

Differences in the Humoral Autoreactivity to Zinc Transporter 8 Between Childhood- and Adult-Onset Type 1 Diabetes in Japanese Patients

Eiji Kawasaki ^a, Kan Nakamura ^a, Genpei Kuriya ^b, Tsuyoshi Satoh ^b, Masakazu Kobayashi ^b, Hironaga Kuwahara ^b, Norio Abiru ^b, Hironori Yamasaki ^c, Nobuo Matsuura ^d, Junnosuke Miura ^e, Yasuko Uchigata ^e, Katsumi Eguchi ^b

^a Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, Nagasaki, Japan, ^b Department of Endocrinology and Metabolism, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, ^c Center for Health and Communicating Medicine, Nagasaki University, Nagasaki, Japan, ^d Department of Early Childhood Education, Seitoku University, Matsudo, Japan, ^e Diabetes Center, Tokyo Women's Medical University School of Medicine, Tokyo, Japan

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Address for correspondence and reprint requests:

Eiji Kawasaki, M.D., Ph.D.

Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital

1-7-1 Sakamoto, Nagasaki 852-8501, Japan

TEL: 81-95-819-7550 FAX: 81-95-819-7270

E-mail: eijikawa@nagasaki-u.ac.jp

Abstract

The aim of this study was to evaluate the humoral autoreactivity to zinc transporter 8 (ZnT8) depending on the clinical phenotype of type 1 diabetes (T1D). ZnT8 autoantibodies (ZnT8A) were determined by radioimmunoassay using carboxy-terminal ZnT8 constructs in 57 childhood-onset (CO), 97 adult-onset (AO), and 85 fulminant T1D. The ZnT8A frequency was higher in CO patients and decreased with increasing age of onset from 70% to 24% ($P_{trend} < 0.005$). None of patients with fulminant T1D were positive for ZnT8A. There were at least two distinct ZnT8A epitope patterns associated with the aa325-restriction, CO patients have aa325-nonrestricted response more frequently compared to the AO group ($P < 0.05$). The level of ZnT8A was inversely associated with the copy number of HLA-*DR4* allele ($P < 0.05$). These results suggest differences in the humoral autoreactivity to ZnT8 depending on the clinical phenotype, which should provide strategy for autoantibody measurement in subjects to allow early diagnosis of autoimmune T1D.

Key words: Autoantibodies, Epitope, Fulminant, HLA, Type 1 diabetes, Zinc transporter-8

1. Introduction

Type 1 diabetes is an autoimmune disease characterized by T cell-mediated destruction of pancreatic β cells and the presence of circulating autoantibodies directed against several β cell autoantigens [1]. Although type 1 diabetes is frequently considered to be a childhood disease, it may develop at any age, and a greater proportion of type 1 diabetic cases are diagnosed later in life [2]. Moreover, there is increasing evidence that type 1 diabetes, especially in adult-onset patients, includes clinically and immunologically heterogeneous type. Those include slow-onset and fulminant type 1 diabetes [3]. Although the different clinical phenotypes may depend on the extent of β cell destruction, the underlying immune mechanisms are largely unknown.

To date, the expression of anti-islet autoantibodies has been the best phenotypic marker of autoimmune type 1 (type 1A) diabetes [1]. Recently, the cation efflux transporter zinc transporter 8 (ZnT8) has been identified as a novel target autoantigen in patients with type 1 diabetes [4]. Zinc transporters are multipass transmembrane proteins that function in the transport of zinc out of the cytoplasm or into vesicles [5]. ZnT8 is specifically expressed in the pancreatic β -cells and plays a major role in insulin maturation [4, 6]. Previous studies have reported that autoantibodies to ZnT8 (ZnT8A) were identified in more than 60% of young patients with type 1 diabetes and the combined measurement of autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), and protein tyrosine phosphatase IA-2 (IA-2A), and ZnT8A raised autoimmunity detection rates to 98% at disease onset in Caucasoid populations [4]. However, the relevance of ZnT8A in patients with adult-onset type 1 diabetes, especially in cases of slow-onset and fulminant type 1 diabetes, has not been clarified. The intent of this study was to evaluate the association of humoral autoreactivity to ZnT8 with clinical heterogeneity in Japanese patients with type 1 diabetes and establish its potential use as an additional marker of autoimmunity and phenotype characterization. We also examined the influence of HLA-DR on reactivities to ZnT8 protein.

