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ORIGINAL ARTICLE

Zinc transporter 8 autoantibodies complement glutamic acid decarboxylase and insulinoma-associated antigen-2 autoantibodies in the identification and characterization of Japanese type 1 diabetes

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Keywords
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ABSTRACT

Aims/Introduction: This study aimed to investigate the significance of zinc transporter 8 autoantibody (ZnT8A) in identifying and characterizing autoimmune-mediated type 1 diabetes in Japanese individuals.

Methods: ZnT8A were determined in 324 patients with type 1 diabetes, 191 phenotypic type 2 diabetes and 288 healthy control individuals using bridging-type enzyme-linked immunosorbent assay in addition to autoantibodies to glutamic acid decarboxylase and insulinoma-associated antigen-2.

Results: We set a cut-off value of 10.0 U/mL, and 25% of the type 1 diabetic patients had ZnT8A levels exceeding this level. The prevalence of ZnT8A was significantly higher in patients with acute-onset type 1 diabetes than in those with slowly progressive and fulminant type 1 diabetes ($P < 0.05$). ZnT8A were more frequent in patients aged ≤10 years, but less frequent in patients with duration ≥5 years ($P < 0.05$). ZnT8A were detected in 5.2% of phenotypic type 2 diabetic patients, with 90% of these being ZnT8A-single-positive. Furthermore, the ZnT8A levels in the phenotypic type 2 diabetes cohort (143.8 ± 194.9 U/mL) were significantly higher than those in the type 1 diabetes cohort (22.9 ± 8.3 U/mL, $P < 0.05$). In the acute-onset and slowly progressive type 1 diabetic patients with duration ≤5 years, additional measurement of glutamic acid decarboxylase autoantibodies significantly increased the disease sensitivity in patients aged ≤10 years, but not in patients aged ≥11 years (11.7 vs 3.6%, $P < 0.05$). Multivariate analysis showed that ZnT8A positivity was independently associated with age at sampling and insulinoma-associated antigen-2 autoantibody positivity.

Conclusions: These results suggest that the bridging-type ZnT8A enzyme-linked immunosorbent assay might provide a valuable additional marker for Japanese patients with type 1 diabetes, which could, in turn, allow for an increase in the number of identifiable cases and differentiate clinical phenotypes.

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INTRODUCTION

Anti-islet autoantibodies directed against insulin, glutamic acid decarboxylase (GADA), insulinoma-associated antigen-2 (IA-2A) and the recently described zinc transporter 8 (ZnT8A) are all useful markers in diagnosing, predicting and differentiating the clinical phenotypes of type 1 diabetes. Among these, GADA is the most frequently used for the diagnosis of autoimmune diabetes[1]. ZnT8 is a 369-amino acid polytopic transmembrane protein localized in the insulin secretory granule that transports zinc iron from the cytosol into the vesicles[2,3]. Previous studies have reported that ZnT8A were associated with acute-onset and childhood-onset patients with type 1 diabetes and, therefore, are considered as a more specific marker of autoimmune-mediated β-cell destruction[3]. Despite this, the relevance of ZnT8A in patients with adult-onset type 1 diabetes, especially in slowly progressive and fulminant type 1 diabetes, and type 2 diabetes has yet to be clarified.

Furthermore, several studies have identified that the major epitopes for ZnT8A are localized in the carboxy-terminal 102 amino acids (aa 268–369) that lie within the cytoplasmic domain. Furthermore, residue at position 325 is a major determinant that is controlled by the single nucleotide polymorphism rs13266634 (Arg325Trp) in the ZnT8 gene, *SLC30A8*[4,5].

In a recent report carried out by our team, we detailed the development and the characterization of bridging-type enzyme-linked immunosorbent assay (ELISA) with dimeric carboxy-terminal ZnT8 domains carrying either 325Trp or 325Arg as antigens[6]. Although this assay achieved high sensitivity and specificity in the Islet Autoantibody Standardization Program or Diabetes Antibody Standardization Program[7], its clinical significance in the Japanese populations remains ambiguous, as the serum samples used in these programs are derived from white people. Therefore, the present study aimed to investigate the significance of ZnT8A on the identification and characterization of autoimmune-mediated type 1 diabetes in the Japanese population.

