Original Article

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

Junko Matsuda1, 2, Ichiro Yokota1, 3
1Department of Pediatrics, The University of Tokushima Graduate School of Medical Sciences, Tokushima, Japan
2Institute of Glycotechnology, Tokai University, Kanagawa, Japan
3Institute for Clinical Research, Kagawa National Children’s Hospital, Kagawa, Japan

Abstract. To clarify the contribution of Cblb to the development of type 1 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
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
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 $2 \times 2$ or $2 \times 3$ contingency-table $\chi^2$ tests, except that Fisher’s exact test was used when the expected number in a $2 \times 2$ or $2 \times 3$ 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
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 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' TTTTTAAATCTTCTGCCCTTTTTAAAGAACT 3'         |                  |
| Exon 2 R (as) | 5' ACAAGTGAATAGTGTGTTTCCGAAC 3'              | 270 bp           |
| Exon 3 F (s) | 5' TTATTTTCTCTGGAATAATAATATTATG 3'           |                  |
| Exon 3 R (as) | 5' TGACCTTTACCAAAACATCTG 3'                 | 348 bp           |
| Exon 4 F (s) | 5' GCATGCATCTAGTGTTTTATCCTTATC 3'           |                  |
| Exon 4 R (as) | 5' CCAACTGGAGGGAGGATACA 3'                  | 270 bp           |
| Exon 5 F (s) | 5' TGAATTTGATCTCAATATATCTTCCCT 3'           |                  |
| Exon 5 R (as) | 5' GAAGAGGGAATGGAAGAATAGC 3'                | 240 bp           |
| Exon 6 F (s) | 5' GTTAAGTGTATATAATGTTGCGATATG 3'           |                  |
| Exon 6 R (as) | 5' CCGGGTTATCTGCAATTTAGG 3'                 | 299 bp           |
| Exon 7 F (s) | 5' GCTTGGGAAGAAACCTCTAACAATGTG 3'           |                  |
| Exon 7 R (as) | 5' CATGAATAAGCAGCCTTTCAACTTTCC 3'          | 260 bp           |
| Exon 8 F (s) | 5' TCTTAAAGGCAATTTATACATTTATATG 3'         |                  |
| Exon 8 R (as) | 5' TTCCAGGACAGGGAGGTAGATTTATATG 3'         | 203 bp           |
| Exon 9 F (s) | 5' CATTACTTTCTCCCTCTCCCTCCC 3'             |                  |
| Exon 9 R (as) | 5' GGACATTATAAGAAGAAGCAGCAGTAAATG 3'       | 203 bp           |
| Exon 10 F (s) | 5' CAAAGAAAACACATCTGCAATTTC 3'             |                  |
| Exon 10 R (as) | 5' CACATCACTTAACTAACCCATGT 3'              | 277 bp           |
| Exon 11 F (s) | 5' CACTCTGCACACGTAAATACAGC 3'              |                  |
| Exon 11 R (as) | 5' GTCTTCAAAATTTCTATCTGTTTC 3'            | 280 bp           |
| Exon 12 F (s) | 5' GTCAGTGTCATGAGGCAACCTTTGAGT 3'         |                  |
| Exon 12 R (as) | 5' TTTAAGGACAGATCTCTTGTGTTC 3'            | 473 bp           |
| Exon 13 F (s) | 5' GAGCAGGTATCGTGAAGGTCACT 3'              |                  |
| Exon 13 R (as) | 5' CTTGATGCAGAAGTACTGCTC 3'               | 206 bp           |
| Exon 14 F (s) | 5' TGTCACATCAGACTTCGCTTCTT 3'              |                  |
| Exon 14 R (as) | 5' CAGATGTAATGGCGAAAATCTGCG 3'            | 251 bp           |
| Exon 15 F (s) | 5' CTGTTTCTCCTCTTCTCAGTTACTTT 3'          |                  |
| Exon 15 R (as) | 5' GCCTTTAAAAATTTGAGGACTATTATG 3'        | 233 bp           |

(s) and (as) denote sense and antisense sequences, respectively.
Gene Polymorphism in Japanese Type 1 Diabetes

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...
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

