at nt 8185 (8185[TGC]$_n$). The polymorphisms identified were all in Hardy-Weinberg equilibrium, except for C5355T, which deviated from the Hardy-Weinberg equilibrium in 32 subjects, but had a minor allele frequency of less than 0.1.

Selected sequence variants were genotyped in a larger number of samples (n = 275) in addition to the aforesaid 32 subjects used for sequencing, and the genotype distribution was compared in a total of 307 subjects with and without type 1 diabetes mellitus. Among 8 sequence variants in intron 1, we chose 834I/D. The C5355T, C5384G, A5638(−), T5707C, and G5745C polymorphisms were not studied because of the low minor allele frequency (<0.1). The C157T, C268G, and 834I/D polymorphisms in intron 1 were in complete linkage disequilibrium in 32 subjects; therefore, 834I/D was chosen as a tag polymorphism among the 3 polymorphisms. The G6437A polymorphism in intron 2 was also excluded because it was in complete linkage disequilibrium with 834I/D in 32 subjects. Among 4 sequence variants in intron 4 and exon 5, we chose C7625T and T7785C. The T7785C

Table 4
Frequency (%) of alleles of selected sequence variants of *PDCD1* in larger number of subjects

Loci		T1D	Control	*P*	Locus		T1D	Control	*P*
834D/I	Genotype	n = 140	n = 147		8185[TGC]$_n$	Genotype	n = 153	n = 150	
	DD	24.3	21.8			TGC 9/10	0.0	0.7	
	DI	50.7	47.6			TGC10/10	92.8	84.7	
	II	25.0	30.6	.56		TGC10/11	7.2	13.3	
	Allele	n = 280	n = 294			TGC11/11	0.0	0.7	
	D	49.6	45.6			TGC10/13	0.0	0.7	.17
	I	50.4	54.4	.33		TGC10/10	92.8	84.7	
7625C/T	Genotype	n = 137	n = 143			TGC10/non10	7.2	14.7	
	CC	27.0	23.8			TGCnon10/non10	0.0	0.7	.07
	CT	49.6	48.3			Allele	n = 306	n = 300	
	TT	23.4	28.0	.64		TGC 9	0.0	0.3	
	Allele	n = 274	n = 286			TGC10	96.4	92.0	
	C	51.8	47.9			TGC11	3.6	7.3	
	T	48.2	52.1	.35		TGC13	0.0	0.3	.10
7785C/T	Genotype	n = 143	n = 143			TGC10	96.4	92.0	
	CC	40.6	49.7			TGCnon10	3.6	8.0	.02
	CT	50.3	38.5						
	TT	9.1	11.9	.13					
	Allele	n = 286	n = 286						
	C	65.7	68.9						
	T	34.3	31.1	.42					

Table 5
Association of triplet repeat polymorphisms in *PDCD1* with type 1 diabetes mellitus relative to HLA-*DRB1* genotypes

	HLA-DRB1 genotype	No. of subjects	TGC10/10 (%)	Others (%)	*P* (vs control)
Type 1 diabetes mellitus	High-risk HLA (+)[a]	36	97.2	2.8	.049
	High risk HLA (−)	93	91.4	8.6	.15
Control		134	85.1	14.9	

[a] *DRB1*0405/0405*, *DRB1*0405/0901*, or *DRB1*0901/0901*.

polymorphism was chosen as a tag polymorphism of these 4 polymorphisms in strong linkage disequilibrium. In addition, we chose the C7625T polymorphism because this polymorphism was in the coding region leading to an amino acid substitution from alanine to valine. The PD1.3A (G7146A) polymorphism, which was previously reported to be associated with type 1 diabetes mellitus in a white population [11], was not detected in the initial 32 samples for sequencing. We therefore sequenced a larger number of samples (n = 137), but none had the polymorphism. Among 3 sequence variants in the 3′ UTR, we chose the 8185[TGC]$_n$ polymorphism because triplet repeat polymorphisms have been reported to be associated with several genetic diseases. 8185[TGC]$_n$ was significantly associated with type 1 diabetes mellitus (P = .02; OR, 2.33; 95% confidence interval, 1.14-4.77) (Table 4). This association was significant in female patients (P = .04 vs controls), but not in male patients (not significant vs controls). Although most of the study patients (>80%) had at least one susceptible *DRB1* allele, *DRB1*0405* or *DRB1*0901*, when the patients were limited to those with genotypes with the combination of 2 doses of these susceptible *DRB1* alleles, *PDCD1* was significantly associated with type 1 diabetes mellitus in patients with high-risk HLA genotypes but not in those without (Table 5). When the patients were divided into 3 groups according to age at onset of the disease so that each group contained the same number of subjects, the association of *PDCD1* with type 1 diabetes mellitus tended to be stronger in patients with younger age at onset than in those with older age at onset (Table 6). When age of onset per se was compared between those with and without the 10/10 genotype of 8185[TGC], however, no significant difference was noted (16.1 ± 12.4 vs 17.4 ± 8.6 years, respectively; P = .74).