METHODS

Participants

In the present cross-sectional, observational and collaborative study, we recruited 870 participants, all of whom were of Japanese origin, including 364 patients with type 1 diabetes, 191 with clinically diagnosed type 2 diabetes and 288 healthy control individuals, from seven hospitals in three major cities (Fukuoka, Osaka and Tokyo). Sera used in the study were stored at −20°C until application. Among the 364 patients with type 1 diabetes, 40 patients complicated by autoimmune thyroid disease at the time of serum sampling were excluded from this study, while the remaining 324 patients with type 1 diabetes were analyzed. These patients were then classified into three subtypes according to the criteria of the Japan Diabetes Society[8-10]; 240 acute-onset, 25 fulminant and 59 slowly progressive type 1 diabetes. A diagnosis of slowly progressive type 1 diabetes was made if patients showed positive for GADA and/or islet cell antibodies at any time during the disease course irrespective of anti-islet autoantibody status at the time of study. The clinical and immunological profiles of all participants are summarized in Table 1.

Autoantibody assays

ZnT8A, GADA and IA-2A were determined using bivalent ELISA kits (RSR Ltd., Cardiff, UK) using biotinylated ZnT8, GAD65 and IA-2, respectively, as described previously[6,11,12]. All of these determinations were based on the sandwich type principle with dimeric carboxy-terminal domains of ZnT8 (aa 275–369) carrying either 325Trp or 325Arg, full-length GAD65 and intracellular domain of IA-2 (aa 604–979) as antigens. In short, the serum samples and unlabeled recombinant antigens (ZnT8, GAD65 or IA-2) coated onto the ELISA plate were incubated in the well. After washing the wells, a corresponding biotinylated antigen was added to each well, with any unbound biotinylated antigens then being removed by washing. Subsequently, streptavidin–peroxidase conjugate was added, and after the addition of tetramethylbenzidine, the absorbance of the plate wells was read at 405 and 450 nm using an ELISA plate reader. The results were then read from a calibration curve constructed in the same run as the calibrators, and expressed in U/mL. The cut-off value for the GADA was 5.0 U/mL, and 0.6 U/mL for the IA-2A, respectively[12,13].

Ethics statement

In accordance with the Declaration of Helsinki, this study's protocol has been approved by the ethics committee of each participating hospital and informed consent was obtained from all participants.

Table 1 | Clinical characteristics

	Acute-onset type 1 diabetes	Slowly progressive type 1 diabetes	Fulminant type 1 diabetes	Phenotypic type 2 diabetes	Healthy controls
n	240	59	25	191	288
Male : female	1.0:1.4	1.0:1.1	1.0:0.8	1.0:0.6	1.0:1.0
Age (years)	31.6 ± 20.1	55.8 ± 15.6	49.3 ± 14.2	61.3 ± 13.7	41.7 ± 11.7
Age at diabetes onset (years)	20.3 ± 17.4	43.6 ± 14.4	42.4 ± 14.3	46.9 ± 14.0	NA
Duration (years)	11.4 ± 11.9	12.2 ± 10.8	7.6 ± 6.3	13.7 ± 10.2	NA

NA, not applicable.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD) or median (range). Autoantibody prevalence was compared using the χ^2-test, Fisher's exact test and the Cochran–Armitage test where appropriate, and differences in non-parametric data were tested using the Mann–Whitney *U*-test. With the results of curve fitting using linear and non-linear regression, the correlation between the duration of diabetes and ZnT8A level was analyzed using the exponential regression curve (Table S1). A *P*-value <0.05 was considered statistically significant. Patient-only logistic regression analysis was carried out to test for the association of ZnT8A positivity using sex, duration, age at sampling, GADA positivity and IA-2A positivity as variables. The optimum cut-off point for age at sampling was determined based on the receiver operating characteristic curve, and statistical analysis was carried out using both StatView statistical software (version 5.0; SAS Institute, Cary, NC, USA) and SigmaPlot software (version 14.0; Systat Software Inc., San Jose, CA, USA).

