Original Article

Zinc transporter-8 autoantibodies can replace IA-2 autoantibodies as a serological marker for juvenile onset type 1 diabetes in India

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ABSTRACT

Introduction: Zinc transporter-8 (ZnT8) is an islet cell secretory granule membrane protein recently identified as an autoantigen in type 1 diabetes (T1D). The aim of this study was to estimate the prevalence of antibodies to ZnT8 (ZnT8A) in juvenile onset T1D and to determine the utility of ZnT8A as an independent marker of autoimmunity either alone in antibody-negative subjects or in conjunction with glutamic acid decarboxylase (GAD) and insulinoma-2 antigen antibodies (GADA and IA2A). **Research Design:** ZnT8A, GADA, and IA2A were measured in sera of consecutive T1D patients (*n* = 88, age range 2-18 years) within 4 years of diagnosis and 88 sex-matched controls. **Results:** The prevalences of GADA, ZnT8a, and IA2A were 64.7%, 31.8% and 19.3%, respectively. In newly diagnosed patients, the frequency of ZnT8A was 45%. ZnT8A were positive in 26% of patients negative for both GADA and IA2A. IA2A were positive only in two patients who were negative for other two antibodies. Combined use of ZnT8A and GADA could detect 97% of antibody positive patients. In receiver operating characteristic (ROC) analysis, the performances of GADA and ZnT8As were better than that of IA2A; and AUCs of GADA, ZnT8A, and IA2A for the prediction of T1D were 0.8, 0.65, and 0.59, respectively. **Conclusions:** ZnT8A complements GADA and increases the diagnostic sensitivity for detection of autoimmunity in juvenile-onset T1D. Inclusion of ZnT8A increases the proportion of patients with antibody positivity to nearly 80%. ZnT8A can replace IA2A as a serological marker for autoimmunity in Indian T1D patients without loss of sensitivity and specificity.

Key words: Autoantigen, autoimmunity, India, juvenile, type 1 diabetes, zinc transporter

Introduction

Zinc transporter-8 (ZnT8) is an islet cell secretory granule membrane protein recently identified as an autoantigen in type 1 diabetes (T1D).[1] Autoantibodies to ZnT8 (ZnT8A) complement the established antibodies to insulin (IAA), GAD (glutamic acid decarboxylase antibodies, GADA), and protein tyrosine phosphatase (insulinoma-2 antigen antibodies, IA2A). Antibodies to ZnT8 have been detected in 60-80% of Caucasian and 33-58% of Asian population with T1D.[2-4] The combined determination of established diabetes autoantibodies together with the ZnT8A increases the diagnostic sensitivity to over 90% for new onset cases of T1D in Caucasians. A high frequency of patients (45%) with presumed diagnosis of T1D in India lack GADA and IA2A autoantibodies, so establishing the autoimmune basis becomes difficult.[5] A low frequency of IA2A in T1D has been reported from different studies from India.[6,7] Inclusion of ZnT8A presumably reduces the proportion of patients with negative autoantibodies. As no data on ZnT8A in Indian population are available, the aim of our study was to estimate the prevalence of ZnT8A in T1D patients of Indian origin and determine the utility of ZnT8A as an independent marker of autoimmunity either alone in antibody-negative subjects or in conjunction with GADA and IA2A.

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Materials and Methods

Eighty-eight patients who were diagnosed with diabetes before 18 years of age according to American Diabetes Association (ADA) criteria[8] and classified as type 1 diabetic patient on clinical grounds were recruited to the study. Type 1 diabetes (T1D) was defined on the clinical grounds by ketosis at presentation, lean phenotype, requirement for insulin, and/or low C-peptide. The duration of T1D after diagnosis was less than 48 months. Twenty-one of the patients were newly diagnosed cases, and the blood sample was obtained from them within a week after diagnosis. Patients were consecutively recruited from two centers in Bangalore, Bangalore Diabetes Hospital and M S Ramaiah Medical College between 2010 and 2012. Sex-matched healthy nondiabetic controls aged 2-18 years ($n = 88$) were also recruited. Children with disease duration > 48 months, evidence of secondary diabetes, and those with hormonal abnormalities were excluded. Abdominal X-ray and/or ultrasound were done to rule out pancreatic calcification. Informed consent was obtained from participants and/or parents. Ethical committee approval of the participating hospitals was obtained. ZnT8A, GADA, and IA2As were estimated in both patients and controls. The sera were stored at - 70°C till analysis.

