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ORIGINAL ARTICLE

Nucleotide substitutions in *CD101*, the human homolog of a diabetes susceptibility gene in non-obese diabetic mouse, in patients with type 1 diabetes

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ABSTRACT

Aims/Introduction: Although genome-wide association studies have identified more than 50 susceptibility genes for type 1 diabetes, low-frequency risk variants could remain unrecognized. The present study aimed to identify novel type 1 diabetes susceptibility genes by newly established methods.

Materials and Methods: We carried out whole-exome sequencing and genome-wide copy-number analysis for a Japanese family consisting of two patients with type 1 diabetes and three unaffected relatives. Further mutation screening was carried out for 127 sporadic cases. The functional consequences of identified substitutions were evaluated by *in silico* analyses and fluorescence-activated cell sorting of blood samples.

Results: Whole-exome sequencing and genome-wide copy-number analysis of familial cases showed co-segregation of the p.V863L substitution in *CD101*, the human homolog of an autoimmune diabetes gene in the non-obese diabetic mouse, with type 1 diabetes. Mutation screening of *CD101* in 127 sporadic cases detected the p.V678L and p.T944R substitutions in two patients. The p.V863L, p.V678L and p.T944R substitutions were absent or extremely rare in the general population, and were assessed as 'probably/possibly damaging' by *in silico* analyses. CD101 expression on monocytes, granulocytes and myeloid dendritic cells of mutation-positive patients was weaker than that of control individuals.

Conclusions: These results raise the possibility that *CD101* is a susceptibility gene for type 1 diabetes.

INTRODUCTION

Type 1 diabetes mellitus is a multifactorial disease in which pancreatic β-cells are destroyed primarily by a T cell-mediated autoimmune reaction[1]. Autoimmunity in type 1 diabetes is facilitated by various cells, including dendritic cells (DCs)[1–3]. A subset of patients with type 1 diabetes shows familial aggregation, suggesting a significant contribution of genetic factors to the etiology of the disease[1]. Type 1 diabetes represents a polygenic disorder, whereas other forms of diabetes mellitus, such

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as maturity-onset diabetes of the young, neonatal diabetes and syndromic diabetes, often result from monogenic mutations or chromosomal abnormalities[1,2]. Genome-wide association studies (GWAS), together with candidate gene approaches, have identified more than 50 susceptibility genes for type 1 diabetes[2]. However, these genes account for just ~80% of the genetic heritability of type 1 diabetes, indicating that additional disease-associated genes remain to be identified[2,4]. In particular, susceptibility variants of low frequency might be undetected by GWAS, as this method focuses primarily on relatively common polymorphisms[5].

CD101 (also known as V7 and IGSF2) is a transmembrane glycoprotein expressed on various types of immune cells[6–9]. *Cd101* has been reported as an 'autoimmune diabetes gene in the non-obese diabetic (NOD) mouse'[10–12]. Murine CD101 is known to modulate the function of regulatory T cells and antigen-presenting cells, with genotype-dependent *Cd101* expression determining the risk of autoimmune diabetes in NOD mice[11,12]. In addition, monoclonal antibodies against CD101 inhibit allogeneic T cell responses[9]. Human CD101 has also been implicated in immune regulation[7,8,13–16]. Previous studies have suggested that human CD101 plays a costimulatory role in T cell activation mediated by T cell receptor/CD3 or skin DCs[7,8]. However, *CD101* mutations have not been associated with diabetes in humans. Here, we report the identification of three *CD101* substitutions in patients with type 1 diabetes. These substitutions were detected through whole-exome sequencing of familial cases and mutation screening of sporadic cases.

MATERIALS AND METHODS

Whole-exome sequencing and genome-wide copy-number analysis of a family with type 1 diabetes

The present study was approved by the institutional review board committee at the National Center for Child Health and Development, and was carried out after obtaining written informed consent. We carried out molecular analyses of a Japanese family (family A) consisting of two patients with type 1 diabetes and three unaffected relatives. The male proband (case 1) and his mother (case 2) developed diabetes at the ages 2.6 and 18 years, respectively (Table 1). At disease onset, case 1 was positive for the insulin autoantibody, whereas case 2 was positive for the islet cell surface antibody. Human leukocyte antigen (*HLA*) typing showed known risk alleles of the Japanese population[17], *DRB1*04:05* and *DQB1*04:01*, in cases 1 and 2 and two unaffected family members, and *DQB1*03:02* in case 1 and his unaffected father (Table 1). The unaffected grandmother of case 1 (the mother of case 2) carried two risk alleles, *DRB1*04:05* and *DQB1*04:01*, together with a protective *DQB1*03:01* allele. Cases 1 and 2 showed no additional clinical features. No family history of other autoimmune diseases was recorded in this family.