RESULTS

Establishment of the cut-off value for ZnT8A in the Japanese population

Table S2 summarizes the results of ZnT8A in the 288 healthy control participants in comparison with those obtained from the manufacturer (RSR Ltd., $n = 297$). The mean and median level of ZnT8A in the 288 healthy control participants was 3.8 ± 2.3 U/mL and 3.7 U/mL (range 0.0–28.0 U/mL), respectively. Although the mean and median levels were higher than those in the RSR normal control participants, the mean + 3SD (10.7 U/mL) and 99th percentile (9.6 U/mL) were lower than those in the RSR controls due to the smaller SD value (2.3 U/mL). Consequently, we set the cut-off value for the Japanese population at 10.0 U/mL. By this criterion, three of the 288 (1.0 %) healthy control participants had positive ZnT8A titers, of 11.3, 13.4 and 28.0 U/mL. However, none of these participants were found to be positive for GADA or IA-2A.

ZnT8A positivity and the distribution of ZnT8A levels

Figure 1 shows the distribution of ZnT8A levels in patients with type 1 diabetes, phenotypic type 2 diabetes and healthy control participants. The prevalence of ZnT8A in the type 1 diabetic patients was 24.7% (80/324), and median level of ZnT8A in the autoantibody-positive participants was 111.5 U/mL (range 10.7–1,871.5 U/mL). Furthermore, the prevalence and median level of ZnT8A were 28.8% (69 /240) and 118.0 U/mL (range 10.7–1,871.5 U/mL) for acute-onset type 1 diabetes, 15.3% (9 /59) and 84.9 U/mL (range 13.7–876.9 U/mL) for slowly progressive type 1 diabetes. Just two of 25 (8.0%) patients with fulminant type 1 diabetes were positive for ZnT8A, with their levels being 11.7 and 14.0 U/mL. The present study showed that the prevalence of ZnT8A in acute-onset type 1 diabetic patients was significantly higher than in slowly progressive or fulminant type 1 diabetic patients ($P < 0.05$; Figure 2). Additionally, ZnT8A were also detected in 10 of the 191 (5.2%) patients with phenotypic type 2 diabetes, where the median level was 54.8 U/mL (range 11.3–607.5 U/mL). Considering the cut-off value suggested by the manufacturer (<15 U/mL), the prevalence of ZnT8A was 26.7% (64/240) for acute-onset type 1 diabetes, 0% (0/25) for fulminant type 1 diabetes, 13.6% (8/59) for slowly progressive type 1 diabetes and 4.7% (9/191) for phenotypic type 2 diabetes. Among these nine cases positive for ZnT8A between 10 and 15 U/mL, three were not associated with any other autoantibodies.

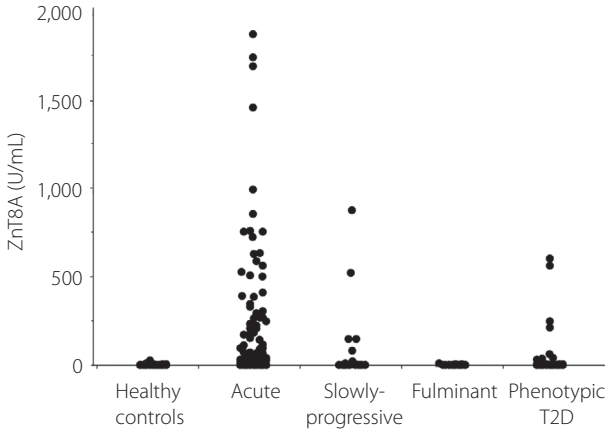

Figure 1 | Distribution of zinc transporter 8 autoantibody (ZnT8A) levels in participants in the present study. Acute-onset type 1 diabetes ($n = 240$): median 118.0 U/mL (range 10.7–1,871.5 U/mL), slowly progressive type 1 diabetes ($n = 59$): median 84.9 U/mL (range 13.7–876.9 U/mL), fulminant type 1 diabetes ($n = 25$): 11.7 and 14.0 U/mL, phenotypic type 2 diabetes ($n = 191$): median 54.8 U/mL (range 11.3–607.5 U/mL). T2D, type 2 diabetes.

