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Short communication

Association between anti-ZnT8 autoantibody specificities and *SLC30A8* Arg325Trp variant in Japanese patients with type 1 diabetes

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Abstract

Aims/hypothesis We analysed the association between humoral autoreactivity to zinc transporter-8 (ZnT8) and the *SLC30A8* rs13266634 polymorphism (Arg325Trp), which is located at the most distal loop in the ZnT8 protein.

Methods Autoantibodies to ZnT8 were determined by RIA in 270 patients with type 1 diabetes using ZnT8 carboxy-terminal constructs (amino acids 268–369) carrying 325Trp(CW) and 325Arg(CR) and a hybrid construct (CW-CR). Forty-four ZnT8 autoantibody-positive sera with genomic DNA were used to examine the association between reactivity to ZnT8 constructs and the rs13266634 genotype.

Results Seventy-five patients reacted to the CW-CR hybrid construct, whereas 37 and 36 patients reacted to the CW and CR constructs, respectively. All sera positive for either CW or CR autoantibodies were positive for CW-CR autoantibodies. Among 19 patients with a 325Arg(CC) genotype, 5% had CW-specific autoantibodies, 42% had CR-specific autoantibodies and 32% had dual reactivity. Conversely, 73% of 15 patients with the 325Trp(TT) genotype had CW-specific autoantibodies, no patients had CR-specific autoantibodies and 13% had dual reactivity. Nine of the ten patients (90%) with the CT genotype reacted with either CR or CW constructs. The titre of CR autoantibodies in patients carrying the C allele was significantly higher than that in TT homozygotes ($p < 0.0001$). In contrast, the titre of CW autoantibodies in patients carrying a T allele was significantly higher

than that in CC homozygotes ($p < 0.005$). No evidence of an association between rs13266634 and type 1 diabetes was observed.

Conclusions/interpretation These results indicate that variant residue at amino acid 325 is a key determinant of humoral autoreactivity to ZnT8 and the *SLC30A8* genotype is an important determinant of autoantibody specificity.

Keywords: Autoantibodies, epitope, genetics, Japanese population, *SLC30A8*, type 1 diabetes, zinc transporter-8

Abbreviations:

aa	amino acids
CR	zinc transporter-8 carboxy-terminal construct carrying 325Arg
CW	zinc transporter-8 carboxy-terminal construct carrying 325Trp
CW-CR	hybrid ZnT8 construct generated by fusion of CW and CR
SNP	single nucleotide polymorphism
ZNT8	zinc transporter-8
ZnT8A	zinc transporter-8 autoantibodies

Introduction

Zinc transporters belonging to the SLC30 protein family are multipass transmembrane proteins with a role in the transport of zinc out of the cytoplasm or into vesicles [1]. One of these, zinc transporter-8 (ZnT8), is specifically produced in the pancreatic beta cells and localised into insulin secretory granules and was recently identified as a major autoantigen in human type 1 diabetes [2, 3]. The gene coding for ZnT8 (solute carrier family 30 member 8; *SLC30A8*) is mapped to chromosome 8q24.11 [3]. The reference gene contains eight exons, spans 37 kb, and encodes a 369 amino acid protein. In genome-wide association studies Sladek and colleagues reported an association between a non-synonymous variant in *SLC30A8* (rs13266634; Arg325Trp) and susceptibility to type 2 diabetes [4]. The fact that major epitope(s) for ZnT8 autoantibodies (ZnT8A) lie within the cytoplasmic domain of the molecule (amino acids [aa] 268–369) [2] and the variant residue at aa325 is located at the most distal extension of the molecule into the cytoplasm [5] prompted us to analyse the association between humoral autoreactivity to ZnT8 and the *SLC30A8* polymorphism.

Methods

Initially, sera from 270 Japanese patients with type 1 diabetes (61% female), including 112 new-onset patients, were used to identify the ZnT8A-positive samples. The median age at onset and median duration of diabetes were 25.0 (range 1.0–77.0) years and 2.0 (range 0.0–42.0) years, respectively. Among these patients, genomic DNA samples were available for 171 patients and were genotyped for the rs13266634 single nucleotide polymorphism

(SNP). To examine the relationship between humoral autoreactivity to ZnT8 and the *SLC30A8* variant, ZnT8A-positive patients with available genomic DNA samples were used. All patients met the criteria of the revised American Diabetes Association for type 1 diabetes [6]. Furthermore, 114 genomic DNA samples from healthy control participants were also genotyped for the rs13266634 SNP to examine the contribution of this SNP to susceptibility to type 1 diabetes in the Japanese population. This study was approved by the appropriate ethical committees and informed consent was obtained from all participants.