We carried out whole-exome sequencing using genomic DNA samples obtained from cases 1 and 2 and three unaffected family members (the father and two older siblings of the proband). DNA libraries were constructed using a SureSelect Kit (51 Mb version 4; Agilent Technologies, Santa Clara, CA, USA), and sequenced using a Hiseq 1000 sequencer (Illumina, San Diego, CA, USA). Nucleotide alterations were called by Avadis NGS 1.3.1 (DNA Chip Research, Yokohama, Japan) or SAMtools 0.1.17 software (https://sourceforge.net/projects/samtools/files/samtools/). We searched for nucleotide alterations shared by cases 1 and 2, but absent from the three unaffected relatives. We focused on exonic mutations that alter protein sequences and intronic substitutions located within a 5-bp region from an exon–intron boundary. Known polymorphisms with an allele frequency of more than 1.0% in the general population (NCBI Browser, http://www.ncbi.nlm.nih.gov/), and mutations whose functional outcomes were predicted as 'benign' by *in silico* analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) were excluded from further analysis. We referred to the OMIM database (https://www.ncbi.nlm.nih.gov/omim) to examine whether the genes identified in the present study were associated with any human disorders. We also searched the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) for previous reports on these genes. Nucleotide alterations in *CD101* (NM_004258) and *GAD2* (NM_000818.2) were confirmed by Sanger sequencing. Primer sequences are available on request. In addition, we analyzed the parental origin of a *CD101* mutation identified in case 2. Copy-number alterations in case 1 were analyzed by array-based comparative genomic hybridization (SurePrint G3 Human Microarray, 2 × 400 k format; Agilent Technologies). We referred to the Database of Genomic Variants (http://projects.tcag.ca/variation/) to exclude known benign variants.

Mutation screening of *CD101* in sporadic cases with type 1 diabetes

We carried out mutation screening of *CD101* in 127 sporadic cases with type 1 diabetes (49 males and 78 females, aged 2.0–18.1 years). All patients were of Japanese origin, and developed diabetes between the age of 0.9 and 15.7 years. The patients were positive for anti-GAD and/or islet antigen 2 antibodies. Patients with syndromic diabetes were excluded from the study.

Nucleotide alterations in *CD101* coding exons and their flanking regions were examined by amplicon-sequencing using Nextera Kits (FC-121-1031 and FC-121-1012; Agilent Technologies) and a Miseq next-generation sequencer (Illumina). All *CD101* nucleotide alterations, except for common polymorphisms found in the NCBI Browser, were confirmed by Sanger sequencing. To assess the presence or absence of the pathogenicity of *CD101* substitutions, we attempted to obtain parental samples of mutation-positive patients.

Functional assessment of *CD101* substitutions

We examined whether the *CD101* substitutions identified in the patients are present in the general population. First, we analyzed 185 DNA samples obtained from healthy Japanese controls (Human Science Research Resources Bank, Tokyo, Japan; present distributer, National Institute of Biomedical Innovation,

Table 1 | Clinical and molecular findings of *CD101*-substitution-positive patients and unaffected family members

Familial/Sporadic Cases	Family A Case 1 (proband)	Case 2 (mother)	Grandmother of case 1	Father of case 1	Older brother of case 1	Older sister of case 1	Sporadic Case 3	Sporadic Case 4
Sex	Male	Female	Female	Male	Male	Female	Male	Female
Clinical findings at the onset of the disease								
Age at onset (years)	2.6	18	–	–	–	–	10.5	13.2
Body mass index (kg/m^2)	14.8 (14.4–17.2)	22 (16.3–23.5)	–	–	–	–	17.1 (15.0–21.5)	16.6 (16.3–23.5)
Blood glucose (mmol/L)	**18.9** (4.1–5.2)	**8.6** (4.4–5.2)	–	–	–	–	**51** (4.5–5.2)	**13** (4.4–5.2)
HbA1c (mmol/mol)	**95** (26–36)	**53** (26–36)	–	–	–	–	**89** (26–36)	**92** (26–36)
HbA1c (%)	**10.8** (4.5–5.4)	**7** (4.5–5.4)	–	–	–	–	**10.3** (4.5–5.4)	**10.6** (4.5–5.4)
Blood C-peptide (nmol/L)	**0.2** (0.3–0.6)	NA	–	–	–	–	**0.2** (0.4–0.8)	NA
Urine C-peptide (μg/day)	**<9** (12–34)	**15** (29–167)	–	–	–	–	NA	90 (29–167)
Anti-GAD antibody (U/mL)	Negative (<1.5)	NA	–	–	–	–	**3.8** (<1.5)	**81** (<1.5)
Insulin autoantibody (%)	**20.8** (<10)	NA	–	–	–	–	NA	NA
Islet cell surface antibody	NA	**Positive**[†] (negative)	–	–	–	–	NA	NA
Molecular findings								
HLA-DRB1	**4:05*	**4:05*	**4:05*	**11:01*	**4:05*	**4:05*	**4:05*	**9:01*
	**11:01*	**4:05*	**12:02*	**13:02*	**13:02*	**13:02*	**9:01*	**9:01*
HLA-DQB1	**3:02*	**4:01*	**3:01*[‡]	**3:02*	**4:01*	**4:01*	**3:03*	**3:03*
	**4:01*	**4:01*	**4:01*	**6:09*	**6:09*	**6:09*	**4:01*	**3:03*
CD101 substitution	p.V863L	p.V863L	p.V863L	None	None	None	p.V678L	p.T944R

