Prevalence of Zinc Transporter 8 Auto Antibodies among Newly Diagnosed Type 1 Diabetic Cases Admitted to Assiut University Children Hospital

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Type 1 diabetes mellitus (T1DM) remains the most common form of diabetes in childhood. The incidence of type 1 diabetes is continuously increased. Zinc transporter protein 8 antibodies (ZnT8A) measurement can be helpful in detection of suspected new cases of type 1diabetes when other islet auto antibodies are negative. We evaluated the role of ZnT8A in diagnosis of new cases of T1DM in comparison to islet cell antibody (ICA), and assessed its prediction value among siblings. 31 of newly diagnosed T1DM patients and 55 age and sex matched healthy siblings were included. Measurements of ZnT8A and ICA was carried out by ELISA. ZnT8A had 45% sensitivity and 69% specificity while ICA had 64.5% sensitivity and 83.64% specificity. 22.6% of diabetic patients had high level of ZnT8A as compared to 20% of siblings (*P*< 0.001 and *P* < 0.001, respectively). 28.6% of diabetic patients with high titer ZnT8A had positive ICA (*P* <0.04) as compared to 63.6% in sibling group (*P*<0.001). It is concluded that ZnT8A and ICA play an important role in diagnosis and prediction of T1DM cases.

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease associated with selective destruction of insulin-producing pancreatic β-cells [1]. Clinical manifestation of T1DM reflects the consequence of an underlying, sustained autoimmune process. For instance, auto antibodies against islet antigens are detected before the clinical onset of T1DM [2]. The onset of clinical disease represents the end stage of β-cell destruction leading to T1DM [1]. The incidence of type 1 diabetes is increasing, particularly in young children under the age of 5 years. The distinguishing feature of islet inflammation in very young children is a high proportion of infiltrating B lymphocytes [3]. B-cell responses to islet auto antigens are characteristic of T1DM and auto antibodies provide important predictive markers of disease. Auto antibodies first appear in the first 5 years of life, most commonly with specificity for insulin which may then be followed by the progressive appearance of autoantibodies to other major islet cell antigens including glutamate decarboxylase (GAD), IA-2 and the zinc transporter ZnT8 [3].

Zinc is an essential trace element that has three major biological roles: catalytic, structural and regulatory. The catalytic and structural role of zinc is well recognized. Zinc is a structural constituent in numerous proteins, including growth factors, cytokines, receptors, enzymes, and transcription factors belonging to cellular signaling pathways. Moreover, it is implicated in numerous cellular processes as a cofactor for an estimated 3000 human proteins including enzymes, nuclear factors and hormones [4].

Zink transporter 8, a 369 amino acid transmembrane protein, is a novel islet

autoantigen in type 1 diabetes mellitus. It is more specifically expressed in insulin-containing secretory granules than that of GAD65 and tyrosine phosphatase-related molecules [5]. Zink transporter 8 is predominantly confined to the pancreatic islet beta cell with more modest expression in the alpha and ductal cells. ZnT8 spans the secretory granule membrane where it catalyzes the import of Zn^{++} ions from the cytosol into the lumen in exchange for protons translocated by the vesicular proton pump. Lumenal zinc concentrations approach 20 mM (the highest in the body) and facilitate insulin processing/maturation, crystallization and hexamer formation and eventual secretion [6]. Zink transporter 8 represents a major player to provide zinc for insulin maturation and/or storage processes in insulin secreting pancreatic β-cells. Therefore, antibodies against ZnT8 may affect several important processes, such as insulin synthesis, storage, secretion and may impair islet cell paracrine/ autocrine communication a well [7].

The β cell response against the zinc transporter 8 protein is unique, as patients generate three variants of ZnT8 autoantibodies (ZnT8A). These autoantibody variants are directed specifically against epitope(s) that include arginine (R), tryptophan (W) or glutamine (Q) at amino acid (aa) position 325 and may be displayed either alone or in combination [7]. The objectives of this study were to evaluate ZnT8A in newly diagnosed type 1 diabetes mellitus compared to ICA and to assess the level of ZnT8A in early prediction of type 1 diabetes mellitus among siblings.

Material and Methods

Patient characteristics

The study was conducted on 31 of newly diagnosed type1 diabetes mellitus patients and 55 age and sex matched healthy siblings of those patients. The patients were selected from Intermediate care unit and Endocrine and diabetes unit where patients with diabetes are managed in Assiut University Children Hospital. Informed consent was obtained from every patient for laboratory studies according to the guidelines of the Committee of Medical Ethics of Assiut University Hospital. They included 17 males and 14 females of diabetic group; their age ranged between 2 and 16 years with mean 7.85 ± 4.36, 28 males and 27 females of sibling group; their age ranged between 0.5 and 18 years with mean 8.07 ± 4.71. All patients were subjected to history taking and clinical examination anthropometric measurement (weight, height) and Signs of other autoimmune disease as thyroid swelling, hepatosplenomegaly and hypopigmentation of skin.