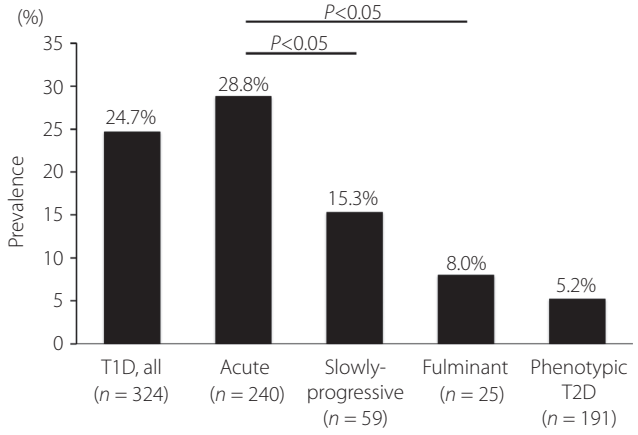

Figure 2 | Prevalence of zinc transporter 8 autoantibodies in participants in the present study. The prevalence of zinc transporter 8 autoantibodies in patients with acute-onset type 1 diabetes was significantly higher than in patients with slowly progressive or fulminant type 1 diabetes ($P < 0.05$). T1D, type 1 diabetes; T2D, type 2 diabetes.

Association between ZnT8A and duration of type 1 diabetes

The 324 patients in the present study with acute-onset and slowly progressive type 1 diabetes, whose duration of diabetes was available, were divided into three groups using the tertile of diabetes duration: T1 (0–3 years, $n = 114$), T2 (4–15 years, $n = 112$) and T3 (16–58 years, $n = 98$). Subsequently, we investigated the association between the prevalence of ZnT8A and duration of diabetes (Figure 3). As a result, we found the prevalence of ZnT8A decreased across the three tertiles according to diabetes duration (37.7, 28.6 and 15.3%; $P_{trend} < 0.001$; Figure 3a). Furthermore, as expected, we observed a significant negative correlation between ZnT8A level and disease duration in ZnT8A-positive patients ($r = -0.350$, $P < 0.001$; Figure 3b).

Combinatorial analysis of ZnT8A, GADA and IA-2A

Combinatorial analysis of GADA and IA-2A in addition to ZnT8A showed that 75.0% (180/240) of patients with acute-onset, 81.4% (48/59) of slowly progressive, 16.0% (4/25) of fulminant type 1 diabetes and 11.5% (22/191) of phenotypic type 2 diabetes were positive for at least one of these autoantibodies (Figure 4). Furthermore, among patients who showed negative for GADA and IA-2A, 9.1% (6/66) of patients with acute-onset, 8.3% (1/12) of slowly progressive, 4.5% (1/22) of fulminant and 5.1% (9/178) of phenotypic type 2 diabetes were positive for ZnT8A. Rather unexpectedly, in ZnT8A-single-positive patients, the mean ZnT8A level in the phenotypic type 2 diabetic patients (143.8 ± 194.9 U/mL) was significantly higher than any in the type 1 diabetes cohort (22.9 ± 8.3 U/mL; $P < 0.05$; Figure S1).

After this, we analyzed the relationship between age at sampling and the prevalence of GADA and/or ZnT8A in the 118 patients with acute-onset and slowly progressive type 1 diabetes within 5 years of diagnosis (Table 2). At this point, the prevalence of ZnT8A was found to be significantly higher in patients aged ≤10 years (52.9%) than in the other age groups ($P < 0.05$). Furthermore, an additional measurement of GADA increased diagnostic sensitivity in patients aged ≤10 years (11.7%, 4/34) in comparison with patients aged ≥11 years (3.6%, 3/84). In addition, we also evaluated the relationship between the presence of ZnT8A and clinical and immunological parameters through multivariate logistic regression analysis (Table 3). In doing so, we found the optimal cut-off point for age at sampling, based on the receiver operating characteristic curve, to be 11 years (sensitivity 46.3%, specificity 77.9%). There were no associations regarding the presence of ZnT8A with sex, duration or the presence of GADA. However, the ZnT8A positivity was associated with a younger age of onset (odds ratio 2.64, 95% confidence interval 1.00–6.97, $P = 0.049$) and the presence of IA-2A (odds ratio 2.97, 95% confidence interval 1.24–7.12, $P = 0.015$).

