Novel Enzyme-Linked Immunosorbent Assay for Bivalent ZnT8 Autoantibodies

Eiji Kawasaki [a], Megumi Tanaka [b], Masaki Miwa [a], Norio Abiru [c], Atsushi Kawakami [c]

[a] Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, Nagasaki, Japan,

[b] Cosmic Corporation, Tokyo, Japan

[c] First Department of Internal Medicine, Nagasaki University Graduates School of Biomedical Sciences, Nagasaki, Japan

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

Autoantibodies to zinc transporter 8 (ZnT8A) is a powerful diagnostic or predictive marker in type 1 diabetes. However, the widely used current ZnT8A radioligand binding assay (RBA) has proved to be difficult for many laboratories to implement. The aim of this study was the development and characterization of the performance of a novel fluid phase ZnT8A enzyme-linked immunosorbent assay (ELISA) in relation to standard radioligand binding assay (RBA) in type 1 diabetes. Sera from 114 patients with type 1 diabetes and 140 blinded Islet Autoantibody Standardization Program (IASP2012) samples were studied. The sensitivity of ELISA-ZnT8A is equivalent or <u>slightly</u> higher than conventional RBA with similar specificity. Furthermore, the <u>median</u> SD score using this ELISA was significantly higher than that obtained with RBA ($P<0.0001$). Multiple logistic regression analysis revealed that ELISA-ZnT8A positivity was associated with younger age of onset (≤ 20 years; OR 15.91, $P=0.0002$), acute-onset form of type 1 diabetes (OR 3.38, $P=0.019$), and the presence of IA-2 autoantibodies (OR 3.75, $P=0.014$). Furthermore, the levels of ELISA-ZnT8A were associated with the reactivity to ZnT8-325Arg, but not ZnT8-325Trp. We conclude that this nonradioactive bivalent ZnT8A assay has high performance and should facilitate large-scale autoantibody screening. Moreover, these results suggest that the humoral autoimmunity against ZnT8 is related to a high risk of faster development of type 1 diabetes and the ZnT8A levels are associated with the known aa325 variants.

Key words: Autoantibodies, ELISA, Radioimmunoassay, Type 1 diabetes, Zinc transporter-8

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]. To date the expression of anti-islet autoantibodies is the best phenotypic marker of autoimmune type 1 (type 1A) diabetes [1]. The cation efflux transporter zinc transporter 8 (ZnT8) has been first described as a novel target autoantigen in patients with type 1 diabetes in 2007 [2]. With successful adaptation to a method for detecting other anti-islet autoantibodies, such as autoantibodies to glutamic acid decarboxylase (GADA) or insulinoma-associated antigen-2 (IA-2A), a high throughput radioligand binding assay (RBA) for ZnT8A was immediately developed and ZnT8A were identified in more than 60% of patients with type 1 diabetes. Furthermore, we and others reported that the most ZnT8-reactive sera recognize the carboxy-terminal 102 amino acids of the molecule (amino acids 268-369) and amino acid encoded by the polymorphic codon 325 is a key determinant of humoral autoreactivity to this protein [3,4].

The first international proficiency evaluation of ZnT8A assays by the Diabetes Antibody Standardization Program (DASP) demonstrated that RBA assays achieved a high degree of sensitivity and specificity [5]. However, the widely used current fluid phase ZnT8A RBA assays have proved to be difficult for many laboratories to implement. Given the need for improved ZnT8A assays, we here describe the development and characterization of the performance of a nonradioactive fluid phase ZnT8A assay in relation to standard ZnT8A RBA in type 1 diabetes. Furthermore, we also investigated the clinical significance of ZnT8A in patients with type 1 diabetes.

Materials and Methods

Subjects

We examined 114 patients with type 1 diabetes (64% female) and 119 healthy control subjects of Japanese origin. Patients with type 1 diabetes consisted of 60 acute-onset patients with type 1 diabetes (73% female, median age 22.5, range 2.0-75.0 years), 42 patients with slow-onset patients

with type 1 diabetes (60% female, median age 36.5, range 12.0-68.0 years), and 12 fulminant type 1 diabetes (33% female, median age 34.0, range 21.0-56.0 years).   Furthermore, 51 of 114 (45%) patients with type 1 diabetes were complicated with autoimmune thyroid disease (AITD) when sera were obtained.   All patients with diabetes analyzed in the present study were diagnosed according to the American Diabetes Association criteria for the classification of diabetes [6].   AITD was defined as Graves' disease, Hashimoto's thyroiditis which was diagnosed based on the finding of either palpable goiter or the presence of chronic thyroiditis with ultrasonography examination in the absence of goiter, abnormal levels of thyroid hormones, or positivity for autoantibodies to thyroid peroxidase (TPO), thyroglobulin (Tg) and/or thyrotropin (TSH) receptor.   All subjects were informed the purpose of the study, and their consent was obtained.   Protocols were approved by the ethics committee of the Nagasaki University.   Sera were stored at −20°C until use.

