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Original Article

cblb Gene Analysis in Japanese Type 1 Diabetes with Younger Age of Onset

Junko Matsuda[1, 2], Ichiro Yokota[1, 3]

[1]*Department of Pediatrics, The University of Tokushima Graduate School of Medical Sciences, Tokushima, Japan*

[2]*Institute of Glycotechnology, Tokai University, Kanagawa, Japan*

[3]*Institute for Clinical Research, Kagawa National Children's Hospital, Kagawa, Japan*

Abstract. To clarify the contribution of Cblb to the development of type1 diabetes (T1D), we investigated Japanese younger-onset T1D patients. We sequenced the *cblb* gene in 10 T1D patients and screened the identified mutations in 109 Japanese T1D patients and 100 normal subjects. In addition to four previously reported synonymous single nucleotide polymorphisms (SNPs), we identified two novel nonsynonymous variants (786 C>T (A155V) and 1718 A>G (N466D)). The A155V mutation was found in one subject with Basedow's disease whose mother also carried both the mutation and Basedow's disease. The N466D mutation was found in 6 T1D cases including a subject who was classified as fulminant T1D. We found no significant differences in the allele frequency of these SNPs among T1D and control subjects, suggesting that the contribution of *cblb* to the genetic susceptibility to T1D might not be high for Japanese younger–onset T1D.

Key words: type 1 diabetes, Cblb, SNPs, autoimmune thyroid disease, fulminant form

Introduction

Type 1 diabetes (T1D) is characterized by insulin deficiency due to the destruction of insulin-producing pancreatic β-cells. According to the recently proposed classification of diabetes by the American Diabetes Association (ADA) and World Health Organization (WHO), T1D is divided into two subtypes: T cell-mediated autoimmune (immune-mediated; type 1A) diabetes and idiopathic (type 1B) diabetes (1, 2). Susceptibility to T1D is determined by a combination of genetic and environmental factors. So far, the major histocompatibility complex (MHC) is the most important susceptibility locus that has been identified for use in human and animal models (3, 4). T1D, especially immune-mediated type (type 1A), is considered a T cell-mediated autoimmune disease (5). From this perspective, the molecules involved in T cell signal regulation, like CTLA-4, could be associated with susceptibility to T1D (6).

Recently, Cblb has been reported to be the gene responsible for rat T1D; i.e., Komeda diabetes-prone (KDP) rat (7, 8). Cblb functions as a negative regulator of T cell activation (9–11). The $Cblb^{-/-}$ KDP rat shows infiltration of lymphocytes into pancreatic islets, the thyroid gland and kidney. Most $Cblb^{-/-}$ KDP rats develop

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Correspondence: Dr. Ichiro Yokota, Institute for Clinical Research, Kagawa National Children's Hospital, 2603 Zentsuji-cho, Zentsuji, Kagawa 765-8501, Japan
E-mail: yokotai@kagawasyoni.hosp.go.jp

Table 1 Clinical and laboratory findings and *cblb* gene SNPs identified in 10 younger-onset T1D cases

Case	Sex	Age of onset	Clinical characteristics	Identified *cblb* gene SNPs
Cases with autoimmune thyroid disease				
1	F	13	chronic thyroiditis (+)	D424D, L447L, T527T, A621A
2	F	4	Basedow's disease (+), mother: Basedow's disease	**A155V**
3	F	13	Basedow's disease (+)	D424D, L447L
Cases with autoimmune disease in a first-degree relative				
4	F	16	younger sister: ITP, father: ALS	ND*
5	F	7	elder sister: MCTD	ND*
Cases with diabetes in a first-degree relative				
6	M	11	T1D (Fulminant form)	**N466D**, A621A
7	M	6		A621 A
8	F	17	younger brother: T1D	ND*
9	M	10		D424D, L447L, **N466D**, T527T
10	M	12		D424D, L447L, T527T

*ND: not detected. ITP; idiopathic thrombocytopenia, ALS; amyotrophic lateral sclerosis, MCTD; mixed connective tissue disease.

overt diabetes through the autoimmune destruction of pancreatic β-cells. Since human T1D patients sometimes have other autoimmune diseases such as autoimmune thyroid diseases (AITDs) (12, 13), Cblb is a good candidate gene for human T1D. Recent studies in the U.K. and U.S. to evaluate the association between Cblb and a human susceptibility to T1D did not find nonsynonymous variants and also failed to find significant evidence of an association between Cblb and T1D (14, 15). In this study, we identified two novel nonsynonymous variants in Japanese T1D patients with a younger age of onset.