2. Materials and Methods

2.1 Subjects

One hundred and sixty-six new-onset patients with type 1 diabetes consecutively recruited at our hospital between 1982 and 2008 with disease duration < 6 months were studied. They consisted of 57 childhood-onset patients (CO, age <15 years) (59.7% female, age 9.7±3.6, median 10.0, range 2.0-14.0 years, median duration 0.40, range 0-6.0 months) and 97 adult-onset patients (AO, age ≥18 years) (63.9% female, age 35.1±16.2, median 28.0, range 18.0-77.0 years, median duration 0.45, range 0-6.0 months) with type 1 diabetes. The remaining 12 patients with type 1 diabetes diagnosed between 15 - 17 years of age (58.3% female) were unclassified.

AO subjects were further divided into three groups according to the mode of diabetes onset (acute-onset, slow-onset, and fulminant). In patients with acute-onset type 1 diabetes, the duration of hyperglycemic symptoms before the start of insulin therapy was less than 3 months. In patients with slow-onset type 1 diabetes, insulin treatment was initiated > 1 year after the diagnosis of diabetes by the positive urine glucose test or the development of hyperglycemic symptoms [7]. Diagnostic criteria for fulminant type 1 diabetes were 1) ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms, 2) plasma glucose level ≥ 16 mM and HbA1c < 8.5% at the first visit, and 3) urinary C-peptide level < 10 µg/day, fasting serum C-peptide level < 0.3 ng/ml or serum C-peptide < 0.5 ng/ml after glucagon or a meal load [3]. Of 97 AO patients, 54 (55.7%) were acute-onset, 28 (28.9%) were slow-onset, and 15 (15.5%) had fulminant type 1 diabetes. To increase the number of patients in this study, we also examined the data for a second set of patients with fulminant type 1 diabetes (n=70), which was provided by the Fulminant Type 1 Diabetes Committee of the Japan Diabetes Society [8]. Therefore, a total of 85 patients with fulminant type 1 diabetes (36.5% female, age 43.3±16.1 years) were used for autoantibody analysis. All CO subjects were considered to be acute-onset forms based on the above mentioned criteria. All patients with diabetes analyzed in the present study were diagnosed according to the American Diabetes Association criteria for the classification of diabetes [9]. All subjects were informed the purpose of the study, and their consent for study participation was obtained. Protocols were approved by the ethics committee of Nagasaki

University and the Japan Diabetes Society. Sera were stored at -20°C until use.

2.2 ZnT8 autoantibody assay

Figure 1 illustrates the secondary structure of full-length human ZnT8 and the constructs used in this study. ZnT8A were determined by radioligand binding assay using a dimeric cDNA construct of the carboxy-terminal domains (aa268–369) carrying 325Trp and 325Arg (CW-CR), which showed higher sensitivity with the same specificity compared with individual monomeric constructs in our previous study [10]. The cut-off value for ZnT8A-CW-CR was an index of 0.007, which was based on the 99th percentile of sera from 139 healthy control subjects. The inter-assay coefficient of variation (CV) and intra-assay CV values were 9.6% and 4.6%, respectively. In this study, ZnT8A were considered as “positive” if sera were ranked as positive for ZnT8A-CW-CR. In the Diabetes Autoantibody Standardization Program 2009 (DASP 2009), this assay had 40% sensitivity and 100% specificity.

Autoantibody reactivities to ZnT8 aa325 variants were also determined using the carboxy-terminal domains (aa268–369) of cDNA encoding the aa325 codon variants CCG (Arg, CR), TCG (Trp, CW), and CAG (Gln, CQ) to analyze an epitope specificity (Figure 1). The cut-off index was 0.018 for ZnT8A-CW, 0.016 for ZnT8A-CR, and 0.006 for ZnT8A-CQ based on the 99th percentile of sera from 139 healthy control subjects. The inter-assay CV and intra-assay CV values were 5.9% and 6.8% (ZnT8A-CW), 10.4% and 5.7% (ZnT8A-CR) and 6.3% and 7.5% (ZnT8A-CQ), respectively. These autoantibodies were determined in 52 CO and 161 AO patients, including 85 patients with fulminant type 1 diabetes because of serum availability.

2.3 Detection of other anti-islet autoantibodies

We used a radioligand binding assay to detect GADA and IA-2A using the cDNA for full-length human islet GAD65 and the complete cytoplasmic region of IA-2 (aa601-979), respectively, as previously described [11]. “Positive” was based on the 99th percentile of sera from 204 healthy

control subjects without family history of diabetes. The cut-off indices were 0.028 for GADA and 0.018 for IA-2A. The inter-assay CV and intra-assay CV were 3.3% and 5.3% for GADA and 1.9% and 2.1% for IA-2A, respectively. In the DASP 2005, the GADA and IA-2A assays had sensitivities of 74% and 68% and specificities of 98% and 96%, respectively.

