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The Diagnostic Value of Zinc Transporter 8 Autoantibody (ZnT8A) for Type 1 Diabetes in Chinese

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Abstract

Background—Zinc transporter-8 (ZnT8) was recently identified as a novel autoantigen in human type 1 diabetes (T1D). Autoantibody to ZnT8 (ZnT8A) were detected in up to 80% of new-onset T1D and 26% of T1D patients otherwise classified as negative on the basis of existing markers. Since no data of ZnT8A in Chinese has been reported, we aim to evaluate the utility of ZnT8A for diagnosis of autoimmune T1D in Chinese relative to other autoantibody markers.

Methods—Radioligand binding assays were performed on 539 T1D sera using human ZnT8 carboxyterminal 325Arg construct or a dimer incorporating 325Arg and 325Trp alongside antibodies to glutamic acid decarboxylase (GADA) or insulinoma-associated protein 2 (IA-2A). The antigenic specificity was analyzed in the context of clinical characteristics of the patients.

Results—ZnT8A were present in 24.1% (130/539) of T1D patients versus 1.8% (10/555) (P<0.001) in type 2 diabetes. At diagnosis, ZnT8A and IA-2A were less prevalent in Chinese T1D subjects than in Caucasian populations (both P<0.001), but similar to Japanese. The diagnostic sensitivity of combined GADA, IA-2A and ZnT8A measurements reached 65.5% with ZnT8A detected in 13.5% (29/215) of GADA and/or IA-2A negative subjects. ZnT8A prevalence was lower in older and fatter patients. ZnT8A+ alone patients were distinguished from Ab- ones (P<0.05~0.001) on the basis of higher insulin requirement and lower systolic blood pressure level.

Conclusion—ZnT8A is an independent marker for T1D in Chinese and combined with GADA and IA-2A enhances diagnostic sensitivity. ZnT8A may be associated with different clinical phenotypes than GADA or IA-2A.

Keywords

ZnT8 autoantibody; GAD autoantibody; IA-2 autoantibody; type 1 diabetes; diagnosis

Introduction

Zinc transporter (ZnT8), the product of the SLC30A8 gene was recently identified as an islet specific gene product localized to the beta cell insulin granule [1]. Its overexpression in

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cultured islet β cells leads to increased insulin secretion [2] and ablation to an insulin sensitization and possibly glucose intolerance [3]. ZnT8 autoantibodies (ZnT8A) are present in 60-80% of new-onset Caucasian type 1 diabetic patients and are predictive of disease [4]. A lower prevalence of ZnT8A (28%) in Japanese type 1 diabetes (T1D) was recently reported by Kawasaki et al [5] though this included patients of variable disease duration.

To date the prevalence of ZnT8A in Chinese T1D patients has not been reported and we thus aimed to perform a comparative analysis of ZnT8A, GADA and IA-2 in different ethnic groups and assess the clinical features of ZnT8A positive T1D patients. Spontaneous IAA has a low prevalence in Chinese patients with T1D [6] and was excluded in these studies since it's difficult to differentiate IAA from insulin antibody (IA) which is induced by insulin therapy.

Materials and methods

Subjects

Five hundred and thirty-nine patients with T1D were enrolled from Oct 1999 to Aug 2009. The average age at diagnosis was 24.0 (range 1-70) years and median duration of diabetes 2 (range 0-348) months with 293 males and 246 females. Five hundred and fifty-five patients with type 2 diabetes (T2D) included 318 males and 237 females with an average age of 49.4 (range 16-80) years and median duration of diabetes of 1.0 (range 0-36) month. They were all negative for GADA and IA-2A. Patients with T1D and T2D were diagnosed according to the revised criteria of the American Diabetes Association for T1D [7]. Healthy volunteers (n=409) who presented normal response in 75-g oral glucose tolerance test were included as controls. They included 141 males and 264 females, with a median age of 37.8 (range 1.0-70.0) years and had no family history of diabetes or overt autoimmune diseases and any chronic diseases. Informed consent was obtained from all study subjects, the protocol was approved by the local Ethics Committee and was carried out in accordance with the Declaration of Helsinki as revised in 2000.