Materials and Methods

Patients and research subjects

We recruited 109 Japanese T1D patients with a younger age of onset (< 18 yr) (41 males and 68 females; median age at T1D onset was 8.2 yr (range 0.2–17 yr)). The diagnosis of T1D was determined according to the ADA classification (1, 2). A total of 100 non-diabetic subjects were used as control subjects. All of the subjects gave their informed consent for participation in this study. This study was approved by the ethics committee of Tokushima University School of Medicine. From among the 109 T1D patients, we selected 10 patients for sequencing of the *cblb* gene (exon 2 to exon 15) to identify mutations of the *cblb* gene in Japanese T1D patients. Next, we screened the other 99 T1D patients and 100 non-diabetic subjects for the identified mutations and compared the allele frequencies. The clinical characteristics of the 10 patients are shown in Table 1. These 10 patients included 3 with AITD, 2 who had a first-degree relative with possible autoimmune disease (idiopathic thrombocytopenia and amyotrophic lateral sclerosis, and mixed connective tissue disease), and 5 who had a first-degree relative with diabetes, including one case of fulminant T1D (16). Anti-glutamic acid decarboxylase (GAD) antibody was positive except in the patient with fulminant T1D (case 6).

Sequencing of the *cblb* gene

Genomic DNA was prepared from peripheral

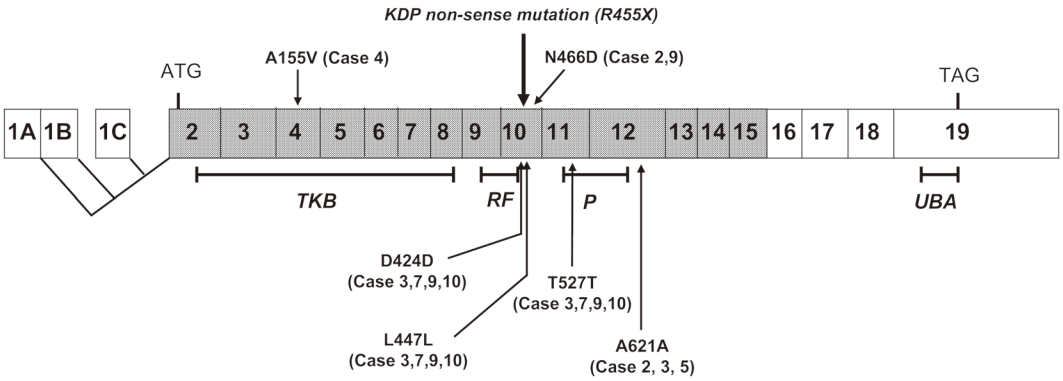

Fig. 1 Schematic representation of human Cblb cDNA. The human *cblb* gene is located on chromosome 3 of 3q13.12. ATG and TAG codons are indicated. The locations of the tyrosine-kinase-binding domain (TKB), RING finger domain (RF), proline-rich region (P), and ubiquitin-associated region (UBA) are indicated. The SNPs identified in this study and the mutation in KDP rat are indicated by arrows.

white blood cells. To identify unknown mutations in the *cblb* gene, exons and exon-intron junctions for exons 2 to 15, which include a tyrosine kinase binding domain, the RING finger domain, and a proline-rich region of Cblb (accession numbers in GenBank; Cblb mRNA: NM_004351, *cblb* genomic DNA: NM_030622) (17) (Fig. 1), were amplified by PCR using suitable primer sets (Table 2). The amplified DNA fragments (from 203 to 473 bp in size) were directly sequenced using a BigDye Terminator v3.1 Cycler Sequencing Kit (PE Applied Biosystems, Foster City, CA) on an ABI PRISM 3100-*Avant* Genetic Analyzer (PE Applied Biosystems).