The insulin autoantibody (IAA) assay was carried out by a micro-IAA assay format as previously described [12]. Based on the difference in cpm between wells without and with cold insulin, an index was determined, with a positivity criterion of 0.010 based on the 99th percentile of sera from healthy control subjects. The inter-assay CV and intra-assay CV were 6.8% and 1.4%, respectively. In the DASP 2005, this assay had a sensitivity of 58% and a specificity of 98%. IAA were determined in sera obtained within 2 weeks after initiation of insulin therapy.

2.4 HLA typing

HLA-DR typing was performed by a standard microcytotoxicity test or PCR-amplified DNA and nonradioactive sequence-specific oligonucleotide probes [11].

2.5 Statistical analysis

Results were expressed as the mean \pm SD unless otherwise indicated. Autoantibody prevalence was compared using the Chi-square test, Fisher's exact test, and Cochran-Armitage's test where appropriate. Differences in nonparametric data were tested by the Mann-Whitney U test or the Kruskal-Wallis test. Comparisons of the ZnT8A levels were made by ANOVA with HLA-DR allele alone and ANOVA with the HLA-DR allele and phenotypic group (CO and AO). The correlation between autoantibody levels was analyzed using the Spearman rank correlation test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1 Humoral autoreactivity to a hybrid ZnT8 construct

ZnT8A (ZnT8A-CW-CR) were detected in 33 of 57 (58%) CO patients with type 1 diabetes, which was significantly higher than that in the AO group (33 of 97, 34%, $P=0.004$). However, the level of ZnT8A in patients positive for ZnT8A was similar between the two groups (CO group, median index = 0.088, range 0.010-0.606; AO group, median index = 0.067, range 0.009-0.669, $P=0.42$). The prevalence of ZnT8A with respect to onset age was also evaluated after the combination of the two groups. CO and AO patients were combined and divided according to the age of onset into four groups (ages <10, 10-14, 18-30, and >30 years); the prevalence of ZnT8A was then evaluated by Cochran-Armitage's trend test. The prevalence of ZnT8A was inversely related to the onset age (70%, 50%, 41%, and 24%, respectively, $P=0.004$). In the AO group, acute-onset patients had a higher frequency of ZnT8A than did slow-onset patients (50% vs. 21%, $P=0.012$). However, none of 85 patients with fulminant type 1 diabetes were positive for ZnT8A. There was no statistical difference between patients' gender and the prevalence or level of ZnT8A (data not shown).

Table 1 shows the clinical and immunogenetic characteristics between ZnT8A-positive and -negative patients with non-fulminant type 1 diabetes. In the CO group, GADA ($P<0.005$), IA-2A ($P<0.0001$) and IAA ($P<0.05$) were positive at higher proportions in the ZnT8A-positive than ZnT8A-negative patients. However, only the prevalence of IA-2A in ZnT8A-positive patients was significantly higher than that in ZnT8A-negative patients in the AO group ($P<0.0001$). There was no correlation between the ZnT8A positivity and the prevalence of two major susceptible class II HLA alleles in the Japanese, *DR4* and *DR9*, in either group. HLA-*DR9* was associated with the presence of IA-2A in our patients (Supplementary Table 1).

3.2 Humoral autoreactivity to ZnT8 aa325 variants

We and others reported that the amino acid encoded by the polymorphic codon 325 is a key determinant and there are three classes of conformational epitopes: one for which 325Arg is an essential determinant, a second that is 325Trp-restricted, and a third that is not affected by aa325 [10, 13]. Therefore, to assess the possible difference on the ZnT8A epitope recognition between CO and

AO patients, we also tested sera for the reactivity to the carboxy-terminal ZnT8 constructs bearing 325Trp (CW), 325Arg (CR), or 325Gln (CQ).