Routine laboratory investigations

-Complete blood cell count (CBC) using (Micros 60 HORIBA Medical)

-Hemoglobin A1C, Kidney function test including urea and creatinine, Blood cholesterol level by using (Cobas Integra 400 Plus).

-Complete urine analysis for detection of ketone bodies using (dip stick).

Special investigations

-Estimation of serum Zinc transporter 8 autoantibody (ZnT8A) was performed by kit supplied by SinoGeneClon Biotech (Cat No: SG- 11004) based on sandwich Enzyme Linked Immunosorbent Assay (ELISA) technique. Human ZnT8 antibody was adopted to coat microtiter plate wells, was made solid phase antibody then ZnT8 to wells was added, ZnT8A was combined with labeled HRP to form antibody – antigen -enzyme –antibody complex, after washing completely, TMP substrate solution was added, TMP substrate became blue color at HRP enzyme –catalyzed, reaction was terminated by addition of stop solution and the color change was measured at a wave length of 45nm on Stat fax-2100 reader. The concentration of ZnT8A in the sample is then determined by comparing the optical denisty (O.D) of the sample of the standard curve, (Detection range 7 ng/L-200 ng/L).

-Detection of Human (ICA) was performed by kit supplied by SinoGeneClon Biotech (Cat No: SG-11098) based on sandwich ELISA technique. Purified antigen was used to coat microtiter plate wells. Solid-phase antigen was made then ICA was added to

wells, antigen with ICA was combined. Non combinative antibody and other components was washed and removed, then antigen was combined with HRP labeled and became antigen – antibody - enzyme- antigen complex. TMB substrate solution was added after washing Completely, TMB substrate became blue color At HRP enzyme catalyzed. Reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm on Stat fax-2100 reader. ICA exist in the sample or not according to CUTOFF value. Calculated critical (CUT OFF): critical =the average of negative control well + 0.15. Negative judgment: sample OD < calculate critical (CUT OFF) is ICA negative. Positive judgment: sample OD > calculate critical (CUT OFF) is ICA positive.

Statistical Analysis

Data was collected and analyzed those using SPSS (Statistical Package for the Social Science, version 20, IBM, and Armonk, New York). Continuous data was expressed in form of mean ± SD or median (range) while nominal data was expressed in form of frequency (percentage). Chi²-test was used to compare the nominal data of different groups in the study while student t-test was used to compare mean of different two groups and ANOVA test for more than two groups. Spearman correlation was used to determine the correlation between ZnT8A and other continuous variables. *P* value was significant if < 0.05.

Results

The study was conducted on 31of newly diagnosed type1diabetes mellitus patients and 55 age and sex matched healthy siblings of those patients. Demographic Data and Baseline laboratory data in both groups are presented in (Table 1). In our study, it was noticed that (41.9%) of diabetic group were less than 5 years old and 40% of sibling group were less than 5 years. Diabetic group had significantly higher urea, creatinine, glycated hemoglobin, cholesterol level and positive urinary ketones in comparison to sibling group (*P*<0.001, 0.01, <0.001, <0.001 and <0.001 respectively). Frequency of ICA was insignificantly higher in sibling group in comparison to diabetic group (16.4% vs. 6.5%; *P*= 0.31) (Table1).

Table 1. Demographic and laboratory data of the studied groups

	Diabetic group (n=31)	Sibling group (n=55)	*P* value
Age (years)	7.85 ± 4.36 (2- 16)	8.07 ± 4.71 (0.5- 18)	NS
Age groups			
Less than 5 years	13 (41.9%)	22 (40%)	NS
5- 10 years	7 (22.6%)	16 (29.1%)	
10- 15 years	9 (29%)	10 (18.2%)	
More than 15 years	2 (6.5%)	7 (12.7%)	
Sex			
Male	17 (54.8%)	28 (50.9%)	NS
Female	14 (45.2%)	27 (49.1%)	
Positive family history	15 (48.4%)	29 (52.7%)	NS
Urea (mmol/L)	4.99 ± 1.97	3.99 ± 1.21	< 0.001
Creatinine (μmol/L)	50.30 ± 14.61	38.30 ± 11.24	0.01
Glycated hemoglobin (%)	11.67 ± 1.84	5.01 ± 0.38	< 0.001
Cholesterol (mg/dl)	102.12 ± 42.19	77.52 ± 38.25	< 0.001
Positive urinary ketones	14 (45.2%)	0	< 0.001
Positive ICA (nmol/L)	2 (6.5%)	9 (16.4%)	NS

Data was expressed in form of mean ±SD, range or frequency (percentage). *P* value was significant if < 0.05.DM, diabetes mellitus. ICA (Islet Cell Autoantibody).