The ZnT8 complementary DNA (cDNA) constructs used in this study were the cytoplasmic carboxy-terminal domains (aa268–369) of human ZnT8 carrying either 325Trp(CW) or 325Arg(CR) and a fusion of the CW and CR with a CLFCEDPCDPSTPPGSSGGGKDFSILLME hinge junction generated by PCR. These cDNA were cloned into a pCDNA3.1 directional TOPO vector (Invitrogen, Carlsbad, CA, USA). The hybrid ZnT8 construct was designated as CW-CR.

Autoreactivity to ZnT8 CW, CR and CW-CR constructs was determined by radiobinding assay as described previously [2]. Positive control and negative control sera were included in each assay, and the antibody titres were expressed as an index defined as follows: (cpm in the unknown sample–cpm in the negative control)/(cpm in the positive control–cpm in the negative control). As a positive control for all constructs, a New Zealand white rabbit antiserum raised against an affinity-purified glutathione *S*-transferase fusion protein containing the cytoplasmic domain (aa268–369) of human ZnT8 was used. Classification of a sample as positive for ZnT8A was based on the 99th percentile of sera from 139 healthy control participants with no family history of diabetes. This corresponded to an index of 0.018 for CW autoantibodies, 0.016 for CR autoantibodies and 0.007 for CW-CR autoantibodies, respectively. The inter- and intra-assay CV were 5.9% and 6.8% for CW autoantibodies, 10.4% and 5.7% for CR autoantibodies and 9.6% and 4.6% for CW-CR autoantibodies, respectively.

The *SLC30A8* rs13266634 C > T polymorphism was genotyped by PCR-RFLP. The PCR products were digested with a PvuII, resolved on a 3% agarose gel and stained with ethidium bromide. No deviations from Hardy–Weinberg equilibrium were observed in control participants ($p=0.36$). Statistical analyses were performed with the StatView 5.0 software (SAS Institute, Cary, NC, USA). The correlation between autoantibody titres was analysed using Spearman's rank-correlation test. Group comparisons of ZnT8A prevalence and titre were analysed by χ^2 test and Mann–Whitney *U* test, respectively. A value of $p<0.05$ was considered statistically significant.

Results

Of 270 patients with type 1 diabetes, 75 (28%) were ranked as positive for ZnT8 CW-CR autoantibodies, whereas the responses to autoantibodies to CW and CR constructs were positive in 37 (14%) and 36 (13%) patients, respectively. All sera positive for either CW or

CR autoantibodies were positive for CW-CR autoantibodies. However, 20 (7%) patients were CW-CR autoantibody-positive but negative for autoantibodies to CW and CR. The prevalence of CW-CR, CW and CR autoantibodies in 112 new-onset patients was 36%, 16% and 20%, respectively. CW-CR autoantibodies were more prevalent in younger (≤ 20 years) new-onset patients compared with patients with age at onset of >20 years (47% vs 28%, $p < 0.05$). Among 171 patients with genomic DNA, 63 patients (37%) were 325Arg (CC) homozygotes in *SLC30A8*, 36 (21%) were homozygotes for the 325Trp (TT) and 72 (42%) were CT heterozygotes. The allele frequency of rs13266634 in patients was not significantly different from that in the 114 healthy control participants (C allele: 57% and T allele: 43%, OR: 1.02, 95%CI: 0.73–1.43).

Forty-four patients were positive for CW-CR autoantibodies among the 171 patients with genomic DNA samples and they were used to examine the association between humoral autoreactivity to ZnT8 and the *SLC30A8* variant. Among 19 patients with the CC genotype eight patients (42%) reacted with the CR construct alone, six patients (32%) reacted with both CR and CW constructs, and only one patient (5%) reacted with the CW construct alone. Conversely, 11 (73%) of 15 patients with the TT genotype reacted with the CW construct alone and no one reacted with the CR construct. Nine of the ten patients (90%) with the CT genotype reacted with either the CR or the CW construct. The prevalence of CR autoantibodies in patients carrying a C allele (CC+CT) was therefore significantly higher than that in TT homozygotes ($p < 0.0001$). In contrast, autoantibodies to the CW construct were more prevalent in patients with a T allele (TT+CT) than in those with the CC genotype ($p < 0.01$) (Table 1). As shown in Fig. 1a, there was no overall correlation between titres of CW autoantibodies and CR autoantibodies ($R = 0.069$, $p = 0.66$) and the autoreactivity to ZnT8 variant forms was closely associated with the *SLC30A8* genotype. The titre of CR autoantibodies in patients with a C allele was significantly higher than that in TT homozygotes (0.139 ± 0.158 vs 0.002 ± 0.014 , $P < 0.0001$) (Fig. 1b). In contrast, the titre of CW autoantibodies was significantly higher in patients with a T allele than in CC homozygotes (0.093 ± 0.103 vs 0.029 ± 0.053 , $p = 0.0024$) (Fig. 1c).