DISCUSSION

In the present study, we showed evidence of five key factors. The first being 25% of patients with type 1 diabetes and 5.2% of phenotypic type 2 diabetes had ZnT8A levels exceeding the cut-off level. Second, there was a higher prevalence of ZnT8A in both patients with acute-onset type 1 diabetes and those aged ≤10 years. Third, a significant inverse correlation between ZnT8A levels and disease duration was observed in ZnT8A-positive patients. Fourth, the levels of ZnT8A in patients with phenotypic type 2 diabetes were significantly higher than in those with type 1 diabetes. Fifth, ZnT8A positivity was independently associated with age at the time of sampling and IA-2A positivity.

As the bridging-type ELISA for ZnT8A used in the present study would be easy to implement and has proven to achieve a

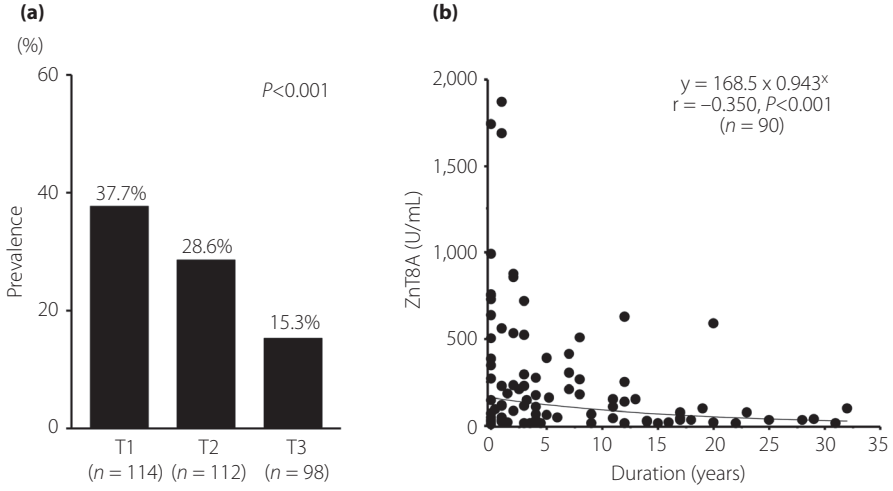

Figure 3 | Correlation of zinc transporter 8 autoantibody (ZnT8A) (a) positivity or (b) levels with duration of diabetes in patients with acute-onset and slowly progressive type 1 diabetes. T1; duration 0–3 years, T2; duration 4–15 years, T3; duration 16–58 years. The correlation between levels of ZnT8A and diabetes duration was analyzed in ZnT8A-positive participants ($n = 90$).