In addition, 140 de-identified blinded samples (90 blood donors and 50 patients with new onset type 1 diabetes) from the 1st Islet Autoantibody Standardization Program (IASP2012) were also analyzed.   According to the decoded data provided by the IASP committee, patients with type 1 diabetes consisted of 28 male and 22 female with the median age-at-onset of 15.0 (range, 9.0–37.0) years.

ZnT8 autoantibody ELISA assay

The human ZnT8 cDNA (dimeric C terminal domain consisting of amino acids 275-369 [325Trp] and amino acids 275-369 [325Arg] with C-terminal 6-His tag) was cloned into an E.coli/Yeast shuttle vector which contains the Uracil Synthase URA3 gene (selectable marker) and the GAL1 (yeast galactokinase) promoter.   Recombinant ZnT8 was expressed in *Saccharomyces cerevisiae* strain c13ABYS86 using galactose for induction.   After the completion of the culture, the cells were harvested by centrifugation and broken by mixing with glass beads on ice.   The breakage mixture was clarified by centrifugation.   ZnT8 was then purified using anion exchange column chromatography, metal affinity column chromatography and size exclusion column chromatography.

Highly purified recombinant ZnT8 was coated onto ELISA plate wells. ZnT8-biotin was prepared using NHS-LC-LC-biotin (Pierce, Etten-Leur, The Netherlands) according to the manufacturer's instructions and was suspended in Tris buffer pH 8.0 containing 0.5g/L sodium azide. In the ELISA, 25µL of test sample was added to ZnT8 coated wells in duplicate and incubated overnight at 4 C. The well contents were aspirated, the wells were washed three times with washing buffer (150mmol/L NaCl, 20mmol/L Tris, pH 7.6 containing 0.05% Tween 20), and 100µL of ZnT8-biotin was added to each well. After 1 hour incubation at 4 C without shaking, unbound ZnT8-biotin was then removed by washing as above. Streptavidin-peroxidase conjugate (100µL; RSR Ltd., Cardiff, U.K.) was added and the plates were incubated for 20 min at room temperature with shaking (500 shakes per min). After the aspiration and washing of the wells as above, 100µL of 3,3',5,5' -tetramethylbenzidine was added to each well and the plates were incubated for 20 min in the dark at room temperature. 100µL of stop solution (0.25 mol/L H_2SO_4) was added to stop the reaction and the absorbance of the plate wells was read at 405 nm and 450 nm using an ELISA plate reader (BioTek Instruments, Inc., Bedfordshire, U.K.).

Positive and negative control sera and a set of calibrators containing different titers of ZnT8A (10 – 2,000 U/mL) were included in each assay. ZnT8A level was estimated using a standard curve prepared from calibrators. Sera were considered as ZnT8A-positive if they contained > 15U/mL of autoantibody, which was based on the 99th percentile (13.6 U/mL) of sera from in 297 healthy blood donors.

ZnT8 autoantibody radioligand binding assay

ZnT8A were also determined by radioligand binding assay (RBA) using human ZnT8 cDNA as described previously [7]. The human ZnT8 cDNA construct used in this assay was a fusion of the cytoplasmic carboxy-terminal domains (amino acids 268-369) of ZnT8 carrying either 325Trp or 325Arg with a linker peptide. The cut-off index for ZnT8A-325TrpArg was an index of 0.007, which was based on the 99th percentile of sera from 139 healthy control subjects. In the Diabetes

Autoantibody Standardization Program 2009 (DASP 2009), this assay had 40% sensitivity and 100% specificity.   In addition, autoantibody reactivities to ZnT8 aa325 variants were determined using the carboxy-terminal domains (amino acids 268–369) of cDNA encoding the 325Arg or 325Trp.   The cut-off index was an index of 0.016 for ZnT8A-325Arg and 0.018 for ZnT8A-325Trp based on the 99th percentile of sera from 139 healthy control subjects.   The inter-assay co-efficient of variation (CV) and intra-assay CV values were 10.4% and 5.7% (ZnT8A-325Arg) and 5.9% and 6.8% (ZnT8A-325Trp), respectively.