ZnT8A (CR and CW-CR autoantibodies) assay

ZnT8A was measured by radioligand assay based on ^{35}S-methionine-labeled human recombinant ZnT8 as described previously [4]. The ZnT8 complementary DNA (cDNA) constructs used in this study were the cytoplasmic carboxy-terminal domains (aa268-369) of human ZnT8 carrying 325Arg (designated as CR) or a hybrid construct of the CR and 325Trp (CW) (designated as CW-CR) with a CLFCEDPCDPSTPPGSSGGGKDFSILLME hinge junction generated by PCR. These cDNA were cloned into a pCDNA3.1 directional TOPO vector (Invitrogen, Carlsbad, CA, USA). Plasmid DNA (2μg) was incubated in 100μl of an invitro-coupled transcription/translation reticulocyte lysate reaction (TNT Quick T7 promoter; Promega, San Luis Osbispo, CA) with 20μCi (1Ci=37GBq) of [^{35}S] methionine (1,000 Ci/mmol; Amersham Bioscience, Madison, WI, USA) and the products were gel-filtered on G25 Sephadex (NAP 5 column; GE Healthcare, UK). Radioactivity incorporated into protein was determined by precipitation with 5% (w/v) trichloroacetic acid after alkaline hydrolysis of any [^{35}S] Met tRNA left in the sample with 1M NaOH (percent incorporation: CR 9.8±1.8; CW-CR 10.2±2.1). Five microliters serum was incubated over night in duplicate with 30,000 cpm of the above translation product in 200μl of Tris-buffered saline with Tween (TBST buffer, 50mM Tris-HCl, 150mM NaCl, 10% v/v fetal bovine serum, 0.15% v/v Tween-20, pH7.2) in 1.5ml Eppendorf tubes and mixed end-over-end for 16h at 4°C, prior to the addition of 30μl of a 50% (v/v) protein A agarose suspension (Invitrogen, CA, USA) After 1hr at 4°C, the immune complexes were isolated by centrifugation, washed for four times with 750μl TBST and finally mixed with 1ml scintillation fluid (Perkin EImer, CT, USA) and radioactivity determined (Micro B Trilux

1450 counter; PerkinElmer, Finland). Values are expressed as: ZnT8A index= (unknown sample cpm- negative standard serum cpm)/ (positive standard serum cpm- negative standard serum cpm). The intra-assay variations of CR autoantibody (CR-A) and CW-CR autoantibody (CW-CR-A) assays were 4.3%-11.0% and 3.9%-9.8% (n=5), and the interassay variations were 5.7%-15.5% and 4.3%-13.8% (n=5) respectively. Based on the 99th percentile of sera from 409 healthy controls, the cutoff value of ZnT8A positivity in our lab was 0.014 for CR-A and 0.011 for CW-CR-A. The sensitivity and specificity of CW-CR ZnT8A assay in our lab in the Diabetes Autoantibody Standardization Program (DASP2009) were 66% and 100% respectively.

GADA and IA-2A assays

GADA and IA-2A were determined as previously described [8]. The cutoff values of positivity for GADA and IA-2A were 18.5U/ml and 3.3U/ml which were determined according to 99th percentile of 188 and 171 healthy controls respectively. The sensitivity and specificity of the GADA assay in our lab were 72% and 98%, and for IA-2A were 66% and 99%, evaluated according to the Fourth Diabetes Autoantibody Standardization Program (DASP2009).

C-Peptide and HbA1c assays

Serum C-peptide (CP) was assessed by chemiluminescence (Bayer AG, Leverkusen, Germany). Intra- and inter- assay coefficients of variation (CVs) were 3.7 and 9.1% (n=3) respectively. The normal range for fasting CP (FCP) was 300-1200pmol/L and the detection limit is 79pmol/L. HbA1c was measured by automated liquid chromatography (Bio-Rad VARIANT II Hemoglobin Testing System, Hercules, CA, USA) with the normal range 4.1-6.1%.