Reference ranges are shown in parentheses. Values above or below the reference range are boldfaced. Glycated hemoglobin (HbA1c) is measured by a National Glycohemoglobin Standardization Program-certified method (%), and converted to International Federation of Clinical Chemistry value (mmol/mol). Human leukocyte antigen (HLA) class II genotypes known as risk alleles of diabetes in Japanese are underlined. [†]Actual value unknown. [‡]Known as a protective allele of diabetes in Japanese. NA, not analyzed.

Osaka, Japan). The presence or absence of the *CD101* substitutions were examined by single-nucleotide polymorphism genotyping (custom-made TaqMan SNP Genotyping Assays; Life Technologies, Carlsbad, CA, USA) or by Sanger direct sequencing. Second, we examined frequencies of the *CD101* substitutions in databases. We referred to the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB/) and the Exome Aggregation Consortium (ExAC) Browser (http://exac.broadinstitute.org/). We also examined the position and evolutionary conservation of affected amino acids in the CD101 protein.

Expression analysis of CD101 in unaffected individuals and substitution-positive patients

To analyze CD101 expression on hematopoietic cells, we carried out multicolor fluorescence-activated cell sorting using LSRFortessa (BD Biosciences, San Jose, CA, USA). Fresh peripheral blood was obtained from five healthy individuals and three *CD101* substitution-positive patients (cases 1, 2 and 4). Fresh blood samples of case 3 were unavailable. Populations of lymphocytes, monocytes and granulocytes were differentiated using forward scatter and side scatter of flow cytometry. For analysis of CD101 expression on DCs, cells expressing human CD45 were gated on HLA-DR$^+$ and lineage$^-$, and divided into two subpopulations in terms of expression of CD11c and CD123; that is, CD11c$^+$/CD123$^-$ cells and CD11c$^-$/CD123$^+$ cells, which were referred to as myeloid and plasmacytoid DCs, respectively (Figure 1a,b). Antibodies used were FITC-conjugated human CD45, APC-Cy7-conjugated HLA-DR, APC-conjugated CD123, PerCP-Cy5.5-conjugated CD11c, PE-conjugated CD101, and brilliant violet 421-conjugated CD3, CD19 and CD14 for lineage markers. The CD101 antibody was purchased from AbD Serotec (Kidlington, Oxford, UK), and other antibodies were purchased from BioLegend (San Diego, CA, USA).

RESULTS

Molecular analyses of family A

Whole-exome sequencing of family A showed that nine hitherto unreported nucleotide changes in nine genes and 15 rare polymorphisms in 14 genes co-segregated with type 1 diabetes (Table 2; Figure 2a,b). Seven of the 24 nucleotide changes were intronic substitutions or an inframe insertion of unknown pathogenicity. Of the 23 mutated genes, *CD101* represented the human homolog of a known diabetes-causative gene in NOD mice[11,12], and *GAD2* encoded a major autoantigen for type 1 diabetes[3]. The p.V863L substitution in *CD101* was hitherto unreported, whereas the p.I228T substitution in *GAD2* was found in 262 of 121,380 alleles in the ExAC Browser. The remaining 21 genes have not been associated with type 1 diabetes or other autoimmune disorders; eight of these genes were associated with some genetic disorders other than diabetes. The p.V863L substitution in *CD101* was shared by the unaffected mother of case 2 (the grandmother of case 1). Mutations in known diabetes-associated genes, including *INS*, *PTPN22*, *IL2RA* and *CTLA4*, were not found in this family. Genome-wide copy-number analysis in case 1 detected no deletions or duplications, except for common copy-number variations.

Mutation screening of *CD101* in sporadic cases with type 1 diabetes

Mutation screening of *CD101* of 127 sporadic patients identified 10 rare nucleotide substitutions (Table S1). These substitutions included p.V678L and p.T944R, which were absent or extremely rare in the general populations, and were assessed as 'probably damaging' by *in silico* analysis (Table S1; Figure 2a, b). Case 3 carrying the p.V678L substitutions and case 4 carrying the p.T944R substitutions developed diabetes at 10.5 and 13.2 years-of-age, respectively (Table 1). At disease onset, these cases were positive for anti-GAD antibodies. Both cases 3 and 4 carried risk *HLA* alleles of the Japanese population. Blood samples from family members of cases 3 and 4 were unavailable for genetic testing.