In the CO group, 29 of 52 (56%) patients reacted to at least one construct, with the highest response recorded in reaction to the CW construct (44%) followed by the CR (38%) and CQ (31%) constructs (Figure 2). Analysis of the overlap in responses shows that 6% and 12% of patients reacted to the CR or CW construct alone, respectively, and rarely to the CQ construct alone (2%); 21% of patients reacted to all three constructs. In the AO group, 24 of 75 (32%) patients reacted to at least one construct, which was significantly lower than the occurrence in the CO group ($P=0.008$). This difference fundamentally results from in the patients who reacted to all three constructs. The prevalence of patients with 325Trp- or 325Arg -restricted response was similar between the two groups. However, the proportion of patients who had ZnT8A not affected by aa325 (aa325-nonrestricted ZnT8A) was frequent in the CO group among patients who reacted to at least one construct (38% vs. 13%, $P<0.05$). None of 85 patients with fulminant type 1 diabetes reacted with any of the ZnT8 variant constructs.

3.3 ZnT8A titer and class II HLA

It has been reported that HLA characteristics were associated with the frequencies and levels of anti-islet autoantibodies [14, 15]. We therefore examined the association between ZnT8A and HLA-DR. Although there were no associations between the positivity of ZnT8A and the frequency of the HLA-*DR4* or *DR9* allele in our subjects, the level of ZnT8A was associated with the copy number of the HLA-*DR4* allele. Among the ZnT8A-positive patients, the mean index of ZnT8A in HLA-*DR4* homozygotes (0.041 ± 0.040 , mean \pm SD) was significantly lower than those in patients carrying no (0.163 ± 0.165) or one copy (0.132 ± 0.097) of *DR4* allele ($P=0.028$ by the Kruskal-Wallis test) (Figure 3A). HLA-*DR9* had no influence on the level of ZnT8A (Figure 3B). A mixed model ANOVA using the HLA-*DR4* allele (4/4, 4/X, X/X) and phenotypic group (CO and AO) as factorial fixed effects revealed no differences in ZnT8A levels between phenotypic groups ($P=0.82$) or

phenotype/allele interactions ($P=0.58$).

3.4 Overlapping prevalence with other anti-islet autoantibodies

Figure 4 illustrates an overlapping prevalence of ZnT8A, GADA, IA-2A, and IAA in patients whose sera were obtained within 2 weeks after the initiation of insulin treatment. The prevalence of GADA, IA-2A, IAA, and ZnT8A was 83%, 78%, 49%, and 61% in the CO group, and 80%, 41%, 57%, and 39% in the AO group, respectively (Figure 4A and 4B), while that for patients with fulminant type 1 diabetes was 9%, 4%, 6%, and 0%, respectively (Figure 4C). In the CO group, the combined analysis of GADA and IA-2A revealed type 1A diabetes in 90% of patients (37 of 41) (Figure 4A). Inclusion of the IAA and/or ZnT8A did not affect the number who tested positive for at least one of these autoantibodies.

In the AO group, the prevalence of patients positive for GADA and/or IA-2A was 89% (54 of 61), and inclusion of the IAA and/or ZnT8A reduced the number of autoantibody-negative subjects from 12% to 5%. Two individuals (40%) from a group of 5 patients who were negative for GADA, IA-2A, and IAA were ZnT8A positive. Of note, the prevalence of patients positive for all four autoantibodies was greater in the CO group (37%) than that in the AO group (11%, $P=0.003$). On the other hand, the prevalence of one or two autoantibody-positive patients was significantly higher in the AO group (52%) as compared with the CO group (24%, $P=0.005$). In fulminant type 1 diabetes, most patients were single-autoantibody positive and only one patient showed an overlap of positivity for GADA and IAA (Figure 4C).

Analyzed in terms of the levels of autoantibodies, ZnT8A correlated with IA-2A in the CO patients ($r=0.434$, $P<0.005$) but not in the AO patients ($r=0.056$, $P=0.67$). There was no correlation between levels of ZnT8A with those of GADA or IAA in either group (data not shown).

4. Discussion

We demonstrated 1) different humoral autoreactivity to ZnT8 between adult-onset and childhood-onset type 1 diabetes, 2) an inverse association between the copy number of HLA-*DR4* and the levels of ZnT8A, and 3) no humoral autoreactivity to the ZnT8 molecule in fulminant type 1 diabetes.