Detection of other autoantibodies

Autoantibodies to GAD65, TPO and Tg were measured using commercially available radioimmunoassay (RIA) kits (Cosmic Corporation, Tokyo, Japan). TSH receptor autoantibodies were measured using an RIA kit provided by Yamasa Corporation (Chiba, Japan).   IA-2A were determined by RBA as previously described [7].

Statistical analysis

Unpaired data were analyzed by the Chi-square test and the Mann-Whitney *U* test.   The correlation between autoantibody levels was analyzed using Spearman rank correlation test. Agreement between the ELISA and RBA was assessed with the Kappa statistic, which is a measure of the strength of agreement on scale from 0-1.   A Kappa statistic value of > 0.75, 0.40 to 0.75, or < 0.40 represents excellent agreement, good to fair agreement, and poor agreement, respectively [8].   A SD score was calculated for each serum: SD score = (antibody level of test serum – mean level of healthy control sera)/SD of the levels of healthy control sera. The SD score provides a measure of the certainty with which individual serum can be deemed different from normal.   The statistical significance of paired differences of SD score between ELISA and RBA was determined by the Wilcoxon signed-rank test.   Patient-only logistic regression analysis was performed to test for the association of the ELISA-ZnT8A positivity with age at onset, gender, mode of type 1 diabetes onset, co-occurrence of

AITD, GADA positivity, and IA-2A positivity as variables. StatView Ver.5.0 (SAS Institute, Cary, NC) was used for these tests. A *P* value less than 0.05 was considered statistically significant.

Results

ZnT8A measured by ELISA assay in patients with type 1 diabetes

For 297 healthy blood donors, the median level of ELISA-ZnT8A was 1.0 U/mL (range 1.0-45.0). The interassay CV and intraassay CV were 2.5% (n=4) and 2.3% (n=5), respectively. Fifty-five percent (63 of 114) of patients with type 1 diabetes had ZnT8A levels exceeding the cut-off level. Analysis of the data stratified with the age of onset, showed that the prevalence of ZnT8A was significantly higher in patients with childhood and adolescent diabetes (88%, 30 of 34) compared to that in adult-onset diabetes (41%, P < 0.0001). The median level of ELISA-ZnT8A-positive sera was 462.8 U/mL (range 18.6-2,261). In contrast, three of samples from 119 healthy control subjects were above 15U/mL.

Table 1 shows the clinical and immunological characteristics between ELISA-ZnT8A-positive and -negative patients with type 1 diabetes. ZnT8A-positive patients, compared with ZnT8A-negative patients, showed a female predominance (P = 0.03), younger age of onset (P = 0.0001), and higher prevalence of acute-onset type 1 diabetes (P < 0.0001). Furthermore, GADA (P = 0.05) and IA-2A (P < 0.0001) were positive in higher proportion of ZnT8A-positive than ZnT8A-negative patients. There was no correlation between the presence of ZnT8A and AITD. We also evaluated the relationship of the presence of ZnT8A with clinical and immunological parameters in patients by multiple logistic regression analysis (Table 2). The optimum cut-off point of onset age, based on receiver operating characteristic curve, was 20 years (sensitivity 47.6%; specificity 92.2%). There were no associations of the presence of ZnT8A with gender, the co-occurrence of thyroid autoimmunity or the presence of GADA. However, the ZnT8A positivity was associated with younger age of onset (OR 15.91, 95%CI 3.79-66.84, P = 0.0002), acute-onset form (OR 3.38, 95%CI 1.22-9.34, P = 0.019), and the presence of IA-2A (OR 3.75, 95%CI 1.31-10.76, P = 0.014).

Comparison of ELISA-ZnT8A and ZnT8A-325TrpArg measured by RBA

Of 114 patients with type 1 diabetes, 65 (57%) were positive for ZnT8A-325TrpArg using RBA. Figure 1 shows the correlation between the levels of ELISA-ZnT8A and those of ZnT8A-325TrpArg in patients with type 1 diabetes, which were obtained without endpoint titration. A close correlation was observed between the assays (r = 0.895, P < 0.0001 for all samples and r = 0.866, P < 0.0001 when the samples below the cut-off were excluded). Only 1% (1 of 114) of patients was ELISA-ZnT8A positive but ZnT8A-325TrpArg negative. On the other hand, 3% (3 of 114) of patients were ZnT8A-325TrpArg positive but ELISA-ZnT8A negative. Considering the agreement of sera as positive or negative, the agreement between the ELISA-ZnT8A and ZnT8A-325TrpArg was 96.5% (Kappa statistic = 0.928, 95%CI 0.860-0.997) (Table 3).