Functional assessment of *CD101* substitutions

The p.V863L, p.V678L and p.T944R substitutions were absent in 185 healthy Japanese individuals. Furthermore, these substitutions have not been registered in the Human Genetic Variation Database. Likewise, the p.V678L and p.V863L substitutions were absent from 121,412 alleles in the ExAC Browser, and the p.T944R substitution has been identified in one of 121,370 alleles.

The p.V678L and p.V863L substitutions resided within the immunoglobulin-like domains of CD101, whereas the p.T944R substitution was located in a connecting region between an immunoglobulin-like domain and the transmembrane domain (Figure 2a). The signal peptide was not affected by these substitutions. The three substitutions involved nucleotides that were evolutionary conserved among humans, rhesus, dogs and elephants (Figure 2b).

Expression analysis of CD101 in unaffected individuals and substitution-positive patients

In keeping with previous reports[7–9,13,14], CD101 was highly expressed on monocytes and granulocytes of control individuals, whereas lymphocytes including T cells, B cells and natural killer cells of these individuals barely expressed it (red lines in Figure 1c). Interestingly, the expression of CD101 on the monocytes and granulocytes of cases 1, 2 and 4 was weaker than that of the control individuals. The reduction of CD101 expression was also observed in myeloid DCs of these cases (Figure 1b and blue lines in Figure 1c). Expression of CD101 was weak or absent on plasmacytoid DCs of both patients and control individuals. Collectively, the *CD101* mutations likely affected protein expression primarily on myeloid lineage cells.

DISCUSSION

Whole-exome sequencing of family A showed that a p.V863L substitution in *CD101*, the human homolog of an autoimmune