No significant differences in the frequencies of alleles and genotypes for other polymorphisms were observed between patients with type 1 diabetes mellitus and control subjects.

4. Discussion

More than 20 *IDDM* loci in total have been reported to be linked to or associated with type 1 diabetes mellitus in humans. Among these, 4 genes, *HLA*, *INS*, *CTLA4* and *PTPN22*, which have been repeatedly shown to be responsible for disease susceptibility, were initially identified by a candidate gene approach, indicating the importance of this approach in identifying susceptibility genes for multifactorial diseases, such as type 1 diabetes mellitus, with a modest effect. *PDCD1* is an important candidate gene because of its function as an inhibitory regulator of immune reaction and regulation of peripheral tolerance [12], as well as its structural homology to *CTLA4*, another inhibitory molecule whose variant is associated with type 1 diabetes mellitus and other autoimmune diseases [4]. Furthermore, a recent study in the NOD mouse, an animal model of type 1 diabetes mellitus, showed that blockade of PDCD-1 and its ligand, PD-L1, accelerates type 1 diabetes mellitus [7], indicating a role of PDCD-1 in the development of type 1 diabetes mellitus in NOD mice.

In the present systematic search for polymorphisms in the gene encoding PDCD-1, we found a total of 16 sequence variants. Among the polymorphisms subjected to case-control association study, an allele with 10 repeats of the 8185[TGC]$_n$ microsatellite was found to be significantly associated with type 1 diabetes mellitus. When the P value was multiplied by the number of polymorphisms tested, the corrected P value of .08 was obtained. This marginal evidence for an association warrants further investigation. The polymorphism is located in the 3′ UTR region, which is important for gene expression and stability of RNA. Triplet repeat polymorphisms are reported to exert detrimental effects through different molecular mechanisms dependent on their location in or outside the coding region in mice [13]. Therefore, the *PDCD1* 8185[TGC]$_n$ microsatellite identified in the present study may be functional, affecting PDCD1 expression and/or function, leading to modification of the susceptibility to type 1 diabetes mellitus.

To assess the possible gene-gene interaction of *PDCD1* with HLA (*IDDM1*), the study patients were stratified by HLA status. An association of the 10/10 genotype of 8185[TGC] with type 1 diabetes mellitus was observed in patients with high-risk HLA genotypes, but not in those without (Table 5), suggesting that the effect of the *PDCD1* 8185[TGC]$_n$ microsatellite is stronger in subjects with high-risk HLA class II genotypes. Given that those with high-risk HLA are expected to have stronger susceptibility to the disease, this subanalysis may indicate that *PDCD1* would enhance the predisposition conferred by *IDDM1*. When the

Table 6
Association of triplet repeat polymorphisms in *PDCD1* with type 1 diabetes mellitus relative to age at onset of disease

Age at onset	No. of subjects	TGC10/10 (%)	Others (%)	*P* (vs control)
Type 1 diabetes mellitus				
Younger	44	95.5	4.5	.07
Intermediate	44	93.2	6.8	.16
Older	44	90.9	9.1	.33
Control	134	85.1	14.9	

patients were divided into tertiles according to age at onset of the disease, the frequency of the 10/10 genotype tended to be higher in patients with younger age at onset (Table 6). These data suggest that *PDCD1* could be involved in disease predisposition by promoting the pathologic process leading to the development of diabetes.

The PD1.3A polymorphism has previously been reported to be associated with type 1 diabetes mellitus in a white population [11]. In the present study, however, this polymorphism was not found among 137 subjects sequenced, indicating that the PD1.3A polymorphism is absent or very rare in the Japanese population. A recent study by Kong et al [14] reported that the PD1.3A polymorphism was not detected among 827 subjects in the Chinese population, suggesting that the polymorphism is absent or very rare in Asian populations.

In conclusion, we identified a total of 16 sequence variants of *PDCD1*. A case-control study showed that 10 repetitions of 8185[TGC]$_n$ were significantly associated with type 1 diabetes mellitus in Japanese. These data, together with acceleration of type 1 diabetes mellitus by blocking or disruption of PDCD1 in NOD mice [7,8], suggest the contribution of *PDCD1* and its gene product to the development of type 1 diabetes mellitus. Further studies with a larger number of subjects as well as subjects in different ethnic groups are warranted to clarify the contribution of *PDCD1* to susceptibility to type 1 diabetes mellitus and other autoimmune diseases.

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