Estimation of autoantibodies

ZnT8, GAD, and IA2 antibodies were estimated using commercial enzyme-linked immunosorbent assay (ELISA) (DLD Diagnotika, GMBH, Germany). ZnT8 autoantibody ELISA kit detects autoantibodies specific to arginine (R) or to tryptophan (W) at residue 325, or to nonspecific variants at residue 325. In ZnT8 antibody ELISA, ZnT8A in test patients' sera, calibrators, and controls were allowed to interact with ZnT8 coated onto ELISA plate wells. After 16 − 20 h incubation, the samples were discarded leaving ZnT8 autoantibodies bound to ZnT8 coated wells. ZnT8 biotin was added in the second incubation step where, through the ability of ZnT8 autoantibodies in the samples to act bivalently (or polyvalently), a bridge would be formed between ZnT8 immobilized on the plate and ZnT8 biotin. Unbound ZnT8 biotin was then removed in a wash step, and the amount of bound ZnT8 biotin determined (in the third incubation step) by addition of streptavidin peroxidase (SA-POD), which bound specifically to biotin. Excess, unbound SA-POD was then washed away and addition of 3,3',5,5'-tetramethylbenzidine (TMB) resulted in formation of a blue color. This reaction was stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 405 and 450 nm was then read using an ELISA plate reader. A higher absorbance indicated the presence of ZnT8 autoantibody in the test sample. Low values (less than 50 U/ml) were read off with the 450 nm calibration curve and high values (more than 50 U/ml) were read off with 405 nm calibration curve. The measuring range was 10-2,000 U/ml. A cut-off value of ≥ 15 U/ml was considered as positive. Cut-off values for positive results for GAD and IA2 antibodies were 20 and 7.5 U/ml, respectively based on our laboratory reference norms.

Statistical analysis

Results were expressed as mean ± SD unless otherwise indicated. Autoantibody frequencies and anthropometric and biochemical parameters between patients and controls were compared using the χ^2 test, Fisher's exact test, analysis of variance (ANOVA) test, and Student's *t*-test (two-tailed, independent) where appropriate. Diagnostic performance of three antibody tests was analyzed using receiver operating characteristic (ROC) analysis and pairwise comparison of areas under curves of ROC plots of the autoantibodies were done by a dichotomous, binary classification of the test results as positive or negative. *P* value less than 0.05 were considered statistically significant.

Results

Comparison of clinical characteristics of patients and controls is presented in Table 1. ZnT8A were detected in 31.8% of patients. In newly diagnosed patients, the prevalence of ZnT8A was 45%. The frequencies of GADA and IA2A were 64.7 and 19.3%, respectively. Sixty-eight (77.3%) patients were positive for at least one antibody and five of them were positive for all three antibodies [Figure 1]. ZnT8A were positive in 33.3% of patients positive for GADA and 40% of patients positive for IA2A. ZnT8A were positive in 26% of patients negative for both GADA and IA2A. GADA were positive in 60% of patients negative for other antibodies, whereas IA2 was positive in only two patients negative for other two antibodies.

Table 1: Clinical characteristics of patients and controls

	Patients (*n*=88)	Controls (*n*=88)	*P* value*
Age (years)	11.04 (4.2)	10.1 (2.1)	0.96
Wt SDS	−0.77 (1.1)	0.15 (1.02)	<0.001
Ht SDS	−0.42 (0.87)	0.11 (1.09)	<0.001
Duration of diabetes (months)	11.5 (14.14)	-	
HbA1c (%)	8.6 (1.27)	-	
ZnT8A	28/88 (31.8%)	2/88 (2.3%)	<0.0001
GADA	57/88 (64.7%)	3/88 (3.4%)	<0.0001
IA2A	17/88 (19.3%)	0/88 (0%)	<0.0001

Wt SDS: Weight standard deviation score, Ht SDS: Height standard deviation score, HbA1c: Hemoglobin A1c, ZnT8A: Zinc transporter-8 antibodies, GADA: Glutamic acid decarboxylase antibodies, IA2A: Insulinoma-2 antigen antibodies. **P* value<0.05 significant

ZnT8A and GADA identified 97.7% of antibody positive patients detected by the combined use of all three antibodies. ZnT8A increased the proportion of patients positive for at least one of the antibodies from 64.5 to 75% when compared to GAD alone, from 69 to 77.3% when compared to GADA and IA2A combined. Exclusion of IA2A from the antibody panel reduced antibody positivity by 2.3% only. Five out of 88 controls were positive for antibodies. None of them were positive for more than one antibody and antibody titers were much lower compared to patients.