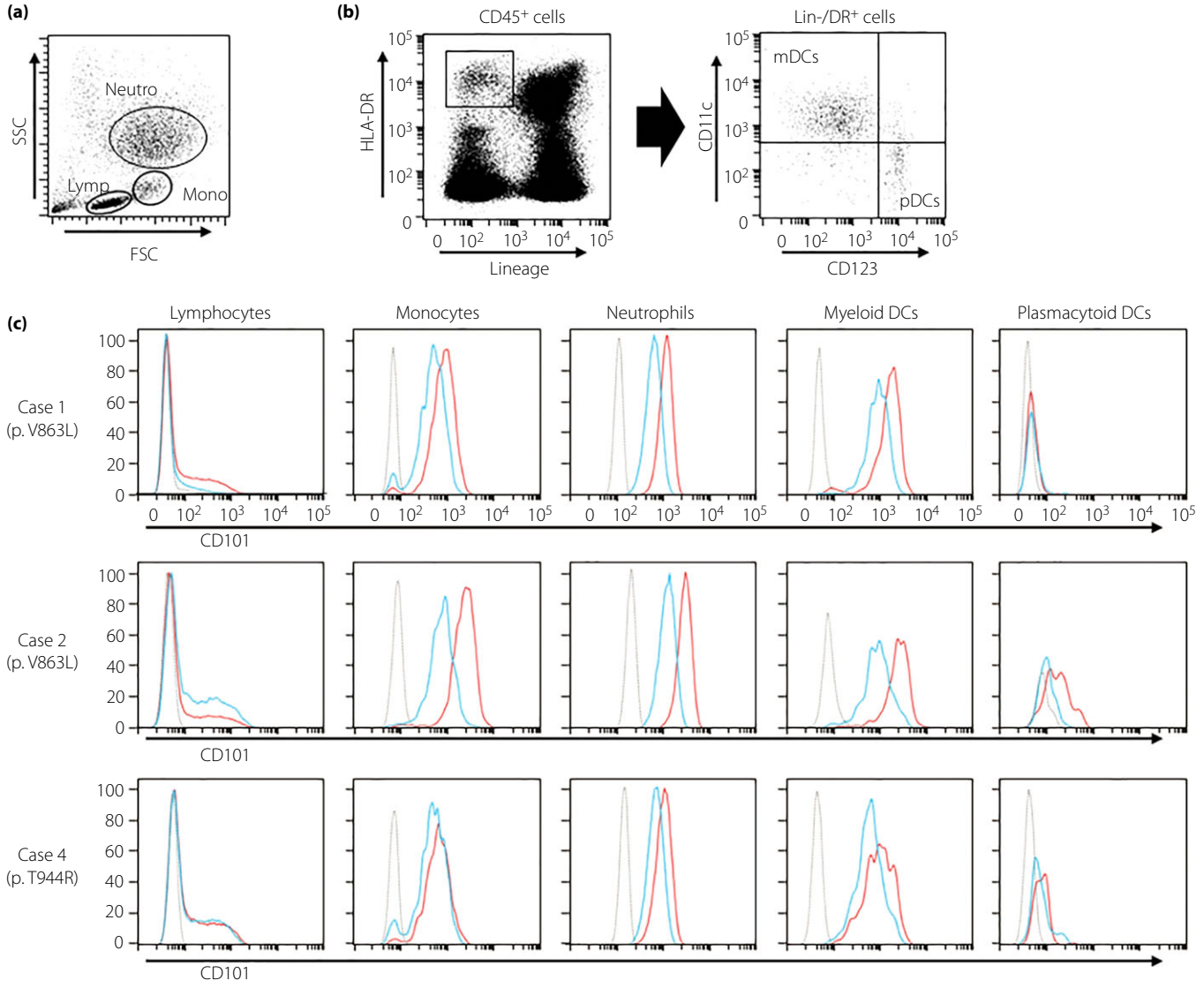

Figure 1 | CD101 expression on peripheral blood cells. (a) Major subpopulations of peripheral blood cells, such as lymphocytes (Lymp), monocytes (Mono) and neutrophils (Neutro), were differentiated based on forward scatter (FSC)/side scatter (SSC) of flow cytometry. (b) Cells expressing human CD45 were gated on human leukocyte antigen-DR positive (HLA-DR$^+$) and lineage$^-$, and divided into two subpopulations; that is, CD11c$^+$/CD123$^-$ myeloid dendritic cells (mDCs) and CD11c$^-$/CD123$^+$ plasmacytoid DCs (pDCs). (c) Results of cases 1, 2 and 4 are shown in blue lines. Representative results of control individuals and the unstained control population of cells are shown with red and gray lines, respectively. Samples of case 3 were not available for this analysis.

diabetes gene in NOD mice[10–12], co-segregated with type 1 diabetes. Notably, type 1 diabetes was seen in cases 1 and 2, but not in the father or the older siblings of case 1, although *HLA* risk alleles were shared by all of these individuals. Whereas 23 other nucleotide substitutions were also linked to the disease phenotype of this family, there were no data supporting an association between these substitutions and diabetes. In fact, many of the 23 substitutions were of unknown pathogenicity, or resided within genes that underlie human disorders other than diabetes. Although cases 1 and 2 carried a p.I228T substitution (rs143186590) in *GAD2* encoding a major autoantigen of type 1 diabetes, a relatively high frequency of *GAD2* p.I228T in the general population argued against the pathogenicity of this mutation. Johnson *et al.*[18] suggested that genetic variations in *GAD2* are unlikely to underlie type 1 diabetes. Actually, *GAD2* variations have been associated with obesity and anxiety disorders, rather than type 1 diabetes[19,20]. Mutations in known diabetes-causative genes, including *INS*, *PTPN22*, *IL2RA* and *CTLA4* were excluded in family A. Furthermore, although several submicroscopic deletions in the genome have been associated with type 1 diabetes[21], such defects were absent in case 1. Subsequently, we carried out *CD101* mutation screening of 127