SNP genotyping by specific restriction enzyme digestion sites (PCR-RFLP)

To screen the 99 T1D patients and 100 non-diabetic subjects for the single nucleotide polymorphisms (SNPs) that had been identified by sequencing of the *cblb* gene in the initial 10 T1D patients, we established a genotyping method using PCR-RFLP. As shown in Table 3, respective primer sets were designed to create specific restriction enzyme sites. Some primers involved mismatched bases in the 3'-end of the primers to create specific restriction enzyme sites. The PCR reaction products were cleaved using the respective restriction enzymes, separated by electrophoresis on agarose gels and photographed under ultraviolet illumination.

Statistical analysis

The statistical significance of associations among the genotypes and alleles in the T1D patients and normal subjects was assessed using 2×2 or 2×3 contingency-table χ^2 tests, except that Fisher's exact test was used when the expected number in a 2×2 or 2×3 contingency-table was less than five.

Results and Discussion

By direct sequencing of the *cblb* gene (exons 2 to 15) in 10 Japanese T1D patients, six SNPs were identified, including four previously reported SNPs (1594 C>T (D424D), 1663 A>C (L447L), 1903 G>A (T527T), 2186 G>A (A621A)) which did not change any amino acid residue, and two novel SNPs (786 C>T (A155V), 1718 A>G (N466D)) which did change amino acid residues (Table 1, Fig. 1). All 6 of these SNPs were confirmed by PCR-RFLP analysis and agarose gel electrophoresis using genomic DNA from the patients. The four previously reported synonymous SNPs were found in the Japanese

Table 2 Sequences of the PCR primers used in the present study to amplify exons of the human *cblb* gene

Name	Sequence	PCR product size
Exon 2 F (s)	5' TTTTTAAATCTTCTGCCTTTTAAAGAACT 3'	
Exon 2 R (as)	5' ACAAAGTGAATAGTGTTTCGCACA 3'	270 bp
Exon 3 F (s)	5' TTTTAATTTTTCTGTGAAATAAAATATTATG 3'	
Exon 3 R (as)	5' TGACCTTTACACCAAAACATCTG 3'	348 bp
Exon 4 F (s)	5' GCATGCATCTAGGTGTTTATTTCTTATC 3'	
Exon 4 R (as)	5' CCAACTGGAGGGAGGATACA 3'	270 bp
Exon 5 F (s)	5' TGATACTTGATTCAATTATTTCCCT 3'	
Exon 5 R (as)	5' GAAGAAGGGAATGGATAGACTAAGC 3'	240 bp
Exon 6 F (s)	5' GTTAAGTGTATTAAATATGGTTCAGTATG 3'	
Exon 6 R (as)	5' GCGGGTATTGCTGACTTACTTAGG 3'	299 bp
Exon 7 F (s)	5' GCTTGGAAGAAACCTCCTAACAAATTGT 3'	
Exon 7 R (as)	5' CATGAATCATAAGCACTCCAACTTC 3'	260 bp
Exon 8 F (s)	5' TCCTTAAATGCAATTAAAACTTGTATATT 3'	
Exon 8 R (as)	5' TTTCAAGGGCATTATGGATACTTAT 3'	203 bp
Exon 9 F (s)	5' CATTACTTTCTCCCTCCTCCCCA 3'	
Exon 9 R (as)	5' GGACATTATAAGAAAGCATACCATGTTGC 3'	203 bp
Exon 10 F (s)	5' GAACAAAACCAATCCATGCATTTT 3'	
Exon 10 R (as)	5' CACATCACCTTAACTAAACCCATGT 3'	277 bp
Exon 11 F (s)	5' CACTCTGCACAGCTAATAAACAGC 3'	
Exon 11 R (as)	5' GTCTTACAAAATTTTCATCTGTGTTTC 3'	280 bp
Exon 12 F (s)	5' GTCAGTGCATGGTACAACCTTTAGT 3'	
Exon 12 R (as)	5' TTTATAAGCAGATTCTCTAGCTTCTGC 3	473 bp
Exon 13 F (s)	5' GAGCAGCTATGGCTTTAAGGTCC 3'	
Exon 13 R (as)	5' CTTGATGCATAGACAAGTGATCTCC 3'	206 bp
Exon 14 F (s)	5' TGTCACATCAGACTTGCCTGTTTTG 3'	
Exon 14 R (as)	5' CAGATGTAATGGGCAAAAATCTGC 3'	251 bp
Exon 15 F (s)	5' CTAGTTCCCTCTACCTCTCAAGTTACTT 3'	
Exon 15 R (as)	5' GCCTTTAAATTCTGACCATTAAGATG 3'	233 bp

(s) and (as) denote sense and antisense sequences, respectively.