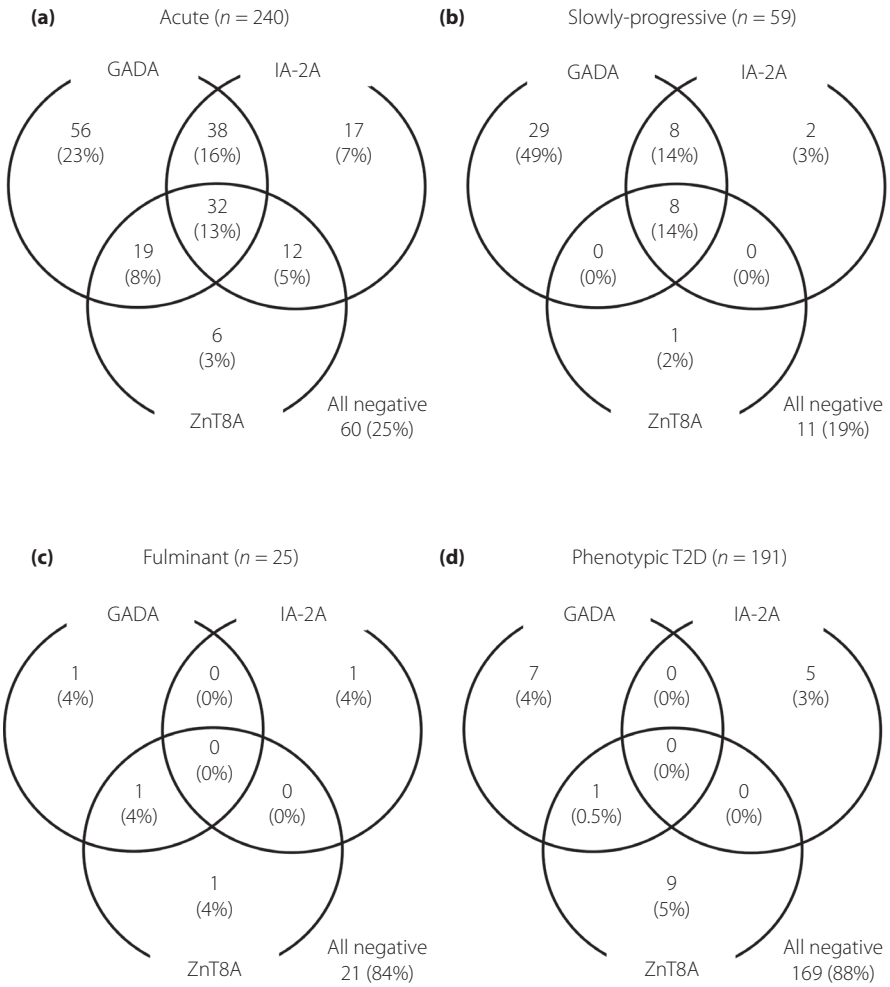

Figure 4 | Combined analysis of zinc transporter 8 autoantibodies (ZnT8A) with glutamic acid decarboxylase autoantibodies (GADA) and insulinoma-associated antigen-2 autoantibodies (IA-2A). (a) Acute-onset type 1 diabetes (n = 240), (b) slowly progressive type 1 diabetes (n = 59), (c) fulminant type 1 diabetes (n = 25) and (d) phenotypic type 2 diabetes (n = 191). T2D, type 2 diabetes.

Table 2 | Relationship between age and the prevalence of glutamic acid decarboxylase and zinc transporter 8 autoantibodies in patients with acute-onset and slowly progressive type 1 diabetes with duration ≤5 years

Age	*n*	GADA			ZnT8A			GADA and/or ZnT8A		
		n (%)	OR	95% CI	*n* (%)	OR	95% CI	*n* (%)	OR	95% CI
≤10 years	34	21 (61.8)	1	—	18 (52.9)	1	—	25 (73.5)	1	—
11–34 years	43	31 (72.1)	1.60	0.61–4.18	12 (27.9)	0.34*	0.13–0.89	33 (76.7)	1.19	0.42–3.36
>35 years	41	32 (78.0)	2.20	0.80–6.06	11 (26.8)	0.33*	0.12–0.86	33 (80.5)	1.49	0.50–4.39

*$P < 0.05$. CI, confidence interval; GADA, glutamic acid decarboxylase autoantibodies; OR, odds ratio; ZnT8A, zinc transporter 8 autoantibodies.

high degree of sensitivity and specificity in the recent proficiency evaluations by the Islet Autoantibody Standardization Program, this assay should be deemed a useful diagnostic method for determining type 1 diabetes. Although the provided cut-off value for this kit (ElisaRSR™ ZnT8 Ab) by the manufacturer is <15 U/mL[6], we have established a distinctive cut-off value of <10 U/mL for Japanese populations based on the 99th percentile and 3SD above the mean value obtained through the 288 healthy Japanese control participants. Although the exact reasons for the cut-off difference between Japanese

Table 3 | Logistic regression analysis for the association of clinical and immunological parameters with zinc transporter 8 autoantibody positivity among acute-onset and slowly progressive type 1 diabetes with duration ≤5 years

Variable	OR	95% CI	*P* value
Sex (female)	1.06	0.44–2.55	0.898
Duration (years)	0.85	0.65–1.11	0.221
Age at sampling (≤11 years)	2.64	1.00–6.97	0.049
GADA positive	2.25	0.75–6.76	0.149
IA-2A positive	2.97	1.24–7.12	0.015

CI, confidence interval; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; OR, odds ratio.