The prevalence of ZnT8A was significantly higher in CO patients than that in AO patients. Furthermore, the prevalence of ZnT8A was inversely related to the onset age with the highest prevalence of 70% in patients aged < 10 years. Thus, ZnT8A exhibit heterogeneity with regard to the age of diabetes onset and are good markers of childhood-onset type 1 diabetes. Notably, the higher frequency of ZnT8A in CO patients fundamentally resulted from an increased number of patients with aa325-nonrestricted ZnT8A (Figure 2). We and others recently reported that the amino acid encoded by the polymorphic codon 325 (Arg, Trp, Gln) is a key determinant of humoral autoreactivity to this protein [10, 13]. Furthermore, Wenzlau and coworkers reported that the C-terminal domain of ZnT8 contains at least three discrete conformational epitopes: 325Trp-restricted, 325Arg-restricted, and aa325-nonrestricted epitopes [10, 13]. The considerably higher proportion of subjects with aa325-nonrestricted ZnT8A among CO patients could be because autoreactivity to ZnT8 reflects a more severe β cell destruction leading to manifestation of the disease early in life, or because the humoral autoreactivity to other cytoplasmic epitopes of ZnT8 is relatively rare in patients who develop type 1 diabetes at an older age.

It has been reported that the HLA characteristics were associated with the frequencies and levels of anti-islet autoantibodies in Caucasoid patients [14, 15]. In the present study, we demonstrated that the copy number of HLA-*DR4* is associated with the ZnT8A production (Figure 3). Furthermore, this association was independent of the clinical phenotype. This novel observation of the HLA-non*DR4* bias of ZnT8A production is one of the interesting findings in this study and is contrary to the previous observations that the level of IA-2A was associated with the HLA-*DR4* allele [14, 16]. This may indicate that ZnT8 peptides are poorly presented by *DR4* class II molecules to the T-cell receptors. Analysis of peptide binding to *DR4*, peptide elution studies from the *DR4*

homozygote, or visualization of *DR4*-peptide binding interaction will be important to test the possibility of reduced or profound binding. Furthermore, this observation needs to be validated in the Caucasoid population, because type 1 diabetes-susceptible HLA-*DR4* in Japanese patients (*DRB1**0405) is different from that in Caucasoid patients (*DRB1**0401).

Measurement of a combination of autoantibody markers has been suggested as a useful tool for determining type 1A diabetes. However, the clinical utility of ZnT8A might be limited over testing GADA, IA-2A, and IAA in CO patients. In the present cohort, 90% of the CO patients were positive for GADA and/or IA-2A, but inclusion of IAA and/or ZnT8A did not increase the sensitivity for identifying type 1A diabetes (Figure 4). Furthermore, GADA, IA-2A, and IAA were positive in a greater proportion of the ZnT8A-positive patients in the CO group (Table 1). In the AO group, inclusion of the ZnT8A reduced the number of autoantibody-negative subjects from 8% to 5% and 2 of 5 (40%) patients who were negative for GADA, IA-2A, and IAA were ZnT8A positive. Furthermore, the prevalence of patients positive for one or two of these four autoantibodies was greater in the AO group as compared with the CO group ($P<0.005$). Such a broader autoantibody response in AO patients implicates that different pathogenic mechanisms may be involved between adult-onset and childhood-onset type 1 diabetes.

Finally, we also demonstrated that none of the sera from patients with fulminant type 1 diabetes reacted to ZnT8A, although ZnT8A are apparently markers for acute-onset patients with type 1 diabetes. Fulminant type 1 diabetes is a subtype of type 1 diabetes characterized by extremely rapid onset with nearly normal HbA1c level, frequent flu-like symptoms just before the disease onset, and virtually no C-peptide secretion at disease onset [8]. Although the underlying pathogenesis of fulminant type 1 diabetes has not been fully clarified, there are increasing evidences to support the involvement of autoimmune mechanisms [17-19]. However, in contrast to type 1A diabetes, both α and β cells are greatly reduced in number and there is a lymphocytic infiltration in the exocrine pancreas tissue in patients with fulminant type 1 diabetes [20, 21]. Furthermore, it has been recently reported that ZnT8A titer declined similarly to C-peptide response after the onset of type 1 diabetes

[22], although anti-islet autoantibodies are considered to be an epiphenomenon resulting from the autoimmune destruction of the β cells. Taken together, our findings suggest that ZnT8A might be more specific markers of autoimmune-mediated β cell destruction and that non-autoimmune mechanisms such as antiviral immunity following viral infection of β cells are the major causes of fulminant type 1 diabetes.

In conclusion, our present data demonstrated the differences in the humoral autoreactivity to ZnT8 between adult- and childhood-onset type 1 diabetes, and the non*DR4* bias of the ZnT8A production. Furthermore, clinical phenotypes of Japanese type 1 diabetes are associated with the appearance of different autoantibodies, which should provide a strategy for autoantibody measurement in subjects to promote the early diagnosis of type 1A diabetes.