Single Nucleotide Polymorphisms (JSNP) database (http://snp.ims.u-tokyo.ac.jp). In the novel nonsynonymous SNPs, A155V represents a C to T substitution at position 786 in exon 4, which changes an alanine to a valine at position 155 in the tyrosine kinase binding domain of Cblb (Fig. 2). The other novel nonsynonymous SNP, N466D, represents an A to G substitution at position 1718 in exon 10, which changes an asparagine to an aspartic acid at position 466, which is just outside the ring-finger domain of Cblb.

All Cbl proteins have a highly conserved N-terminal region which contains two domains that are critical for Cbl protein function. The first, a tyrosine kinase binding (TKB) domain, recognizes and binds to phosphorylated tyrosine residues in tyrosine kinase. The second domain is a RING finger domain which is the catalytic domain for the ubiquitin protein ligase activity of Cbl proteins (17). The A155V SNP was located in the TKB domain and the N466D SNP was located near the RING finger domain. Both domains are highly conserved in a variety of mammalian and non-mammalian species and are thought to be functionally important for Cblb

Table 3 Sequences of primers for detecting SNPs by PCR-RFLP

SNPs	Primers Name	Sequence	Restriction enzyme	PCR product size after digestion
A155V	Exon 4 mis F (s)	5' ACTGTCCCTTATCTTCAGTCACATACTAG 3'	Spe I	201 bp> 177 bp + 24 bp
	Exon 4 R (as)	5' CCAACTGGAGGGAGGATACA 3'		
D424D	Exon 10 F (s)	5' GAACAAAACCAATCCATGCATTTT 3'	BamHI	277 bp> 103+174 bp
L447L	Exon 10 R (as)	5' CACATCACCTTAACTAAACCCATGT 3'	TaqI	277 bp> 173+104 bp
N466D			AatII	277 bp> 233+44 bp
T527T	Exon 11F2 (s)	5' TGACCCACTCCAGATCCCA 3'	AluI	124 bp> 100+24 bp
	Exon 11mis R (as)	5' AAATTGATCTACCTTTGGTGAAGC 3'		
A621A	Exon 12F2 (s)	5' TGTTTGGGACTATCAGCTTGTG 3'	Hind III	95 bp> 71+24 bp
	Exon 12 mis R (as)	5' GTGCCTTCCATTGACATTTAAGCT 3'		

(s) and (as) denote sense and antisense sequences, respectively. The underlined bases are mismatched bases.