Figure 2 illustrates the SD score ((level of test sera – mean level of healthy control sera)/ SD of healthy control sera) for individual sera for the ELISA-ZnT8A compared with those of ZnT8A-325TrpArg. The median SD score obtained by ELISA-ZnT8A (14.0, range −0.34-588.3) was significantly higher than those obtained using ZnT8A-325TrpArg (median 5.2, range −8.0-223.7, P < 0.0001).

Humoral autoreactivity to ZnT8 aa325 variants

In this study, sera were also tested for the reactivity to the carboxy-terminal ZnT8 constructs bearing 325Arg or 325Trp. Among 114 patients 14 patients (12.2%) reacted with 325Arg construct alone, 19 patients (16.7%) reacted with both 325Arg and 325Trp constructs, and 18 patients (15.8%) reacted with 325Trp construct alone, respectively. Figure 3 shows the concordance between the ELISA-ZnT8A and ZnT8A-325Arg or ZnT8A-325Trp. Of 63 ELISA-ZnT8A-positive patients with type 1 diabetes, 31 and 35 of patients reacted to the 325Arg or 325Trp construct. There were 28.1% and 24.6% of patients positive for ELISA-ZnT8A but negative for ZnT8A-325Arg or ZnT8A-325Trp, respectively. Furthermore, the agreement between the ELISA-ZnT8A and RBA to ZnT8 aa325

variants was 70.2% (Kappa statistic = 0.429, 95%CI 0.268-0.590) for ZnT8A-325Arg and 73.7% (Kappa statistic = 0.429, 95%CI 0.337-0.648) for ZnT8A-325Trp, which were significantly lower than that for ZnT8A-325TrpArg (P < 0.0001).   Of interest, the level of ELISA-ZnT8A was associated with the positivity of ZnT8A-325Arg, but not with that of ZnT8A-325Trp.   The level of ELISA-ZnT8A in patients who have ZnT8A-325Arg (median 964.5 U/mL, range 88.6-2261.0) was significantly higher than that in ZnT8A-325Arg negative patients (median 300.0 U/mL, range 18.6-1235.9, P < 0.0001). In contrast, the median ELISA-ZnT8A level was not statistically different between ZnT8A-325Trp positive (median 556.8 U/mL, range 45.8-1683.1) and ZnT8A-325Trp negative patients (median 318.7 U/mL, range 18.6-2261.0, P = 0.17).   Furthermore, the prevalence of patients with ELISA-ZnT8A titer $\geq$ 1,000U/mL was significantly higher in ZnT8A-325Arg-positive patients (14 of 31, 45%) than that in ZnT8A-325Arg-negative patients (2 of 32, 6%; P=0.0004).   In contrast, it was similar between ZnT8A-325Trp-positive and -negative patients (26% vs. 25%).

Assay sensitivity/ specificity in Islet Autoantibody Standardization Program

A blinded IASP2012 workshop set of 90 control subjects and 50 new-onset patients with type 1 diabetes was tested for ELISA-ZnT8A assay, and ZnT8A levels were also well correlated with our ZnT8A-325TrpArg RBA (r = 0.776, P < 0.0001), while ELISA-ZnT8A assay had a higher sensitivity of 72.0% vs. 62.0% in the RBA with the similar specificity of 96.7% for ELISA vs. 98.9% for RBA. Excellent agreement was seen in Kappa statistics between ELISA and RBA (90.0% agreement, Kappa statistic =0.776, 95%CI 0.595-0.958) (Table 3).   Of 5 samples from new-onset patients who were ELISA-ZnT8A-positive and ZnT8A-325TrpArg-negative, 4 sera were positive for either GADA or IA-2A in our assay.

Discussion

We demonstrated that 1) the sensitivity of ELISA-ZnT8A assay is equivalent to or slightly higher than conventional RBA assay, 2) ELISA-ZnT8A assay demonstrates better performance