Discussion

This study has demonstrated that (1) the *SLC30A8* variant residue at aa325 is critically involved in the binding of ZnT8A; (2) the *SLC30A8* genotype is an important determinant of autoantibody specificity; (3) the hybrid CW-CR construct is a superior probe for the detection of ZnT8A; and (4) rs13266634 may not contribute to the genetic susceptibility to type 1 diabetes in the Japanese population. The first two points were recently proven in American patients [7]. However, the prevalence of CW-specific autoantibodies in patients with either CW or CR autoantibodies was higher in this study (35% vs 13%, $p < 0.0005$) because of the difference in the frequency of TT homozygotes between Japanese and American. Currently, it is not known whether Arg325Trp SNP affects the functional properties of ZnT8. However, the

amino acid chain that includes 325Arg has a protein kinase A and protein kinase C recognition motif (R-X-S/T) and rs13266634 SNP disrupts this motif, indicating the possible alteration of zinc transporter function. As differences in the epitope specificity of autoantibodies to GAD65 and insulinoma-associated antigen-2 are linked to the progression or the clinical heterogeneity of type 1 diabetes in Japanese patients [8, 9], it is worth investigating the clinical role of ZnT8 CW and CR autoantibodies in the Japanese population.

Although the molecular mechanisms for the production of anti-islet autoantibodies in type 1 diabetes are largely unknown, the interactions of autoantibody with protein antigen usually depend on the conformational structure of the epitopes [10]. Tryptophan (W) encoded by TGG is a hydrophobic amino acid that tends to cluster on the inside of the protein to avoid contact with the aqueous environment and so it is unlikely to form the same bonding arrangement as the hydrophilic amino acid, arginine (R) encoded by CGG. This may explain the observed differences in binding where the majority of ZnT8A in patients carrying the TT genotype did not recognise the CR construct. However, the findings that 37% of CC homozygous patients reacted with the CW construct and 13% of TT homozygous patients reacted with the CR construct indicate that the amino acids neighbouring aa325 are also involved in the ZnT8A binding.

The strength of this study is its use of a hybrid ZnT8 molecule (CW-CR) to identify ZnT8A-positive individuals; this allowed a 15–20% increase of sensitivity with the same specificity in our patients. Limitations are that about 60% of samples were obtained from long-standing cases and a minimum OR to obtain sufficient power (>80%) under the assumption of $\alpha=0.05$ and observed allele frequency among controls was 1.9 in our case-control study.

In conclusion, the present study confirmed that a variant residue at aa325 is a key determinant of humoral autoreactivity to ZnT8 and that the *SLC30A8* genotype is an important determinant of autoantibody specificity. Furthermore, the utility of the hybrid ZnT8 construct should be considered for ZnT8A screening in the relatives of patients with type 1 diabetes and in the general population.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Table 1 The prevalence of autoantibodies to the ZnT8 CR or CW construct in ZnT8 CW-CR autoantibody-positive patients with type 1 diabetes classified by *SLC30A8* genotype

<i>SLC30A8</i> genotype	<i>n</i>	Prevalence of autoantibodies to:	
		CR	CW
CC	19	14 (73.7)	7 (36.8) ^a
CT	10	9 (90.0)	6 (60.0)
TT	15	2 (13.3) ^b	13 (86.7)

Data are *n* (%)

^a $p < 0.01$ vs CT+TT genotype; ^b $p < 0.0001$ vs CC+CT genotype

Fig. 1 Correlation between the reactivity to ZnT8 CR and CW construct (**a**) and the comparison between the titres of ZnT8 autoantibodies and *SLC30A8* genotype (**b**, **c**) in Japanese patients with type 1 diabetes. (**a**) White circles, CC genotype; black circles, CT genotype; triangles, TT genotype. CC+CT vs TT: (**b**) $p < 0.0001$, (**c**) $p < 0.0024$

Fig. 1