In present study, (22.6%) of diabetic group, (20%) of the sibling group had high titer of ZnT8A more than 200 ng/L (Table 2).

In diabetic children 28.6% with high ZnT8A had positive islets cells auto-antibodies with significant (*P* value <0.04). In sibling group (63.6%) children of those with high ZnT8A and (4.5%) children of those with normal ZnT8A had positive islets cells auto-antibodies with significant *P* value (*P*<0.001*)* (Table 2).

Table 2. Levels of ZnT8A (ng/L) and ICA (nmol/L) in the studied groups.

	Diabetic group		*P*	Sibling group		*P*
	High ZnT8A	Normal ZnT8A		High ZnT8A	Normal ZnT8A	
N (%)	7 (22.6%)	24(77.4%)	NS	11 (20%)	44 (80%)	NS
Range	200-406	5- 60.50		204.50-757	5- 190.50	
Median	211	18.50	< 0.001	363	33.50	< 0.001
Mean±SD	255.07 ± 79.63	26.40 ± 19.74		419.5±198.26	42.48 ± 40.23	
Positive ICA	2 (28.6%)	0	0.04	7 (63.6%)	2 (4.5%)	< 0.001

Data was expressed in form of mean ±SD, median, range, frequency (percentage). *P* value was significant if < 0.05. ICA, islets cell autoantibody.

ZnT8A had 45% sensitivity and 69% specificity in diagnosing diabetes mellitus with area under the curve was 0.58 at cutoff point with *P*= 0.04. Islet cell autoantibody had 64.5% sensitivity and 83.64% specificity in diagnosing diabetes mellitus with 74.07% accuracy (Table 3, Fig 1).

Table 3. Diagnostic values of ZnT8A (ng/L) and ICA (nmol/L) in diagnosing T1DM

	ZnT8A	ICA
Sensitivity	45%	64.5%
Specificity	69%	83.64%
Positive predictive value	45%	18.18%
Negative predictive value	69%	61.33%
Cutoff point	> 28	-
Area under the curve	0.58	-
Accuracy	-	74.07%

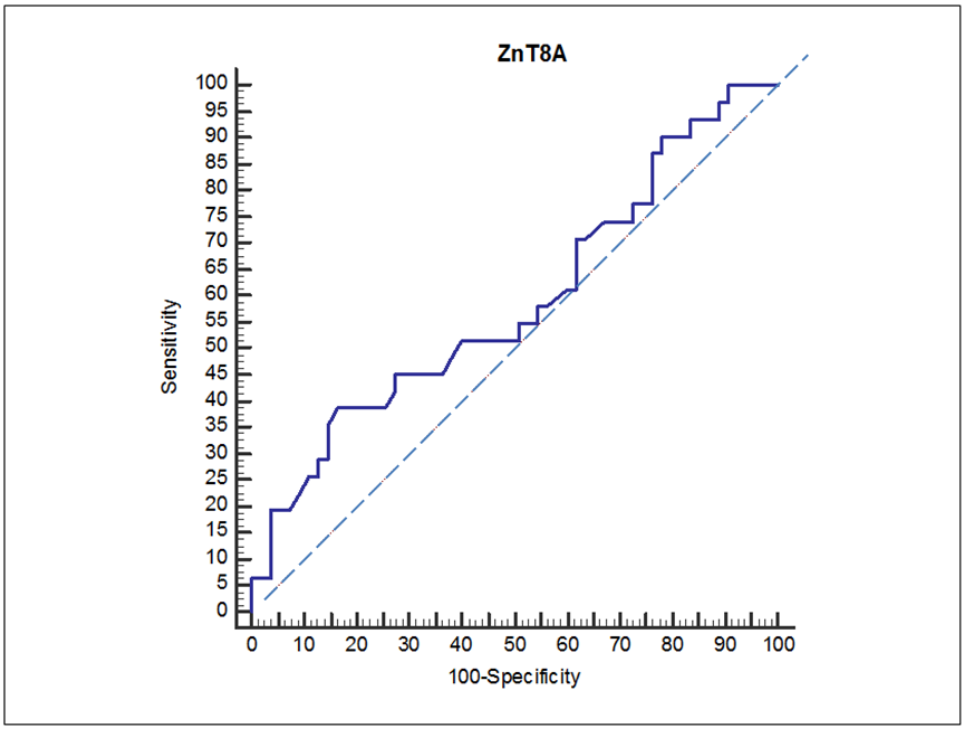

Figure 1.Diagnostic value of Zn T8A (Zinc transporter 8 autoantibody), in diagnosing T1DM. cut off >28, and area under the curve=0.58. *P* value < 0.04

Discussion

Type 1 diabetes (T1DM) is the most common autoimmune disease that develops at any age but most frequently in children and young adults. Autoantibodies against islet antigens are typically present before and for a variable time following diagnosis. Once initiated β cell damage classically leads to progressive loss of insulin secretion and a need for lifelong insulin treatment [8].