compared to RBA assay, 3) humoral autoreactivity to ZnT8 is associated with faster development of type 1 diabetes, and 4) the ZnT8A levels are associated with the known aa325 variants. Although the role of ZnT8 in the pathogenesis of type 1 diabetes is still unknown, ZnT8A is known to be a promising diagnostic and predictive marker for the disease. In the present study, we evaluated the new ELISA assay with dimeric carboxy-terminal domains of ZnT8 carrying either 325Trp or 325Arg as antigens. This assay employed a modified ELISA format which is based on the ability of the autoantibodies to form a bridge between ZnT8 coated on the ELISA plate and ZnT8-biotin, and the detection of autoantibody bound antigen rather than immunoglobulin itself. Our current study shows that the sensitivity for detecting ZnT8A by this ELISA is not only equal to that of RBA in our samples but is <u>slightly</u> higher in IASP2012 samples (72.0% vs. 62.0%) with the similar specificity (96.7% vs. 98.9%). Because the majority of IAPS2012 samples were obtained from childhood-onset patients, ELISA-ZnT8A assay may have advantage for the diagnosis and prediction in the children. Furthermore, in ZnT8A-positive sera, ELISA-ZnT8A assay showed over 15 times higher SD score than the conventional RBA-ZnT8A-325TrpArg assay (Figure 2). We speculated that the better performance of this bivalent ZnT8A assay may relate to the lower non-specific binding among healthy control sera, which may originate in the difference of the conformational structure of the antigens produced by yeast and in *in vitro* transcription/translation system. Another likely explanation is that this ELISA assay permits both antigen binding sites of a single IgG molecule to engage a single ZnT8 antigen, thereby significantly enhancing the strength of the antigen-antibody interaction. Based on not requiring the use of radioisotopes and semi-automated high throughput assay format, the ELISA-ZnT8A assay should be suitable for general application. However, this ELISA has a disadvantage for the amount of sera needed (25µL) compared to RBA (2.5-5µL), which would be prohibited for the autoantibody screening in infants. Also important is the observation that the ZnT8A positivity was associated with younger age of onset, acute-onset form, and the presence of IA-2A (Table 2), supporting the evidences that ZnT8A may mark a critical turning point in the process leading to β cell destruction [2].

One of the interesting findings of the current study is that the higher titer of ELISA-ZnT8A was associated with the humoral autoreactivity to ZnT8A-325Arg. Skärstrand and coworkers recently reported that the titer of peptide antibodies induced by the immunization of ZnT8-325Arg 15-mer peptide was higher than that by ZnT8-325Trp peptide [9]. Furthermore, it was reported that autoantibodies to ZnT8A variants differ in the prognosis of β cell function [10,11]. Although we and others reported that there is a strong genetic specificity between the ZnT8A variants and the *Slc30A8* gene SNPs [3,4], taken together these data highlight the different strength of humoral autoimmune response to the ZnT8 variants, which may be associated with the rate of β cell destruction. However, a weakness to the study is the limited number of subjects. A larger study population should be ascertained to fully answer the question to what extent autoantibodies against the ZnT8-325Arg variant are more specific markers of autoimmune-mediated β cell destruction.

We conclude that this nonradioactive fluid phase ZnT8A assay has high performance and should facilitate large-scale autoantibody screening. Moreover, these results suggest that the humoral autoimmunity against ZnT8 is related to a high risk of faster development of type 1 diabetes and the ZnT8A levels are associated with the known aa325 variants.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1   Correlation between the levels of ZnT8A measured by ELISA (ELISA-ZnT8A) and RBA assay (ZnT8A-325TrpArg) in patients with type 1 diabetes

-------, cut-off value for each assay. A significant correlation was found between ELISA-ZnT8A level and ZnT8A-325TrpArg level (r = 0.895, P < 0.0001 for all samples and r = 0.866, P < 0.0001 when the samples below the cut-off were excluded.)

Figure 2   The comparison of SD score for each sample of ZnT8A measured by ELISA and RBA in patients with type 1 diabetes.

The median SD score obtained by ELISA-ZnT8A was significantly higher than that of ZnT8A-325TrpArg. *P < 0.0001.

Figure 3   Concordance between ELISA-ZnT8A and autoantibodies to ZnT8 aa325 variants in patients with type 1 diabetes.

The agreement between the ELISA-ZnT8A and RBA to ZnT8 aa325 variants was 70.2% (Kappa statistic = 0.429, 95%CI 0.268-0.590) for ZnT8A-325Arg or 73.7% (Kappa statistic = 0.429, 95%CI 0.337-0.648) for ZnT8A-325Trp.

Figure 1

Figure 2

Figure 3

ZnT8A-325Arg   ELISA-ZnT8A

2 (1.8%)   31 (27.2%)   32 (28.1%)

Both negative
49 (43.0%)

ZnT8A-325Trp   ELISA-ZnT8A

2 (1.8%)   35 (30.7%)   28 (24.6%)

