Characterization of patients with diabetes who were incidentally found to be glutamic acid decarboxylase autoantibody-positive by bridging-type enzyme-linked immunosorbent assay

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ABSTRACT

This study aimed to characterize diabetic patients incidentally found to be positive for glutamic acid decarboxylase autoantibodies (GADA) in general practice. Using bridging-type enzyme-linked immunosorbent assay, we screened 1,040 patients with phenotypic type 2 diabetes for GADA, finding 25 (2.4%) to be positive. However, on retesting, with a median interval of 19 days, 44% of GADA-positive patients turned negative (Disappearing Group). The mean age at diabetes onset was significantly higher ($P < 0.05$) and GADA titers at first determination were significantly lower ($P < 0.001$) in the Disappearing Group compared with the Persistent Positive Group. On initial screening, all patients in the Disappearing Group had GADA titers of <6.5 U/mL. The current study showed that a portion of phenotypic type 2 diabetic patients incidentally identified as GADA-positive were falsely positive, and that to avoid the misclassification, remeasurement of GADA is essential in cases showing very low titers.

INTRODUCTION

One of the hallmarks of type 1 diabetes is the presence of islet-associated autoantibodies, including glutamic acid decarboxylase autoantibodies (GADA). The presence of GADA serves as a marker for the development of autoimmune diabetes in adults, and helps to discriminate between slowly-progressive type 1 diabetes (SPIDDM) and type 2 diabetes. In Japan, a recent change in the GADA assay kit from radioimmunoassay (RIA) to enzyme-linked immunosorbent assay (ELISA) has proven to yield mismatched GADA test results between the two kits. It has been reported that bridging-type GADA-ELISA has a higher sensitivity in the low-titer range compared with the RIA kit, which improves the diagnostic accuracy in acute-onset type 1 diabetes[1,2]. Because of this change, a number of low-titer GADA-positive patients have been incidentally discovered by GADA screening in adulthood, leading to confusion in being able to make an accurate diagnosis. Furthermore, previous studies have shown that GADA is also present in a proportion of non-diabetic individuals who do not develop type 1 diabetes for many years, and it has been argued that these cases should be regarded as false positives[3,4]. In the present study, we investigated the potential clinical implications of low-titer GADA-positive diabetic patients who were incidentally identified by GADA-ELISA screening.

METHODS

We consecutively screened for GADA in all diabetic patients who visited Shin-Koga Hospital, Kurume, Japan, for the first time between January 2016 and November 2018. After exclusion of all patients with previously diagnosed type 1 diabetes and any other types of diabetes, a total of 1,040 Japanese patients with phenotypic type 2 diabetes (697 men, 343 women; mean age 63.9 ± 13.2 years) were used for the present study. GADA-positive patients were then retested and divided into two groups (Disappearing Group and Persistent Positive Group) according to their GADA results. The timing of GADA

retesting was determined by the attending physician. This study's protocol was approved by the ethics committee of Shin-Koga Hospital (approval no. 2018-015, approval date 9 November 2018).

Blood samples were drawn from peripheral veins into the collection tube containing clot activator and separator gel, and serum was obtained after centrifugation and directly used for GADA assay without freezing. GADAs were determined at a commercial laboratory (SRL Inc., Tokyo, Japan) using the bridging-type ELISA kits (RSR Ltd., Cardiff, UK), as described previously[5,6]. The results were read from a calibration curve constructed in the same run as the calibrators and expressed in U/mL. The cut-off value was 5.0 U/mL, which was in the 99th percentile of 300 healthy blood donor sera[5], the lower detection limit was 0.57 U/mL, and the intra- and interassay coefficients of variation were 3.5–8.5% and 5.2–6.4%, respectively[6]. Furthermore, autoantibodies to insulin and insulinoma-associated antigen-2 were also determined by Yamasa's RIA kit (Chiba, Japan; normal range <125 nU/mL) and RSR's ELISA kit (normal range <0.6 U/mL), respectively, at GADA initial screening.

Results are expressed as the mean ± standard deviation or median (range). The differences in non-parametric data were tested using the Mann–Whitney U-test, and categorical variables were compared using the χ^2-test or Fisher's exact test where appropriate. Statistical analysis was carried out using StatView (version 5.0; SAS Institute, Cary, NC, USA), and a P value <0.05 was considered statistically significant.