Table 2 | Nucleotide substitutions identified by whole-exome sequencing of family A

Gene	Nucleotide change	Amino acid change	Allele frequency in the general population		*In silico* functional prediction[‡]	Human disease associated with the gene[§]
			HGVD	ExAC		
Rare polymorphism						
COL3A1[†]	IVS25 + 5	–	0.004	74/121,400	Unknown	Ehlers–Danlos syndrome
NEDD4L	IVS14 + 5	–	0.002	13/103,616	Unknown	Unknown
GK	IVS17 + 4	–	0.008	0	Unknown	Glycerol kinase deficiency
SMARCAD1	IVS1-2	–	0	1/119,520	Unknown	Adermatoglyphia
GAD2	T683C	I228T	0.005	262/121,380	Possibly damaging	Unknown
COL3A1[†]	G3133A	A1045T	0.003	71/121,296	Probably damaging	Ehlers–Danlos syndrome
OR52D1	G604A	G202R	0.004	878/121,376	Probably damaging	Unknown
OBSCN	T8774G	L2925R	0.002	6/119,982	Probably damaging	Unknown
LYST	T9898C	Y3299C	0.002	3/21,498	Probably damaging	Chediak–Higashi syndrome
VCPIP1	G2513C	P837A	0.001	0	Probably damaging	Unknown
MYF5	A431G	N144S	0.001	0	Possibly damaging	Unknown
ZNF469	C7381T	R2461W	0.001	0	Probably damaging	Brittle cornea syndrome
FOXS1	G878C	P292A	0.007	15/118,480	Possibly damaging	Unknown
C1QTNF8	C140T	D46N	0.001	1/109,732	Possibly damaging	Unknown
PDE11A	G481C	S160X	0.002	0	No data	Pigmented nodular adrenocortical disease
CREB3L1	IVS11 + 1	–	0	0	Unknown	Unknown
GOLGA8S	IVS16-5	–	0	0	Unknown	Unknown
TPRN	1650insCCG	550_551insA	0	0	Unknown	Hearing loss
CD101	G2587T	V863L	0	0	Possibly damaging	Unknown
PREX2	T2699C	I900T	0	0	Possibly damaging	Oncogene
LOH12CR1	G451C	E151Q	0	0	Probably damaging	Unknown
ZNF532	C449G	P150R	0	0	Probably damaging	Unknown
SDF2L1	C185A	S62Y	0	0	Probably damaging	Unknown
SHROOM2	C3890T	S1297F	0	0	Probably damaging	Ocular albinism