Statistical analysis

Results are shown as mean±SD or as median (interquartile range), or otherwise documented as positive cases, constituent ratio or ratio. The unpaired *t* tests, analysis of variance (ANOVA) for multiple comparisons were used for normally distributed data and nonparametric tests were used for non-normally distributed data. The difference between classified variables was tested using Chi-squared test or Fishers exact test if the expected number of subjects in any cell was less than 5. *P* values less than 0.05 were considered significant.

Results

The prevalence of ZnT8A

The prevalence of CW-CR-A in patients with T1D was 24.1% (130/539) and significantly higher than in patients with T2D [vs 1.8% (10/555), $P<0.001$] and healthy controls [vs 1.0% (4/405), $P<0.001$] respectively. ZnT8A detected with CR-A in type 1 diabetic individuals was lower at 8.6% (37/429), yet concordant with CW-CR-A in 95% of cases and thus not further analyzed. The T1D cohort was divided into A+ and A- subgroups according to GADA and/or IA-2A status showed that ZnT8A positivity was significantly higher in A+ than in A- group (31.2% vs 13.5%, $X^2=11.318$, $P<0.001$).

Association of ZnT8A with GADA and IA-2A

ZnT8A obviously overlap with GADA and IA-2A (Fig.1a). Individually, GADA, IA-2A and ZnT8A were detected in 53.4%, 25.8% and 24.1% of patients. The highest overlapping prevalence produced from GADA and/or IA2-A (19.1%), followed by GADA and/or ZnT8A (16.0%), then IA-2A and/or ZnT8A (12.3%). ZnT8A measurements, if substituted

for IA-2A, detected a similar number of diabetic patients (61.7%). When 3 antibodies combined, the sensitivity reached 65.5% and the inclusion of the ZnT8 assays reduced the number of diabetic autoantibody-negative individuals from 39.9% to 34.5%. Linear regression analysis (Fig.1b) showed a weak correlation between ZnT8 CW-CR titer and IA-2A titer (non-parametric Spearman correlation coefficient R=0.222, P=0.000, n=539) but not GADA (R=0.071, P=0.115, n=539).

ZnT8A frequency in Chinese in comparison with Caucasian and Japanese

To compare the prevalence of ZnT8A in T1D patients with the data reported by Wenzlau and Kawasaki in Caucasian and Japanese respectively, patients were matched in duration of disease and age at onset (Table 1). The prevalence of ZnT8A in Chinese patients with T1D was comparable to that of Japanese (P>0.05), but was much lower than that of a Caucasian cohort (P<0.001). ZnT8A positivity in 146 new-onset younger patients was higher (32.9% vs 24.1%, P<0.05) than the original diabetic cohort that included subjects with varying disease duration. This was still lower than reported in Caucasians at onset [2] which was also the case for GADA and IA-2A (P<0.001).

Distribution of ZnT8A in type 1 diabetic patients stratified with ages of diagnosis, durations of disease, BMI and FCP

Analysis of the data stratified with age at diagnosis, showed that the prevalence of ZnT8A peaked at onset around 9yr then declined with a significant difference detectable between the 10-19yr subgroup as compared to a >40yr subgroup (28.2% vs 16.5%, P<0.05) (Fig.2a). ZnT8A was more prevalent in leaner (25.7%) than more obese (BMI>25.0) subjects (9.3%) [P<0.05] (Fig.2b). No differences in ZnT8A prevalence were observed in subjects stratified with sex, duration and FCP.

Clinical and biochemical characteristics of ZnT8A+, GADA+ and IA-2A+ alone and all Ab- patients

Table 2 indicated that patients with ZnT8A+ alone had a longer median duration of disease, higher insulin requirement and lower systolic blood pressure level when comparing to 3 Abs negative group (6.0 vs 1.0mon; 35.6 vs 27.6U/d and 103.6 vs 110.6mmHg, all P<0.05). Whereas, they showed better controlled glycemia, higher FCP level and were not so lean as compared to individuals with GADA+ and IA-2A+ alone (P<0.05~0.001). Furthermore, significantly impaired β-cell function, lower triglyceride levels were also demonstrated in GADA+ or IA-2A+ alone group as contrast to Ab- group (P<0.05~0.001).