RESULTS

In initial screenings, 25 of 1,040 patients with phenotypic type 2 diabetes (2.4%) were found to be positive for GADA-ELISA. As shown in Table 1, the GADA-ELISA positive patients consisted of 14 men and 11 women, and the mean age at diabetes onset and mean duration of diabetes were 49.0 ± 14.4 years and 11.3 ± 12.4 years, respectively. The median titer of GADA was 7.3 U/mL (range 5.3–586.0 U/mL), and 20 of 25 patients (80%) showed GADA-ELISA levels of <37.5 U/mL, corresponding with the lower GADA-RIA limit of 1.3 U/mL[7]. None of the patients were positive for either autoantibodies to insulin or insulinoma-associated antigen-2.

Among 25 GADA-positive patients, 16 patients for whom we could retest GADA positivity were used for further analyses (Figure 1). As shown in Figure 2, seven of 16 patients (44%), classified as the "Disappearing Group" showed negative results for GADA-ELISA on retesting. The median interval between the first GADA determination and retesting for this group was 19 days (range 5–390 days). The remaining nine patients, classified as the "Persistent Positive Group," were still positive for GADA at retesting, with the median interval between their first GADA determination and retesting being 172 days (range 9–418 days).

After this, we compared the clinical characteristics of the two groups, finding that the mean age at diabetes onset was significantly higher in the Disappearing Group than in the Persistent

Table 1 | Clinical characteristics of 25 patients with phenotypic type 2 diabetes incidentally identified positive for glutamic acid decarboxylase autoantibodies at initial screening

Male (%)	14 (56.0)
Age at diabetes onset (years)	49.0 ± 14.4
Duration of diabetes (years)	11.3 ± 12.4
BMI (kg/m^2)	22.1 ± 4.9
HbA1c (%)	8.3 ± 2.1
Fasting C-peptide (ng/mL)	1.47 ± 0.87
GADA titer (U/mL)[†]	7.3 (5.3–586.0)
IA-2A-positive (yes/no)	0/20
IAA-positive[‡] (yes/no)	0/14
Glucose-lowering agents	
Insulin use (yes/no)	8/17
Insulin secretagogues[§] use (yes/no)	19/6
Others[¶] use (yes/no)	11/14
None (yes/no)	3/22

Data are shown as the mean ± standard deviation or n (%) unless otherwise indicated. BMI, body mass index; GADA, glutamic acid decarboxylase autoantibodies; HbA1c, glycated hemoglobin; IA-2A, insulinoma-associated antigen-2 autoantibodies; IAA, insulin autoantibodies. [†]Median (range). [‡]Determined in individuals who had no history of insulin treatment. [§]Insulin secretagogues include sulfonylureas, glinides, dipeptidyl peptidase-4 inhibitors and glucagon-like peptide-1 receptor agonists. [¶]Others include biguanides, thiazolidines, α-glucosidase inhibitors and sodium–glucose cotransporter 2 inhibitors.
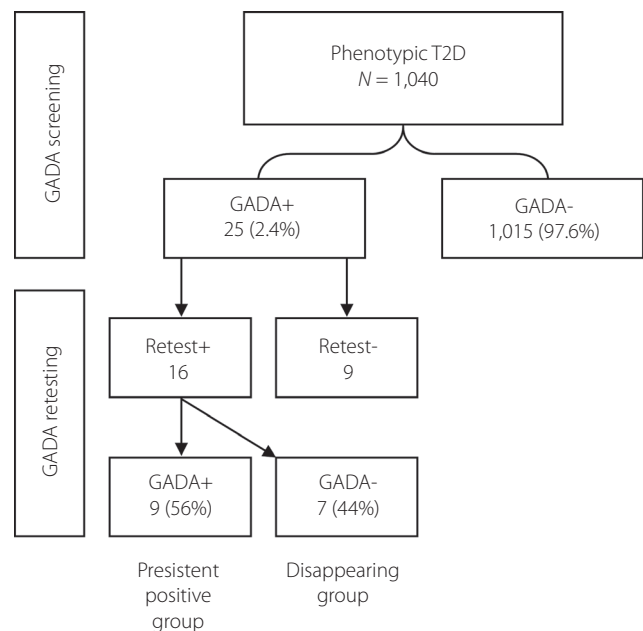

Figure 1 | Flowchart showing the number of phenotypic type 2 diabetic patients (T2D) at glutamic acid decarboxylase autoantibodies (GADA) screening and during the follow up according to GADA positivity.