Diagnostic characteristics of three antibody assays in objectifying autoimmunity in type 1 diabetes are presented in Table 2. In ROC analysis, the performances of GADA and ZnT8As were better than that of IA2A, and AUCs of GADA, ZnT8A, and IA2A for the prediction of T1D were 0.8, 0.65 and 0.59 respectively [Table 2 and Figure 2]. In pair wise comparison of AUCs of ROC curves, AUC of GADA was significantly greater than that of ZnTA and IA2A. AUC of ZnT8A was greater than that of IA2A but didn't reach statistical significance [Table 3].

Discussion

The present study shows that ZnT8 antibody is a specific marker of juvenile onset T1D in line with previous studies. ZnT8A were positive in nearly one-third of the patients, and their prevalence was much higher than that of IA2A. In newly diagnosed cases, the prevalence of ZnT8As was 45%. ZnTA were found in one-quarter of T1D subjects classified as autoantibody-negative based on GADA and IA2A.

The frequency of ZnTA in our subjects was much lower compared to that reported among Caucasians. In a study by Wenzlau *et al.*, ZnT8A were detected in 63% of T1D patients at the diagnosis and 2% of healthy controls, and they were positive in 26% of T1D patients who were negative for other islet autoantibodies.[9] In another study involving Swedish children and adolescents with T1D, ZnTA were detected in 65% of subjects.[10] In a Japanese study, ZnT8A frequency was 58% in childhood onset patients and 34% in adult onset T1D patients.[3] The same authors reported ZnT8A prevalences of 58% patients with acute onset and 20% with slow onset type 1 diabetes.[11] In newly diagnosed cases, the prevalence of ZnT8A in our subjects was higher than that reported from China (32.9%).[4] Genetic factors, specifically

Table 2: Diagnostic performance of the antibody tests in receiver operating characteristic analysis

	Sensitivity	Specificity	AUC	SE	95% CI
GADA	64.77	96.6	0.807	0.034	0.74-0.86
IA2A	19.32	100.00	0.597	0.043	0.52-0.67
ZnT8A	31.82	97.73	0.648	0.041	0.57-0.71

AUC: Area under curve, SE: Standard error, CI: Confidence interval, ZnT8A: Zinc transporter-8 antibodies, GADA: Glutamic acid decarboxylase antibodies, IA2A: Insulinoma-2 antigen antibodies

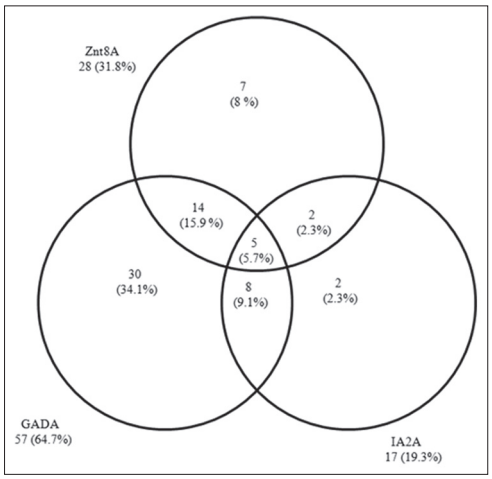

Figure 1: Venn diagram of frequencies and codetection of autoantibody combinations in in antibody positive patients with T1D

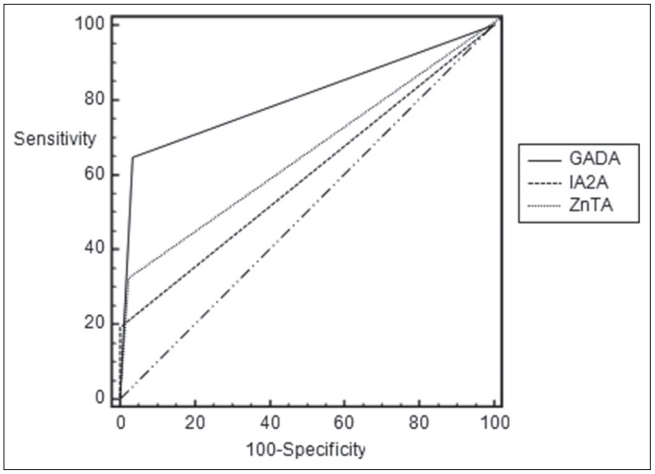

Figure 2: ROC curve analysis of three autoantibody tests Area under curve (AUC) of GADA-0.647, AUC of ZnT8A-0.32, AUC of IA2A-0.19