5. Acknowledgments

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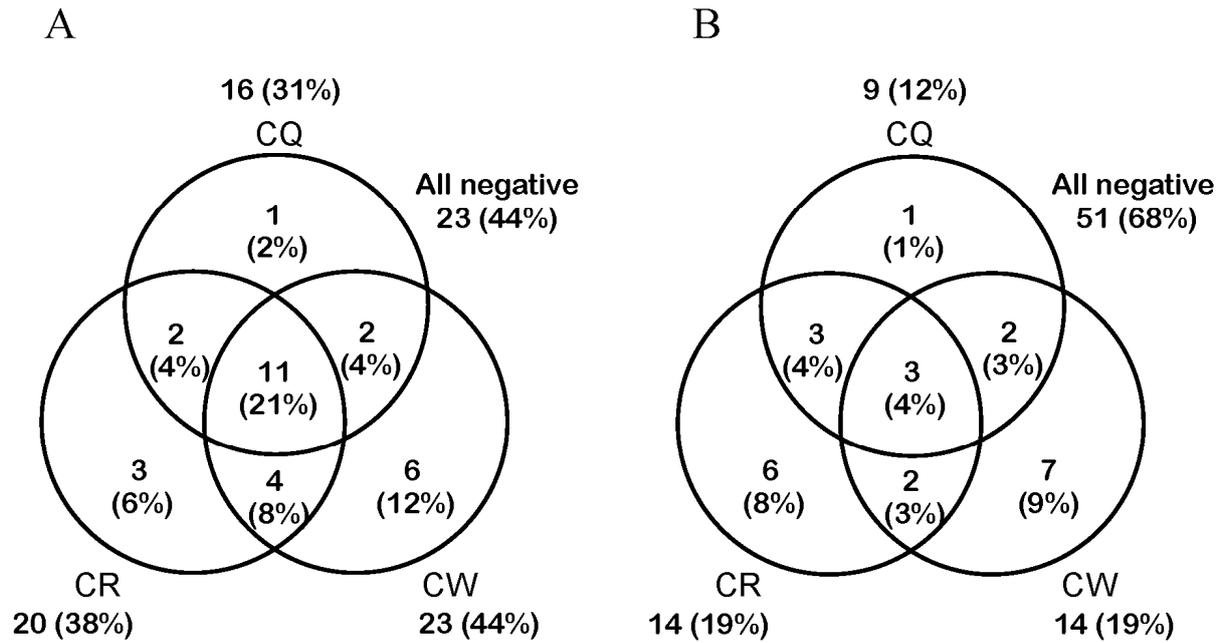


Figure 2 Humoral autoreactivity to 325Trp (CW), 325Arg (CR), and 325Gln (CQ) constructs in patients with childhood-onset (A) and adult-onset type 1 diabetes (B).

Venn diagram illustrates the overlap of autoantibody detection with each of the polymorphic construct in 52 childhood-onset and 75 adult-onset patients with type 1 diabetes.