ExAC, the Exome Aggregation Consortium Browser; HGVD, Human Genetic Variation Database. [†]Two polymorphisms were identified in *COL3A1*. [‡]Based on the data of PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). [§]Based on the data of OMIM (http://www.ncbi.nlm.nih.gov/omim).

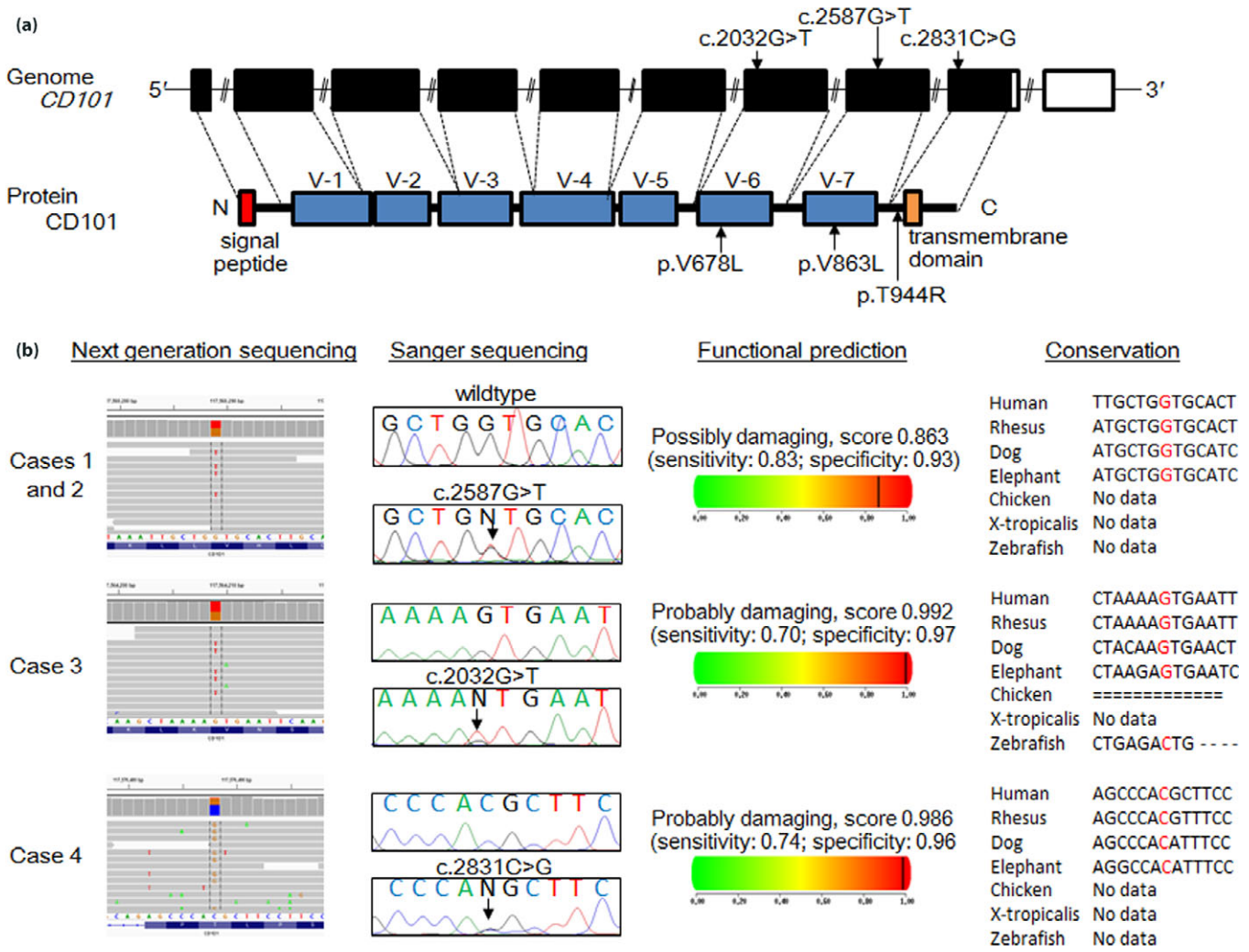

Figure 2 | Nucleotide substitutions of *CD101* identified in the present study. (a) The structure of wild-type *CD101* DNA and CD101 protein, and the position of three substitutions. The black and white boxes on genomic deoxyribonucleic acid denote the coding regions and the untranslated regions, respectively. The p.V678L and p.V863L substitutions resided within the immunoglobulin-like domains (blue boxes), while the p.T944R substitution was located in a connecting region between an immunoglobulin-like domain and the transmembrane domain (orange box). The signal peptide (red box) was not affected by these substitutions. (b) Three substitutions in *CD101*. These substitutions were identified by next-generation sequencing and confirmed by Sanger sequencing (left panel). The black arrows indicate mutated nucleotides. Representative results of an *in silico* analysis (PolyPhen-2) are shown (middle panel). Evolutionary conservation of each substitution is shown (right panel).

sporadic patients with type 1 diabetes and identified additional missense substitutions. The p.V863L, p.V678L and p.T944R substitutions in *CD101* were absent or extremely rare in the general population, and were scored as possibly or probably damaging. Furthermore, the three substitutions were well-conserved among most placental mammals. Collectively, the present findings, in conjunction with prior observations that CD101 plays a significant role in T cell regulation[6–16,22,23], imply an association between *CD101* mutations and type 1 diabetes. *CD101* mutations might increase genetic predisposition to diabetes only in individuals at risk of the disease, because cases 1–4 invariably carried one or more of the *HLA* risk alleles. While the mother of case 2 was free from the disease despite having the same *CD101* substitution as cases 1 and 2, this normal phenotype might reflect the presence of the protective *DQB1*03:01* allele. Considering the small number of participants in the present study, our findings need to be validated in future studies.

Fluorescence-activated cell sorting analysis detected CD101 expression on hematopoietic cells in both control individuals and mutation-positive patients. The percentage of CD101-positive cells among monocytes, neutrophils and myeloid DCs was somewhat lower in cases 1, 2 and 4 compared with that in five control individuals. The underlying mechanism of the reduced *CD101* expression in these cases remains unknown. Actually, the signal peptide and the transmembrane domain are not

affected by the p.V678L or p.T944R substitutions. Furthermore, it is unclear whether these minor differences in the expression levels are of clinical significance. We cannot exclude the possibility that *CD101* mutations identified in the present study are functionally neutral variants. Nevertheless, altered CD101 expression has been associated with various autoimmune disorders. Jovanovic *et al.*[16] showed that the fraction of CD101-positive cells among the CD8+ T cell population was reduced in patients with rheumatoid arthritis. Åkesson *et al.*[23] found that CD101 expression in regulatory T cells was moderately increased in children with celiac disease. In addition, reduced surface expression of murine CD101 was associated with the risk of infection-induced liver autoimmunity[22]. Altered expression of CD101 on DCs could influence the progression of pancreatic insulitis, because DCs are known to play a critical role in the recruitment of lymphocytes in insulitis of the NOD mouse[24]. Further studies, such as viral vector-mediated transduction of the mutant *CD101* to immune cells, will clarify the functional consequences of the p.V678L, p.V863L and p.T944R substitutions.

Previous GWAS did not suggest any association between *CD101* and diabetes. This can be explained by the rarity of *CD101* substitutions in the general population. It is known that disease-associated variants with an allele frequency of less than 0.5% in the general population are barely detectable by GWAS, unless the variants underlie monogenic Mendelian disorders[5]. As we identified *CD101* substitutions in just two of the 127 sporadic patients, such substitutions appear to be rare, even in patient cohorts. The present results show that next-generation sequencing of familial cases is useful for identifying rare risk variants that have been missed by GWAS.

In conclusion, we identified rare *CD101* mutations in familial and sporadic cases of type 1 diabetes. The present findings, in conjunction with the results of previous studies[6–16,22–24], raise the possibility that *CD101* is a susceptibility gene for type 1 diabetes.