### 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      |        |
| (Exon 10, D424D) | 64/107 (60) | 4/107 (4) | 0.74 (0.59) |
| T1D           | C/T      |        |
| Control       | 59/100 (59) | 7/100 (7) | - |
| **1663A>C**   | A/A      |        |
| (Exon 10, L447L) | 64/107 (60) | 4/107 (4) | 0.74 (0.59) |
| T1D           | A/C      |        |
| Control       | 59/100 (59) | 7/100 (7) | - |
| **1903A>G**   | A/A      |        |
| (Exon 11, T527T) | 64/108 (59) | 7/108 (6) | 0.98 (0.03) |
| T1D           | A/G      |        |
| Control       | 59/100 (59) | 7/100 (7) | - |
| **2186G>A**   | G/G      |        |
| (Exon 12, A621A) | 77/106 (73) | 2/106 (2) | 0.89 (0.24) |
| T1D           | G/A      |        |
| Control       | 73/100 (71) | 4/100 (3) | - |

| Novel SNPs | Genotype | Allele |
|------------|----------|--------|
| **786C>T** | C/C      |        |
| (Exon 4, A155V) | 108/109 (99) | 0/109 (0) | >0.99 |
| T1D         | C/T      |        |
| Control     | 98/100 (98) | 0/100 (0) | - |
| **1718A>G** | A/A      |        |
| (Exon 10, N466D) | 101/107 (94) | 0/107 (0) | 0.25 |
| T1D         | A/G      |        |
| Control     | 99/100 (99) | 0/100 (0) | - |

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

Acknowledgments

We thank Dr. Y. Kotani, K. Shinahara and S. Satomura for their assistance. All experiments were performed in compliance with the current laws of Japan.

References

1. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20:1183–97.
2. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1. Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539–53.
3. Davies JL, Kawaguchi Y, Bennett ST, Copeman JB, Cordell HJ, Pritchard LE, et al. A genome-wide search for human type 1 diabetes...
susceptibility genes. Nature 1994;371:130–6.

4. Abiru N, Kawasaki K, Eguchi K. Current knowledge of Japanese type 1 diabetic syndrome. Diabetes Metab Res Rev 2002;18:357–66.

5. Azar ST, Tamim H, Beyhum HN, Habbal MZ, Almawi WY. Type I (insulin-dependent) diabetes is a Th1- and Th2-mediated autoimmune disease. Clin Diagn Lab Immunol 1999;6:306–10.

6. Takara M, Komiya I, Kinjo Y, Tomoyose T, Yamashiro S, Akamine H, et al. Association of CTLA-4 gene A/G polymorphism in Japanese type 1 diabetic patients with younger age of onset and autoimmune thyroid disease. Diabetes Care 2000;23:975–8.

7. Yokoi N, Komeda K, Wang HY, Yano H, Kitada K, Saitoh Y, et al. Cblb is a major susceptibility gene for rat type 1 diabetes mellitus. Nat Genet 2002;31:391–4.

8. Yokoi N, Namee M, Fuse M, Wang HY, Hirata T, Seino S, et al. Establishment and characterization of the Komeda diabetes-prone rat as a segregating inbred strain. Exp Anim 2003;52:295–301.

9. Bachmaier K, Krawczyk C, Kozieradzki K, Kong YY, Sasaki T, Oliveira-dos-Santos A, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature 2000;403:211–6.

10. Chiang YJ, Kole HK, Brown K, Naramura M, Fukuhara S, Hu RJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. Nature 2000;403:216–20.

11. Krawczyk C, Bachmaier K, Sasaki T, Jones GR, Snapper BS, Bouchard D, et al. Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. Immunity 2000;13:463–73.

12. Kordonouri O, Klinghammer A, Lang EB, Gruters-Kieslich A, Grabert M, Holl RW. Thyroid autoimmunity in children and adolescents with type 1 diabetes: a multicenter survey. Diabetes Care 2002;25:1346–50.

13. Hanukoglu A, Mizrachi A, Dalal I, Admoni O, Rakover Bistritzer YZ, et al. Extrapancreatic autoimmune manifestations in type 1 diabetes patients and their first-degree relatives: a multicenter study. Diabetes Care 2003;26:1235–40.

14. Payne F, Smyth DJ, Pask R, Barratt BJ, Cooper JD, Twells RC, et al. Haplotype tag single nucleotide polymorphism analysis of the human orthologues of the rat type 1 diabetes genes ian4 (lyp/iddm1) and cblb. Diabetes 2004;53:505–9.

15. Kosoy R, Yokoi N, Seino S, Concannon P. Polymorphic variation in the CBLB gene in human type 1 diabetes. Genes Immun 2004;5:232–5.

16. Nau MN, Lipkowitz S. Comparative genomic organization of the cbl genes. Gene 2003;308:103–13.

17. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. N Engl J Med 2000;342:301–7.

18. Liu YC, Gu H. Cbl and Cbl-b in T-cell regulation. Trends Immunol 2002;23:140–3.

19. Kawabata Y, Ikegami H, Kawaguchi Y, Fujisawa T, Shintani M, Ono M, et al. Asian-specific HLA haplotypes reveal heterogeneity of the contribution of HLA-DR and -DQ haplotypes to susceptibility to type 1 diabetes. Diabetes 2002;51:545–51.