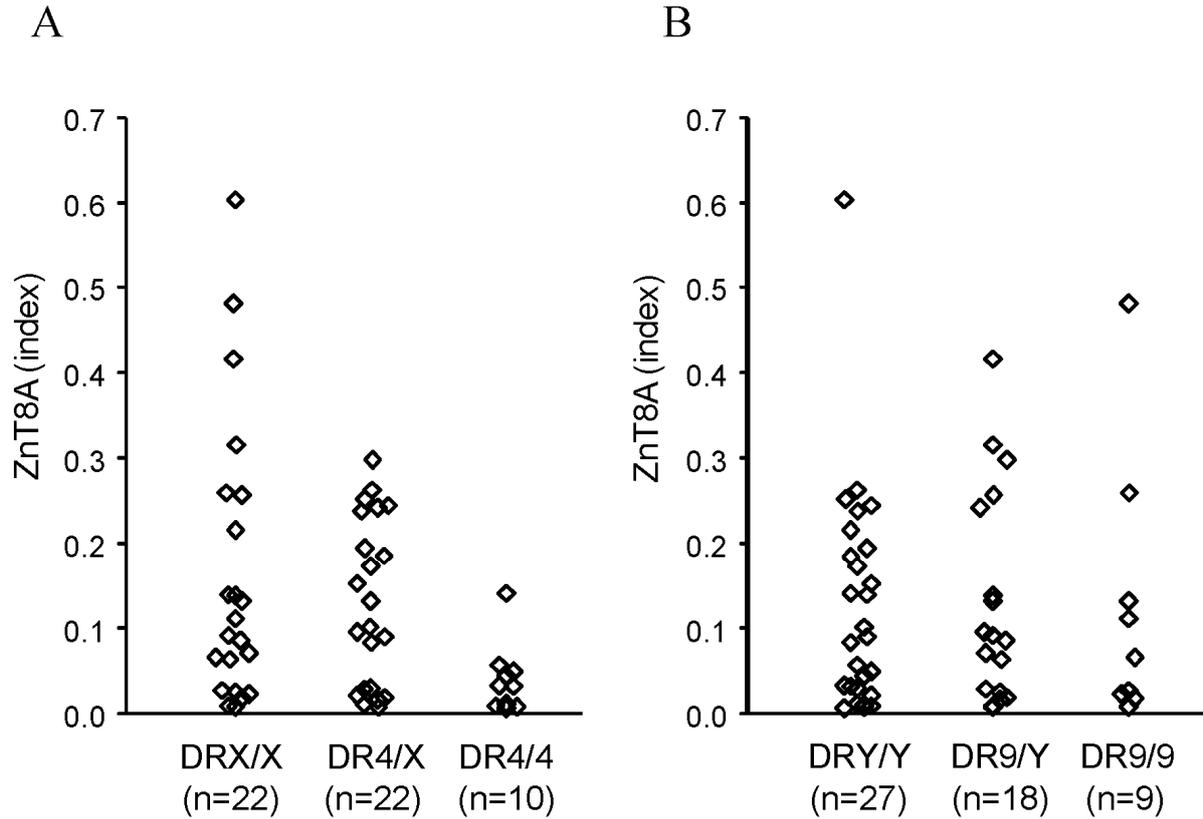


Figure 3 Comparisons of the level of ZnT8A with the copy number of the HLA-DR4 (A) and -DR9 (B). “X”, nonDR4 allele; “Y”, nonDR9 allele (B)

The levels were compared among the ZnT8A-positive individuals. The mean (\pm SD) index of ZnT8A is 0.041 (\pm 0.040) for HLA-DR4 homozygotes, 0.132 (\pm 0.097) for DR4/X, and 0.163 (\pm 0.165) for DRX/X ($P=0.043$ by ANOVA). The mean index is 0.127 (\pm 0.155) for HLA-DR9/9, 0.130 (\pm 0.124) for DR9/Y, and 0.127 (\pm 0.129) for DRY/Y ($P=0.99$).