Both negative
49 (43.0%)
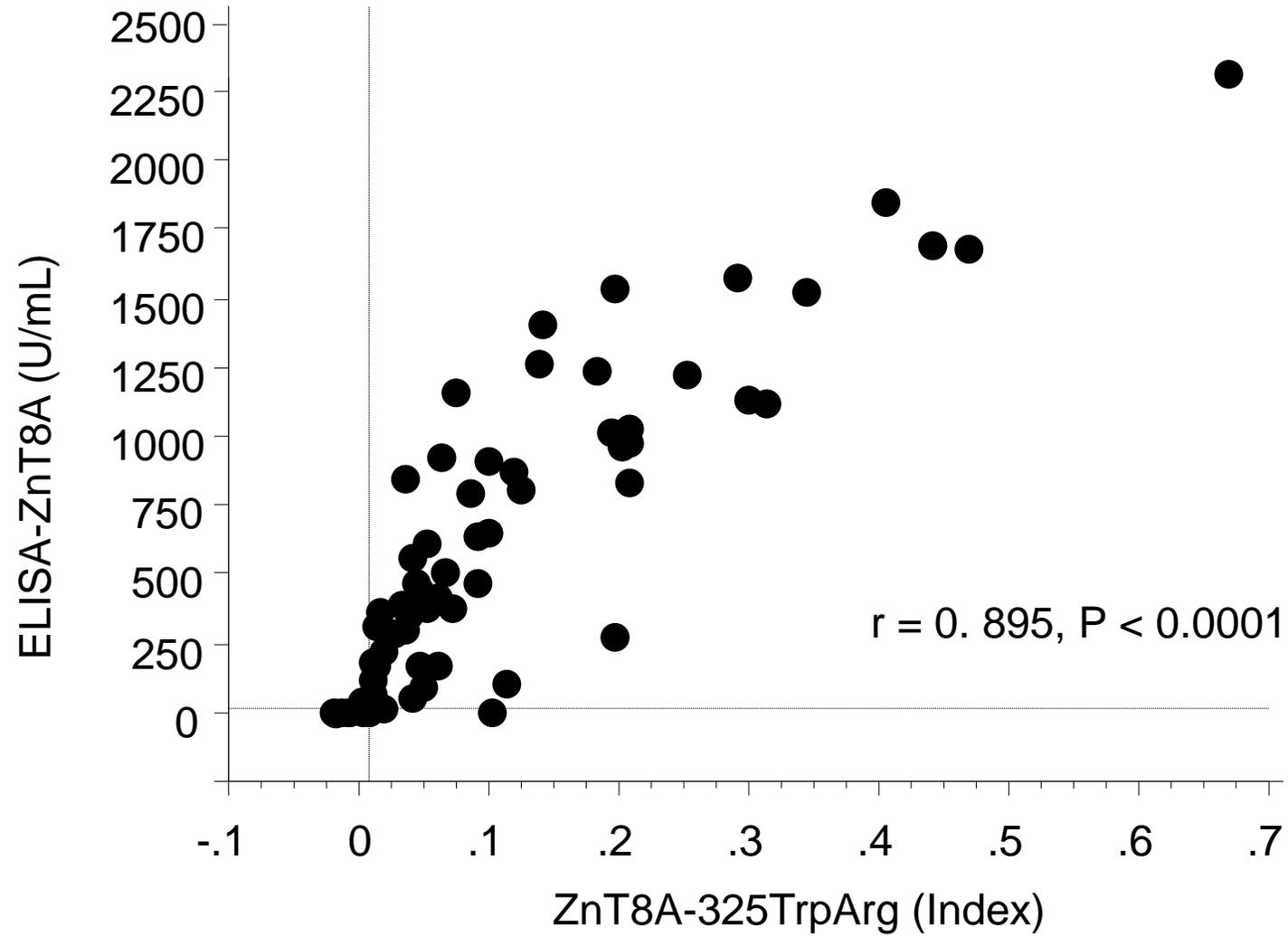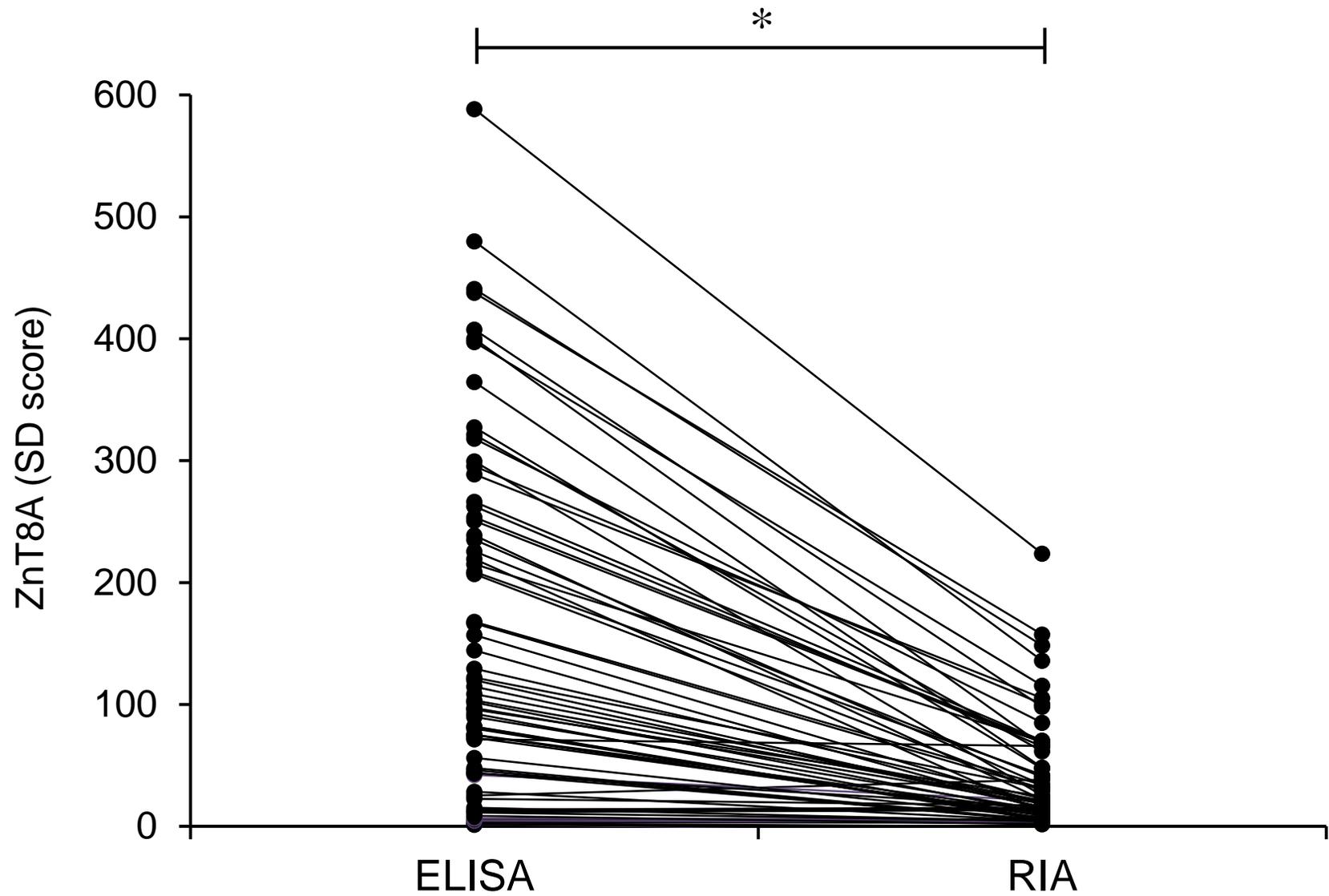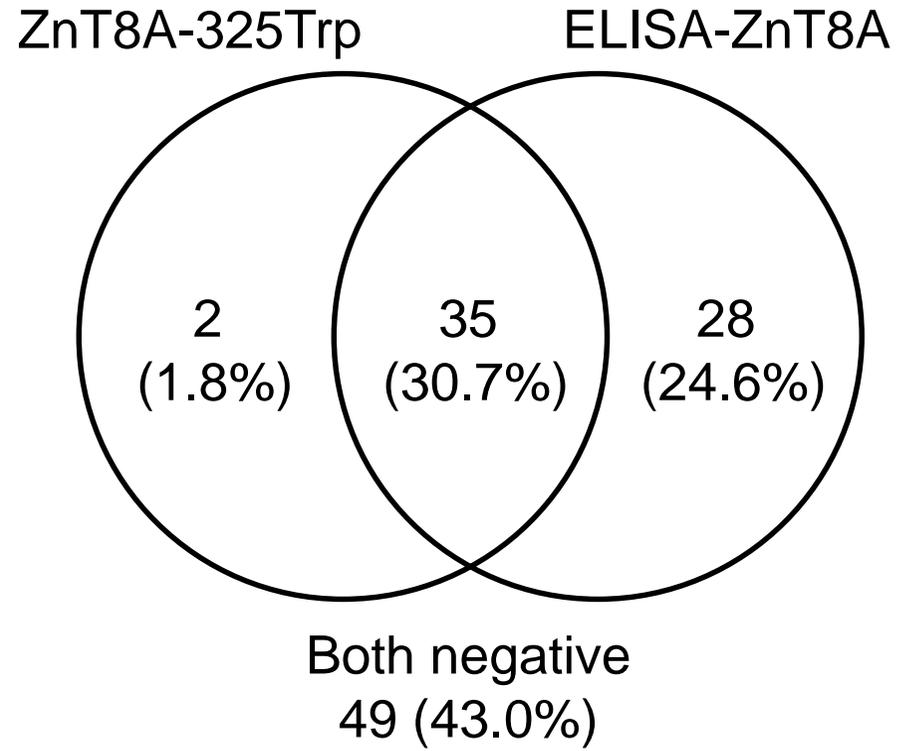