Discussion

Recent description of ZnT8A in combination with GADA, IA-2A and IAA could increase the sensitivity of autoimmunity detection to 98% making it a promising new marker for T1D [4]. The prevalence of ZnT8A in this T1D cohort was 24.1%, which is markedly higher than that observed in T2D; indicating ZnT8A is a specific marker for T1D. The prevalence of ZnT8A was more than two-fold higher in individuals positive for GADA or IA-2A than otherwise autoantibody negative subjects (31.2% vs. 13.5%) possibly reflecting the reported higher association of antibody number than antibody titer with disease [9]. In this context the value of ZnT8A measurement is to detect patients that would otherwise be considered antibody negative (13.5% subjects) and perhaps more importantly individuals (28.4%) with 2 rather than one autoantibody. In either case the data clearly indicate that ZnT8A is an independent marker for T1D in Chinese populations.

The finding of positive correlation between ZnT8A and IA-2A (R=0.165, P=0.001) was consistent with previous studies [4,10], and might be related to ZnT8 and IA-2 being both β-

cell secretory granule membrane proteins and released as particulate matter when β cells were damaged [1,11]. Since ZnT8A showed similar positivity with IA-2A, and both were lower than GADA, it might be assumed that ZnT8A and IA-2A are less useful in the diagnosis of autoimmune mediated diabetes than GADA in Chinese subjects. Nonetheless, the combined use of the three markers increases the detection of autoimmunity to 65.5%, indicating that ZnT8A, IA-2A and GADA are independent markers and provide complementary indices of disease.

In agreement with our previous observations, newly diagnosed Chinese T1D children presented with similar prevalences of GADA and IA-2A with Japanese subjects [6,12]. The current data suggests that ZnT8A follow a similar pattern. However, when compared to the study of Caucasian population, ZnT8A and IA-2A prevalences were both much lower in Chinese T1D subjects whether they were matched for age or disease duration or not. It will be of interest in future studies to explore the relationship of this phenomenon to HLA genotypes or other inter-ethnic genetic markers.

Prospective studies in Caucasian populations suggest that autoimmune damage of islet β cell in patients with T1D typically precedes disease onset by years or decades. The observed peak in ZnT8A in 0-9yr in the current study indicates a similar pattern of disease emergence. It was accordance with the finding that ZnT8A usually preceded disease by many years and frequently appeared by 3 yr of age and peaked at 8-14yr and tended to decline thereafter [4,13]. We similarly observed in our Chinese cohort ZnT8A prevalence declined with increasing of age of onset. The observed negative association of ZnT8A with BMI may in part relate to this however larger numbers of cases are required to clarify this issue.

The comparison between patients with a single autoimmune marker (ZnT8A, GADA or IA-2A alone) to an autoantibody negative group may indicate some phenotypic variation in T1D related to target autoantigens. When compared to Ab− group, ZnT8A+ patients exhibited more features of insulin insufficiency including higher daily insulin requirements and lower blood pressure. It implied patients with ZnT8A alone involved in a slightly greater autoimmune response and is associated more to insulin deficiency in contrast to T1D patients without immune markers. They exhibited fewer signs of insulin deficiency than individuals with GADA or IA-2A alone. These results were in accordance with previous studies in which T1D patients were divided into A+ and A− groups according to GADA and IA-2A [14-15]. It would seem that ZnT8A may also permit stratification of clinical phenotypes of patients. As a recent study reported, combining measurement of ZnT8A, GADA and IA-2A may identify a subset with greater insulin insufficiency in adult-onset diabetic patients [16-17]. Lastly, it seemed that ZnT8A presented a little late relative to GADA and IA-2A in some patients as reflected in the median durations of patients with ZnT8A, GADA and IA-2A (6.0, 3.0 and 3.0 months respectively), a finding that supports the longitudinal study of prediabetics by Wenzlau et al [4]. However, as for the small number of ZnT8A+ alone subjects in this study, which needs further larger group size to clarify it.