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DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Morran MP, Vonberg A, Khadra A, *et al.* Immunogenetics of type 1 diabetes mellitus. *Mol Aspects Med* 2015; 42: 42–60.
2. Santin I, Eizirik DL. Candidate genes for type 1 diabetes modulate pancreatic islet inflammation and β-cell apoptosis. *Diabetes Obes Metab* 2013; 15(Suppl 3): 71–81.
3. Precechtelova J, Borsanyiova M, Sarmirova S, *et al.* Type I diabetes mellitus: genetic factors and presumptive enteroviral etiology or protection. *J Pathog* 2014; 2014: 738512.
4. Groop L, Pociot F. Genetics of diabetes–are we missing the genes or the disease? *Mol Cell Endocrinol* 2014; 382: 726–739.
5. Manolio TA, Collins FS, Cox NJ, *et al.* Finding the missing heritability of complex diseases. *Nature* 2009; 461: 747–753.
6. Ruegg CL, Rivas A, Madani ND, *et al.* V7, a novel leukocytesurface protein that participates in T cell activation. II. Molecular cloning and characterization of the V7 gene. *J Immunol* 1995; 154: 4434–4443.
7. Soares LR, Tsavaler L, Rivas A, *et al.* V7 (CD101) ligation inhibits TCR/CD3-induced IL-2 production by blocking Ca2+ flux and nuclear factor of activated T cell nuclear translocation. *J Immunol* 1998; 161: 209–217.
8. Bouloc A, Bagot M, Delaire S, *et al.* Triggering CD101 molecule on human cutaneous dendritic cells inhibits T cell proliferation via IL-10 production. *Eur J Immunol* 2000; 30: 3132–3139.
9. Fernandez I, Zeiser R, Karsunky H, *et al.* CD101 surface expression discriminates potency among murine FoxP3+ regulatory T cells. *J Immunol* 2007; 179: 2808–2814.
10. Penha-Gonçalves C, Moule C, Smink LJ, *et al.* Identification of a structurally distinct CD101 molecule encoded in the 950-kb Idd10 region of NOD mice. *Diabetes* 2003; 52: 1551–1556.
11. Yamaji K, Ikegami H, Fujisawa T, *et al.* Evidence for *Cd101* but not *Fcgr1* as candidate for type 1 diabetes locus, *Idd10*. *Biochem Biophys Res Commun* 2005; 331: 536–542.
12. Rainbow DB, Moule C, Fraser HI, *et al.* Evidence that Cd101 is an autoimmune diabetes gene in nonobese diabetic mice. *J Immunol* 2011; 187: 325–336.
13. Rivas A, Ruegg CL, Zeitung J, *et al.* V7, a novel leukocyte surface protein that participates in T cell activation. I. Tissue

distribution and functional studies. *J Immunol* 1995; 154: 4423–4433.
14. Soares LR, Rivas A, Ruegg C, *et al.* Differential response of CD4+ V7+ and CD4+ V7- T cells to T cell receptor-dependent signals: CD4+ V7+ T cells are co-stimulation independent and anti-V7 antibody blocks the induction of anergy by bacterial superantigen. *Eur J Immunol* 1997; 27: 1413–1421.
15. Maier LM, Smyth DJ, Vella A, *et al.* Construction and analysis of tag single nucleotide polymorphism maps for six human-mouse orthologous candidate genes in type 1 diabetes. *BMC Genet* 2005; 6: 9.
16. Jovanovic DV, Boumsell L, Bensussan A, *et al.* CD101 expression and function in normal and rheumatoid arthritis-affected human T cells and monocytes/macrophages. *J Rheumatol* 2011; 38: 419–428.
17. Sugihara S, Ogata T, Kawamura T, *et al.* HLA-class II and class I genotypes among Japanese children with type 1a diabetes and their families. *Pediatr Diabetes* 2012; 13: 33–44.
18. Johnson GC, Payne F, Nutland S, *et al.* A comprehensive, statistically powered analysis of GAD2 in type 1 diabetes. *Diabetes* 2002; 51: 2866–2870.
19. Choquette AC, Lemieux S, Tremblay A, *et al.* GAD2 gene sequence variations are associated with eating behaviors and weight gain in women from the Quebec family study. *Physiol Behav* 2009; 98: 505–510.
20. Unschuld PG, Ising M, Specht M, *et al.* Polymorphisms in the GAD2 gene-region are associated with susceptibility for unipolar depression and with a risk factor for anxiety disorders. *Am J Med Genet B Neuropsychiatr Genet* 2009; 150B: 1100–1109.
21. Grayson BL, Smith ME, Thomas JW, *et al.* Genome-wide analysis of copy number variation in type 1 diabetes. *PLoS One* 2010; 5: e15393.
22. Mohammed JP, Fusakio ME, Rainbow DB, *et al.* Identification of Cd101 as a susceptibility gene for *Novosphingobium aromaticivorans*-induced liver autoimmunity. *J Immunol* 2011; 187: 337–349.
23. Åkesson K, Tompa A, Rydén A, *et al.* Low expression of CD39(+)/CD45RA(+) on regulatory T cells (Treg) cells in type 1 diabetic children in contrast to high expression of CD101 (+)/CD129(+) on Treg cells in children with Coeliac disease. *Clin Exp Immunol* 2015; 180: 70–82.
24. Nikolic T, Geutskens SB, van Rooijen N, *et al.* Dendritic cells and macrophages are essential for the retention of lymphocytes in (peri)-insulitis of the nonobese diabetic mouse: a phagocyte depletion study. *Lab Invest* 2005; 85: 487–501.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1| Frequency of single nucleotide polymorphisms in *CD101* in our sporadic patients and the Japanese general population.