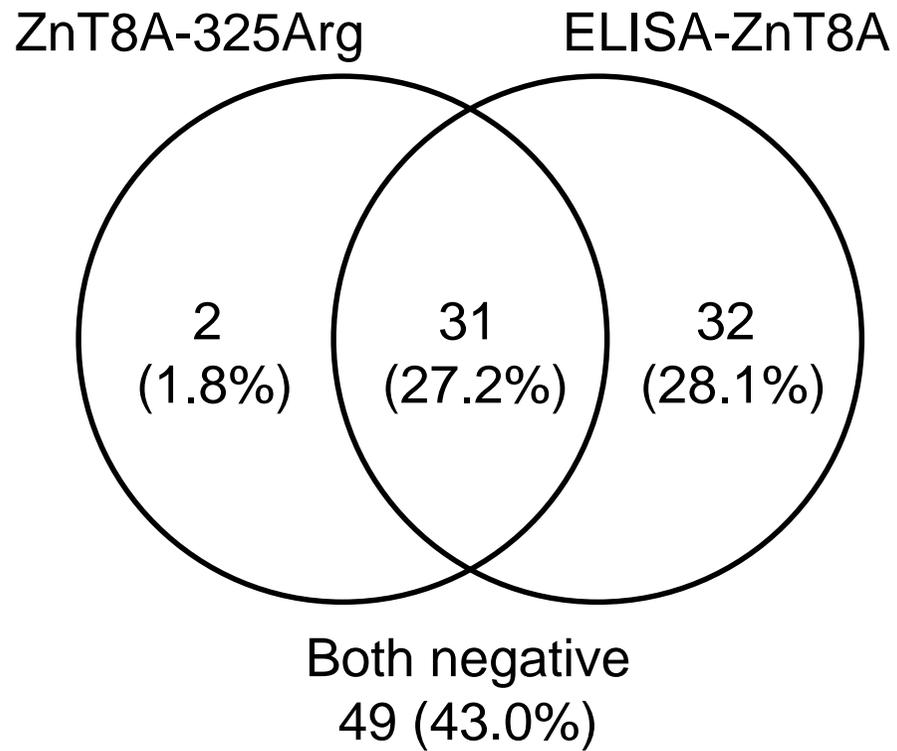
Table 1 Clinical and immunological characteristics in ELISA-ZnT8A positive and negative patients