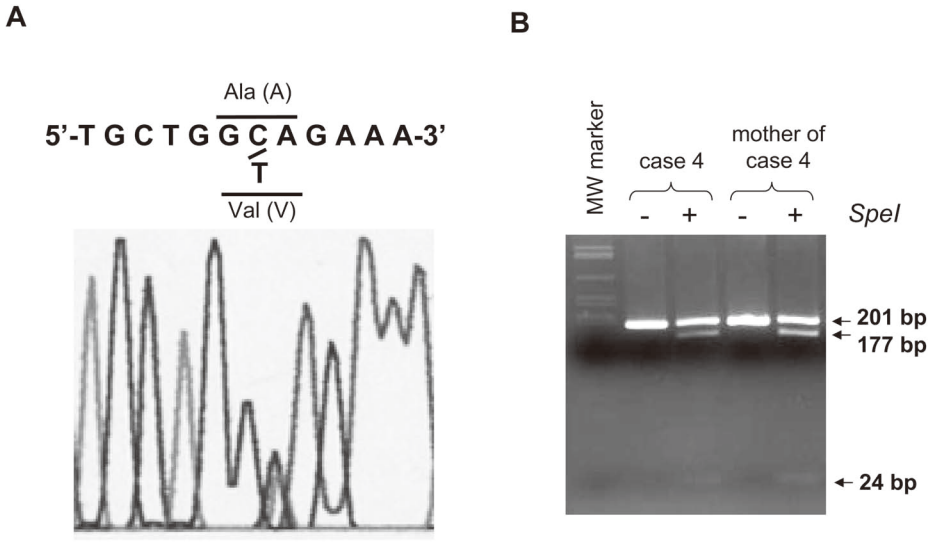

Fig. 2 Detection of the 786 C>T (A155V) mutation in case 2 and her mother. (A) In case 2, a heterozygous mutation of A155V(786 C>T) was identified in exon 4 of the *cblb* gene by direct sequencing. (B) PCR-RFLP analysis using the set of primers in Table 2 revealed that both case 2 and her mother with Basedow's disease showed the A155V mutation. After *Spe*I digestion of PCR products, three bands (201, 177 and 24 bp) were observed in the heterozygous state in case 2 and her mother.

signaling (17). Changes in the amino acid residues at these important sites could change the protein primary structure and functions of Cblb (17, 18).

We screened the above SNPs in 109 Japanese younger-onset T1D patients (M/F=41/68) and compared the allele frequencies with those in 100 non-diabetic subjects using a PCR-RFLP

Table 4 Frequencies of the *cblb* gene SNPs and alleles in type 1 diabetic patients and normal subjects

Reported SNPs		Genotype				Allele	
1594C>T		C/C	C/T	T/T	P (χ^2)	T allele frequency	P (χ^2)
(Exon 10, D424D)	T1D	64/107 (60)	39/107 (36)	4/107 (4)	0.74 (0.59)	47/214 (22)	0.71 (0.14)
	Control	59/100 (59)	34/100 (34)	7/100 (7)	-	48/200 (24)	-
1663A>C		A/A	A/C	C/C	P (χ^2)	C allele frequency	P (χ^2)
(Exon 10, L447L)	T1D	64/107 (60)	39/107 (37)	4/107 (4)	0.74 (0.59)	47/214 (22)	0.71 (0.14)
	Control	59/100 (59)	34/100 (34)	7/100 (7)	-	48/200 (24)	-
1903A>G		A/A	A/G	G/G	P (χ^2)	G allele frequency	P (χ^2)
(Exon 11, T527T)	T1D	64/108 (59)	37/108 (34)	7/108 (6)	0.98 (0.03)	51/216 (24)	0.85 (0.04)
	Control	59/100 (59)	34/100 (34)	7/100 (7)	-	48/200 (24)	-
2186G>A		G/G	G/A	A/A	P (χ^2)	A allele frequency	P (χ^2)
(Exon 12, A621A)	T1D	77/106 (73)	27/106 (25)	2/106 (2)	0.89 (0.24)	31/212 (15)	0.75 (0.11)
	Control	73/100 (71)	23/100 (26)	4/100 (3)	-	31/200 (16)	

Novel SNPs		Genotype				Allele	
786C>T		C/C	C/T	T/T	P	T allele frequency	P
(Exon 4, A155V)	T1D	108/109 (99)	1/109 (1)	0/109 (0)	>0.99	1/218 (0.5)	0.61
	Control	98/100 (98)	2/100 (2)	0/100 (0)	-	2/200 (1.0)	-
1718A>G		A/A	A/G	G/G	P	G allele frequency	P
(Exon 10, N466D)	T1D	101/107 (94)	6/107 (6)	0/107 (0)	0.25	6/214 (2.8)	0.12
	Control	99/100 (99)	1/100 (1)	0/100 (0)	-	1/200 (0.5)	-

Data are n (%). P values are vs. normal control subjects.