Zinc transporter 8 (ZnT8) is the most recently discovered diabetes-associated auto antigen. Zinc transporter 8 autoantibody is highly β-cells specific exclusively expressed in insulin containing secretory granules of β-cells and it is a target of the autoimmune process [9].

Islet cell autoantibody is a strong predictor of T1DM, and it has been a basis for the selection of participants in major prevention trials that have been performed [10].

In the present study we found that the prevalence of high level of ZnT8A in diabetic group was 22.6% with significant *P* value (<0.001). This show similarity to some researchers who suggested that ZnT8A positivity in T1DM was lower as in Chinese patients (24.1%) with new onset T1DM patients age group from 1 to 70 years old, Huang *et al.* and Yang *et al.* who concluded that ZnT8A were present in 24.1% (130/539) of T1DM patients which show agreement with our study [7,11]. In contrast to our study, Turkish children with new onset T1DM show a high ZnT8A prevalence 58% between 1-18 years of age, diagnosed with T1DM [12].

In present study we found that the prevalence of high level of ZnT8A in sibling group was 20% with significant *P* value

(<0.001). This is similar to Grijse *et al.* who found that the antibody prevalence at baseline among the (409) first degree relatives of type 1 diabetic patients (n=80) was 20% [13]. In contrast to our study, DAISY study reported that the predictive value of ZnT8A for the development of T1DM was found in 62.9% of individuals before the development of T1DM which detected by A radioimmunoprecipitation assay [14].

We found in this study that Znt8A titers tend to be higher in sibling group (Mean±SD, 419.5±198.26 ng/L and Median 363 ng/L, range, 204.50-757 ng/L) compared to the titers of the ZnT8A in diabetic group (Mean±SD, 255.07±79.63 ng/L and Median 211 ng/L, range 200-406 ng/L) which show agreement with Niechciał *et al.,* [9]. In contrast to our results Elmaoğulları *et al.,* observed that ZnT8A titers in ZnT8A positive cases in T1DM group were significantly higher (median, 271.37 U/mL, range, 23.28-501.00 U/mL) [12].

In present study we found that the frequency of ICA was higher in sibling group in comparison to diabetic group although the result was statically insignificant (16.4% vs. 6.5%; *P*= 0.31). Our result is similar to study done by Verkauskiene *et al.* and, Andersson *et al.,* who found that ICAs were positive in 7% at onset of type 1 diabetic cases and frequency of positive ICAs declines following diagnosis, and remains positive in less than 5–10% of type 1 diabetes patients after 10 years [15,16]. However, Domuschiev concluded that Islet cell antibody was found 61.9% (13/21) of patients with newly onset T1DM children aged from 5 to 17 years [17].

Salah *et al.* found that 28.75% of first-degree relatives of T1DM patient showed positive ICA and this show similarity to our study [18]. In accordance to our result, Domuschiev found that 21 % of first-degree relatives of patients with T1DM were positive to ICA [17].

In contrast to this result, Long *et al.,* reported that the ICA in first degree relatives was 7% [19]. Similarly, Kimpima *et al.,* found that there were 9.4% siblings of newly onset T1DM tested positive for ICA in the first sample [20].

We found in the present study that 28.6% of diabetic group with high ZnT8A had positive ICA with significant *P* value (*P* value <0.04). This finding is similar to study reported by Fabris *et al.* who concluded that ICA positive type 1 diabetic patients were associated with positive ZnT8A in 16.7 % [21].

In this study we found that 63.6% sibling group with high ZnT8A had positive ICA with significant *P* value (*P*<0.001). In agreement to our result, Long *et al.* found out 526 ICA-positive first-degree relatives 221 (42%) were ZnT8A positive, ZnT8A were associated with increased risk of diabetes in ICA-positive relatives [19].

From this study we concluded that both ZnT8A and ICA play an important role in the identification of individuals at high risk for T1DM. ICA was more specific and sensitive in diagnosis of T1DM than ZnT8A. ZnT8A had more Positive predictive value when compared to ICA. ZnT8A had more benefit in prediction of T1DM in age less than 5 years.

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