	ZnT8A positive	ZnT8A negative	*P* value
n	63	51	
Male/Female	17/46	24/27	0.03
Onset age (years)	27.9 ± 19.5	39.9 ± 16.1	0.0001
Mode of diabetes onset			
Acute	45 (71)	15 (29)	<0.0001
Slow	18 (29)	24 (47)	0.04
Fulminant	0	12 (24)	<0.0001
Co-occurrence of AITD	32 (51)	20 (39)	N.S.
GADA positive	52 (83)	34 (67)	0.05
IA-2A positive	42 (67)	11 (22)	<0.0001

Data are n (%) or mean ± SD.   N.S., not significant

Table 2 Logistic regression analysis for the association of clinical and immunological parameters with ELISA-ZnT8A positivity among patients with type 1 diabetes

Variable	OR	95% CI	*P* value
Age at onset ≤ 20 years	15.91	3.79-66.84	0.0002
Female	1.76	0.54-5.72	N.S.
Acute-onset	3.38	1.22-9.34	0.019
With AITD	2.95	0.93-9.34	N.S.
GADA positive	1.64	0.42-6.48	N.S.
IA-2A positive	3.75	1.31-10.76	0.014

AITD, autoimmune thyroid disease; N.S., Not significant

Table 3　Agreement between the ELISA-ZnT8A and RBA-ZnT8A-325TrpArg with sera from patients with type 1 diabetes

ELISA-ZnT8A	RBA-ZnT8A-325TrpArg		Agreement (%)	Kappa statistic[a] (95%CI)
	Positive	Negative		
Our samples				
Positive	62	1	96.5	0.928
Negative	3	48		(0.860-0.997)
IASP2012 samples				
Positive	31	5	90.0	0.776
Negative	0	14		(0.595-0.958)

[a] A Kappa statistic value of > 0.75, 0.40 to 0.75, or < 0.40 represents excellent agreement, good to fair agreement, and poor agreement, respectively (8).