people and white people are unknown, one possibility might be attributed to the effect of ethnicity. Seeing as the incidence of type 1 diabetes in white people is much higher than in Japanese people, the frequency of incidental recruitment of anti-islet autoantibody-positive healthy individuals might also be higher in the white population. With this newly determined cut-off value, the prevalence of ZnT8A in the 324 patients with type 1 diabetes was 24.7%, which was lower than in previous reports[6,14,15]. As the higher prevalence of ZnT8A in acute-onset type 1 diabetic patients in comparison with the slowly progressive form observed in the present study was in accordance with previous reports, the possible reason for this discrepancy could be due to the differences in age, the duration of diabetes or the proportion of the subtype of type 1 diabetes[16]. Kawasaki *et al.*[16] previously reported that none of the patients with fulminant type 1 diabetes showed positive for ZnT8A using radioligand binding assay. Although we were able to detect two ZnT8A-positive patients with fulminant type 1 diabetes in our current study, the level of ZnT8A in these patients was 11.7 and 14.0 U/mL, which is under the cut-off value provided by the manufacturer, indicating that there is fundamentally no humoral autoreactivity to the ZnT8 molecule in fulminant type 1 diabetes. These results are consistent with previous studies showing that ZnT8A reflect an autoimmune-mediated destruction of β-cells, and that non-autoimmune mechanisms, such as innate immunity after viral infection of β-cells, are the major causes of fulminant type 1 diabetes.

The prevalence of ZnT8A in patients with type 1 diabetes decreases across the three tertiles according to diabetes duration, and there was a negative correlation between the levels of ZnT8A and the duration of diabetes, as was previously reported[17]. In addition, we found the rate of ZnT8A positivity to be prevalent in young patients with type 1 diabetes. In previous reports, ZnT8A positivity has been shown to have a more rapid decline than GADA, whereas the C-peptide responses after onset of type 1 diabetes decline at a rate paralleling the titers of ZnT8A[18,19], indicating a possible link between humoral autoreactivity to ZnT8 and a decline of β-cell mass. Although the association of ZnT8A with IA-2A has already been shown to be stronger than that with GADA in new-onset patients with type 1 diabetes[2,20], we found that this association remains strong in long-standing patients, as well. These findings could be related to the fact that both ZnT8 and IA-2 are transmembrane proteins located within the insulin secretory granules and the ability of autoantibodies to recognize the cytoplasmic domain of the molecule.

Herein, we have shown the interaction between age at sampling and ZnT8A positivity in type 1 diabetic patients with a duration of ≤5 years. Gomes *et al.* reported a negative association between levels of ZnT8A and age at sampling, but not with the age of onset for diabetes[17]. These results suggest that age at sampling rather than age of onset might receive priority in determining ZnT8A in long-standing patients with type 1 diabetes. In support of this, our multivariate analysis showed that both younger age and IA-2A positivity were independently associated with ZnT8A positivity. Despite these findings, the association between age and ZnT8A positivity remains controversial, as some studies have reported ZnT8A as being more frequent in older patients, whereas other studies based on white populations found no age-dependent difference[6,18,20]. However, in support of the present findings, a study involving Chinese patients with type 1 diabetes reported a similar prevalence of ZnT8A in younger patients[21], suggesting that its association might differ across ethnic groups.

In patients with phenotypic type 2 diabetes, the prevalence of ZnT8A was 5.2% (10/191), and 90% (9/10) of those were positive for ZnT8A alone. The median level of ZnT8A-positive type 2 diabetes was 54.8 U/mL (range 11.3–607.5 U/mL), and 80% (8/10) of the patients had ZnT8A levels exceeding 10SD of the normal control participants. Given these results, it is difficult to regard the elevation of ZnT8A in patients with phenotypic type 2 diabetes as a non-specific reaction. Although we believe these results to be robust, further studies are required to characterize ZnT8A-single-positive patients with phenotypic type 2 diabetes, and whether or not these patients have low-affinity autoantibodies, a similar phenomenon like GADA and insulin autoantibodies[22,23], show progressive decline of β-cell function similar to slowly progressive type 1 diabetes or have the distinct clinical features of GADA-single-positive patients.

In summary, the current study showed that in addition to GADA, the bridging-type ZnT8A ELISA is a valuable marker for Japanese patients with type 1 diabetes, and is likely to increase the number of cases identified while enabling clinical phenotypes to be differentiated in the Japanese population. Investigation into the clinical features and natural history of ZnT8A-single-positive patients initially diagnosed as type 2 diabetes should warrant accurate diagnosis and suspicion of immune-mediated type 1 diabetes in the future.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Distribution of zinc transporter 8 autoantibodies in patients positive for zinc transporter 8 autoantibodies alone.
Table S1 | Comparing the curve fitting data of the different models.
Table S2 | Zinc transporter 8 autoantibody levels in healthy controls.