Table 3: Pairwise comparison of receiver operating characteristic curves of three antibody tests

	Difference between areas	SE	95% CI	Z-statistic	Significance level (*P* value)
GADA~IA2A	0.210	0.054	0.105-0.32	3.92	0.0001
GADA~ZnT8A	0.159	0.053	0.055-0.26	2.99	0.0028
ZnT8A~IA2A	0.051	0.058	−0.064-0.16	0.87	0.384

SE: Standard error, CI: Confidence interval, ZnT8A: Zinc transporter-8 antibodies, GADA: Glutamic acid decarboxylase antibodies, IA2A: Insulinoma-2 antigen antibodies. *P* value<0.05 significant

histocompatibility leukocyte antigen (HLA) haplotypic diversification and SLC30A8 gene polymorphism could be responsible for the differences in frequencies noted among different ethnic populations as shown in previous studies.[10,12-16]

A high frequency of type 1B diabetes (45%) has been reported among children and adolescents with recent onset T1D from north India based on negativity for GADA and IA2A.[5] The most significant finding from our study was that the inclusion of ZnT8A increased the antibody detection rate to nearly 80%. This could aid in better classification and risk stratification of early onset diabetes and help differentiating from T1D mimicking forms of diabetes seen in India. In the present study, diagnostic performances of the three assays were compared using ROC analysis. The results from this analysis suggest that ZnT8A is a better diagnostic tool than IA2A for diagnosis of T1D with higher sensitivity, only a slightly lower specificity, but a higher diagnostic accuracy. The combined presence of both the antibodies in serum compared with ZnT8A alone increases the specificity, but contributes little to sensitivity and overall accuracy.

Higher prevalence of type 1B diabetes in India is predominantly due to lower positivity of IA2A antibodies as has been reported from different studies.[6,7,17,18] Our study findings support these earlier reports. Most of the IA2A positive patients in our study were positive for either GADA or ZnT8A and combined use of GADA and ZnT8A could detect > 97% of antibody positive patients detected with the integrated use of all three antibodies, implying that the utility of IA2A was limited and to a large extent redundant. Whereas, inclusion of ZnT8A reduced the proportion of patients with negative autoantibodies, IA2A did not contribute any further in this regard. These findings suggest that ZnT8A have a potential to replace IA2A for screening autoimmunity in Indian T1D patients without loss of sensitivity and specificity. Antibody screening could be done in a cost-effective manner and testing for IA2A reserved for only those patients who are GADA and ZnT8A negative.

Our study has a few limitations. We recruited newly diagnosed T1D patients and those with a shorter duration of diabetes (<4 years) only. The reason for doing this was because ZnT8A titers have been shown to decline exponentially from the clinical onset of T1D with a t1/2 similar to that of C-peptide.[9] We excluded adults with T1D because the frequency of ZnT8A has been shown to be much lower when compared to children and adolescents with T1D.[19,20] We did not examine the relation of ZnT8A to glycemic control and progression of T1D and the prevalence of ZnT8A in adult onset T1D. Further studies are warranted on the kinetics of post onset decline of ZnT8A and their prevalences in patients with different disease durations. Studies examining how HLA haplotype variation and SLC30A8 gene polymorphism modulate humoral autoreactivity to ZnT8 are also required.

In summary, this is the first study on prevalence of ZnT8 antibodies in juvenile onset T1D from India. Frequency of ZnT8A was higher in newly diagnosed cases. ZnT8A complements GADA and increases the diagnostic sensitivity for detection of autoimmunity in juvenile onset T1D. Inclusion of ZnT8A increases the proportion of patients with antibody positivity to nearly 80%. ZnT8A can replace IA2A as a serological marker for autoimmunity in Indian T1D patients without loss of sensitivity and specificity.

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