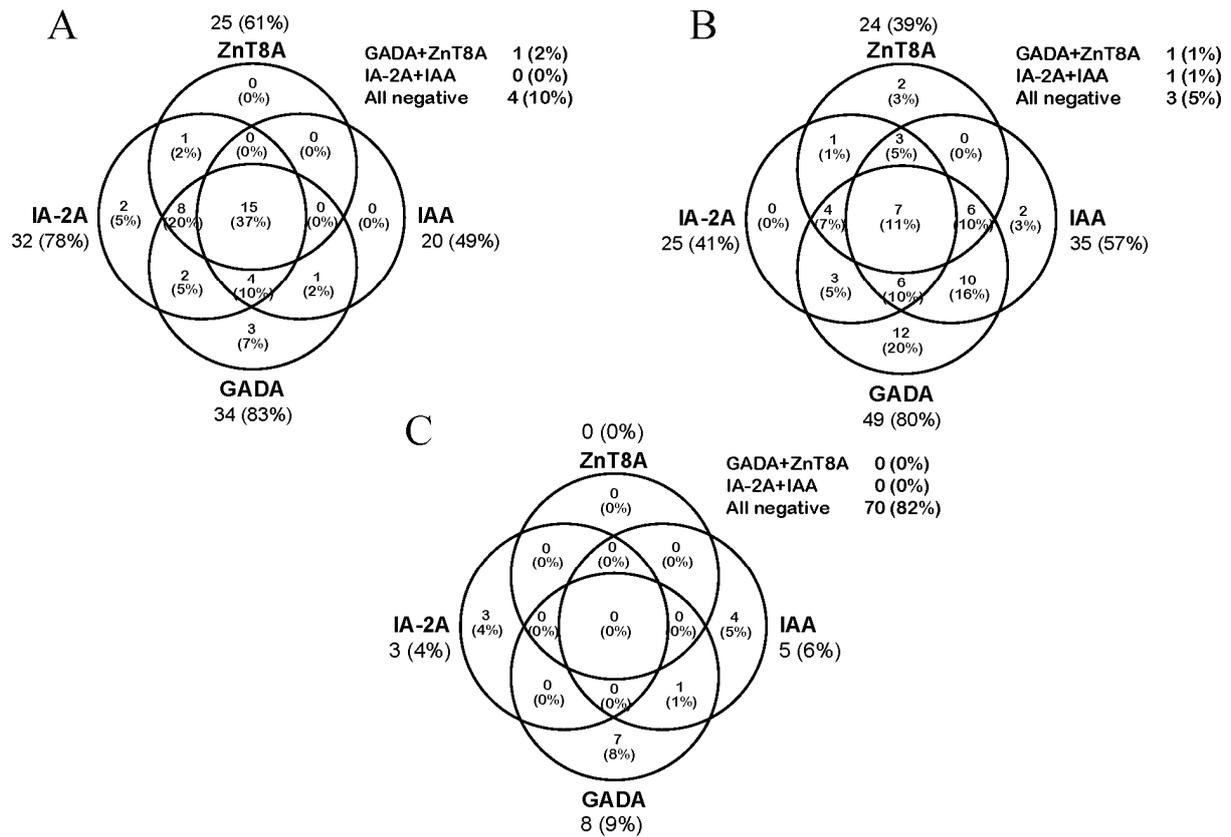


Figure 4 Combinatorial analysis of autoantibodies to ZnT8, GAD65, insulin, and IA-2

A, Childhood-onset type 1 diabetes (n=41); B, Adult-onset type 1 diabetes (n=61); C, Fulminant type 1 diabetes (n=85)

Patients' sera obtained within two weeks after the initiation of insulin treatment were used.

Table 1 Comparisons of clinical and immunogenetic features between type 1 diabetic patients with and without ZnT8A

	Childhood-onset					Adult-onset				
	n	ZnT8A +ve	n	ZnT8A -ve	<i>P</i> value ^a	n	ZnT8A +ve	n	ZnT8A -ve	<i>P</i> value ^a
Male, n (%)	33	14 (42)	24	10 (42)	NS	33	8 (24)	49	18 (37)	NS
Age at onset (years)	33	9.1 ± 3.8	24	10.6 ± 3.3	NS	33	32.5 ± 16.3	49	36.1 ± 17.5	NS
GADA +ve, n (%)	33	30 (91)	24	13 (54)	<0.005	33	27 (82)	49	37 (76)	NS
IA-2A +ve, n (%)	33	31 (94)	24	10 (42)	<0.0001	33	21 (64)	49	13 (27)	<0.001
IAA +ve, n (%) ^b	26	16 (62)	15	4 (27)	<0.05	26	18 (69)	39	20 (51)	NS
HLA- <i>DR4</i> +ve, n (%)	21	12 (57)	19	12 (63)	NS	27	17 (63)	44	24 (55)	NS
HLA- <i>DR9</i> +ve, n (%)	21	11 (52)	19	10 (53)	NS	27	14 (52)	44	18 (41)	NS

Patients with fulminant type 1 diabetes were excluded from this analysis.

Data are means ± SD or n (%); NS, not significant

^a χ^2 test for proportions; Mann-Whitney *U* test for continuous data

^b IAA were evaluated in sera which were obtained within 2 weeks after initiating insulin therapy

Supplementary Table 1 Frequencies of anti-islet autoantibodies and HLA *DR* phenotype in patients with type 1 diabetes

	<i>DR4/9</i>	<i>DR4/X</i>	<i>DR9/Y</i>	<i>DRX/Y</i>	χ^2 df=3	<i>P</i> value
GADA +ve, n (%)	17/20 (85)	39/52 (75)	29/37 (78)	8/13 (62)	2.54	0.47
IA-2A +ve, n (%) ^a	14/20 (70)	29/52 (56)	24/37 (65)	2/13 (15)	11.49	0.0093
IAA +ve, n (%) ^b	9/15 (60)	23/41 (56)	18/31 (58)	3/9 (33)	2.00	0.57
ZnT8A +ve, n (%)	8/20 (40)	24/52 (46)	19/37 (51)	3/13 (23)	3.34	0.34

Patients with fulminant type 1 diabetes were excluded from this analysis.

X=other than *DR9*, Y=other than *DR4*.

^a *P* < 0.05 for *DR9* vs. non-*DR9* patients

^b IAA were evaluated in sera which were obtained within 2 weeks after initiating insulin therapy