analysis (Table 3). There were no differences in the allele frequency of the four previously reported SNPs among the T1D patients and controls (Table 4). With regard to the two novel SNPs, we found the A155V mutation in 1 allele (0.5%) among the T1D patients and in 2 alleles (1.0%) in the controls (P=0.61). Six N466D alleles (2.8%) were found in T1D and 1 allele (0.5%) was found in the controls (P=0.12) (Table 4). Although more N466D SNP was identified in T1D than in the control, the allele frequencies of these two novel SNPs were not significantly different. These results were basically consistent with those of recent studies in the U.K. and U.S. evaluating the association of Cblb with human T1D (14, 15), suggesting that Cblb may not strongly contribute to the genetic susceptibility to T1D in Japanese younger–onset T1D. However, our study may be unique in that we identified two novel nonsynonymous variants which could possibly affect *cblb* gene function. Expression studies to test whether A155V SNP and N466D SNP affect the function of Cblb protein might be needed.

The A155V SNP was found in a patient with T1D. She was diagnosed T1D at 4 yr of age. She was carrying an HLA class II genotype of DRB1*0405/DRB1*0901, which confers a genetic risk for T1D in Japanese (19). She showed symptoms of hyperthyroidism in Basedow's disease at the age of 5. Since her mother also had a history of Basedow's disease, we performed

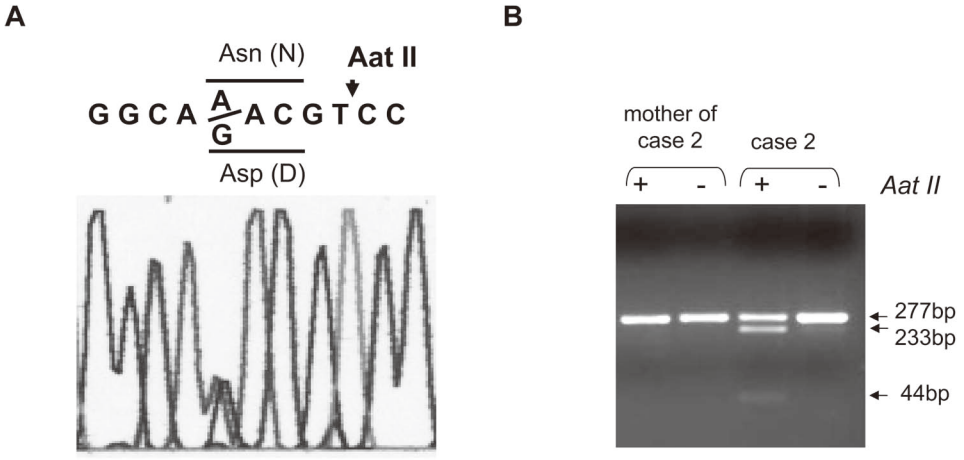

Fig. 3 Detection of the 1718 A>G (N466D) mutation in case 6. (A) In case 6, a heterozygous mutation of N466D (1718 A>G) was identified in exon 10 of the *cblb* gene by direct sequencing. (B) PCR-RFLP analysis using the set of primers in Table 2, revealed that case 6, but not his mother with diabetes, showed the N466D mutation. After *Aat*II digestion of the PCR products, three bands (277, 233 and 44 bp) were observed in the heterozygous state in case 6.

genotyping of A155V SNP in the mother, and found that the A155V SNP was inherited from the mother (Fig. 2).

The N466D SNP was identified in 6 T1D patients. Five of them showed the usual immune-mediated type: type 1A phenotype. One patient with N466D SNP was classified as fulminant T1D (case 6) (Fig. 3). He was diagnosed as having T1D at 11 yr of age. Hyperglycemic symptoms in this patient persisted for only 4 d with abdominal pain. At the time of onset, he had a significantly high plasma glucose concentration (719 mg/dL) and diabetic ketoacidosis, despite lower initial glycosylated hemoglobin values (5.8%), and lower urinary C-peptide excretion (<1.4 μg/day). The serum pancreatic amylase concentration was slightly elevated (151 U/L). Serum GAD antibody was not detected.

In summary, we have identified two novel nonsynonymous variants (A155V and N466D) in the *cblb* gene. Further expression studies will be needed to clarify whether these variants affect the function of Cblb. We did not find evidence of a significant association between the *cblb* gene variants and human T1D, suggesting that the contribution of *cblb* to the genetic susceptibility to T1D might not be high for Japanese younger–onset T1D.

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