In summary, ZnT8A is a distinct marker of β-cell autoimmunity; it can detect about 13.5% cases of autoimmunity from otherwise Ab- Chinese T1D patients and can improve the diagnostic sensitivity of autoimmunity when combined with GADA and IA-2A. Its measurement is important in providing evidence of autoimmunity in patients with T1D and possibly in predicting disease although prospective studies are required to confirm this.

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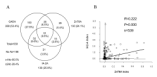

Figure1.
Association of ZnT8A with GADA and IA-2A in 539 T1D patients. (A) Venn diagram of autoantibody combinations in autoantibody positive patients with T1D; (B) Correlation between ZnT8A and IA-2A index (non-parametric Spearman correlation coefficient R=0.222, P=0.000).

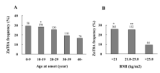

Figure 2.
Prevalence of ZnT8A in T1D patients relation to age at diagnosis (A) and BMI (B); Data are expressed as n (%); (A)When compared with age at diagnosis >40yr, **P*<0.05; (B) When compared with BMI>25.0kg/m^2, **P*<0.05, ***P*<0.01.

Table1

Comparison of ZnT8A prevalence among Caucasian, Chinese and Japanese patients with T1D in different studies.

Ethnicity	Reference	Subjects (age at onset, duration)	ZnT8A positivity (%)	GADA positivity (%)	IA-2A positivity (%)
U.S.A	Wenzlau [4]	11.3 (0.6-58.0) <2 weeks	61.5 (259/421)	68.2 (152/223)	72.2 (161/223)
China	Yang	15.0 (1.0-58.0) <2 months	32.9*,# (48/146)	52.1* (76/146)	38.4*,# (56/146)
Japan	Kawasaki [5]	25.0 (1.0-77.0) no limit	27.8 (75/270)	-	-
China	Yang	24.0 (1.0-70.0) no limit	24.1[a] (130/539)	53.4[a] (288/539)	25.8[a] (139/539)

Data are expressed as median (range) or n (%)

*When compared with U.S.A, $P<0.001$

#When compared with our study without match, $P<0.05$.

Table. 2

Clinical characteristics of T1D patients with single autoantibodies

	ZnT8A+ alone (n=29)	GADA+ alone (n=150)	IA2-A+ alone (n=21)	Ab− (n=186)
Sex (m/f)	15/14	85/65	11/10	103/83
Age at diagnosis (yr)	25.2±11.9	26.7±13.3	16.5±15.9**	26.9±12.7
Duration (month)	6.0** (0.8-27.0)	3.0* (0.5-12.5)	3.0 (0.4-32.0)	1.0 (0.3-12.0)
BMI (kg/m^2)	20.7±3.3	19.3±3.3***#	17.4±4.1***##	20.9±4.2
WHR	0.85±0.07	0.83±0.08***#	0.87±0.06	0.87±0.07
HbA1c (%)	9.4±3.7	11.8±3.6**##	10.6±2.4	10.5±4.0
Insulin dose (U/day)	35.6±10.6**	27.9±12.6	28.0±18.5	27.6±14.1
FCP (pmol/L)	90.4 (14.6-213.2)	72.4*** (24.2-143.0)	79.0** (20.0-131.4)	110.1 (68.8-204.2)
PCP (pmol/L)	181.1 (15.3-603.3)	125.1* (49.8-237.8)	114.0** (59.0-219.8)	205.3 (79.0-383.8)
SBP (mmHg)	103.6±14.0*	108.6±18.1*	105.9±18.9	110.2±16.2
DBP (mmHg)	67.4±11.0	71.1±11.4	68.6±9.3	71.1±11.4
Triacylglycerol (mmol/L)	1.1 (0.6-1.7)	1.0*** (0.7-1.7)	1.0 (0.8-1.2)	1.5 (0.9-2.2)
Total cholesterol (mmol/L)	4.3±1.4	4.4±1.5	4.9±1.4	4.9±1.5
HDL cholesterol (mmol/L)	1.1±0.3	1.2±0.5	1.4±0.4	1.1±0.3

Data are expressed as mean±SD, median (interquartile range) or n (%)

* When compared with Ab−, $P<0.05$

** $P<0.01$

*** $P<0.001$

When compared with ZnT8A+ alone, $P<0.05$.