Positive Group ($P < 0.05$), as shown in Table 2. Furthermore, the GADA titers at first determination were significantly lower in the Disappearing Group than in the Persistent Positive
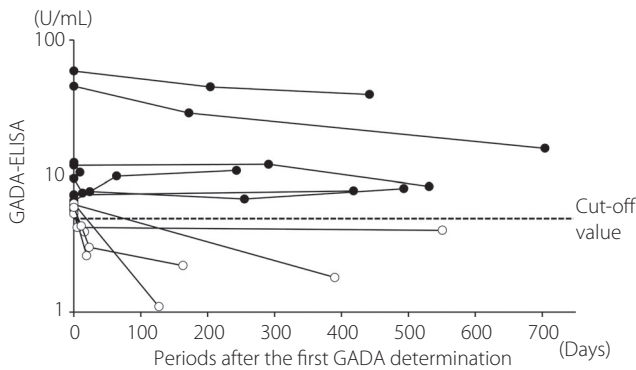
Figure 2 | Glutamic acid decarboxylase autoantibody (GADA) titers in serum samples at first screening (0) and retesting are plotted for patients with phenotypic type 2 diabetes. Patients whose GADA disappeared at retesting are identified with the open circles. ELISA, enzyme-linked immunosorbent assay.

Group ($P < 0.001$). Of note, all patients in the Disappearing Group had GADA titers of <6.5 U/mL on first determination. However, there was no significant difference between the two groups in terms of mean body mass index, glycated hemoglobin, fasting C-peptide immunoreactivity and glucose-lowering agents used for diabetes treatment.

DISCUSSION

In the present study, we showed that: (i) 2.4% of diabetic patients initially diagnosed as having type 2 diabetes are positive for GADA-ELISA; (ii) approximately 40% of GADA-positive patients incidentally identified through GADA screening

could in fact be false positives; and (iii) patients whose GADA disappeared within a short period showed higher age at onset and lower GADA titers when compared with persistent GADA-positive patients.

In general practice, testing for GADA helps a physician make diagnoses and provide appropriate treatment for patients with SPIDDM. However, once a patient has been identified as positive for GADA and diagnosed as SPIDDM, the ability to reassess GADA measurements is limited in Japan as a result of health insurance regulations. Thus, assuring accuracy in the measuring and specificity of autoantibody assay is critical in avoiding the misclassification of diabetes. In the present study, we showed that >40% of GADA-positive patients turned negative with a median retesting interval of 19 days, and that all patients in the Disappearing Group had GADA titers of <6.5 U/mL. Furthermore, the mean age of the Disappearing Group, which is similar to that of GADA-negative type 2 diabetes[8], was higher than that of the Persistent Positive Group. These results suggest that to assure accurate diagnosis, the cut-off value for the GADA-ELISA kit might need to be re-examined using a substantial number of healthy Japanese control individuals, with particular focus on older adults. Furthermore, physicians should not be hasty in diagnosing SPIDDM when patients have GADA titers of 5.0–6.5 U/mL.

Although the reason for false positives involving GADA-ELISA test in the present study remains unclear, Nilson *et al.*[9] reported that the same ELISA kit used in the present study gave a considerable number of false positive GADA results when plasma samples from both type 1 diabetic patients and healthy controls were used. They pointed out the possibility

Table 2 | Clinical characteristics of the glutamic acid decarboxylase autoantibody disappearing group and persistent positive group

	Disappearing group ($n = 7$)	Persistent positive group ($n = 9$)	*P*-value
Male (%)	5 (71.4)	4 (44.4)	NS
Age at diabetes onset (years)	52.0 ± 5.7	38.4 ± 9.1	0.011
Duration of diabetes (years)	16.4 ± 14.3	9.0 ± 10.1	NS
BMI (kg/m^2)	20.7 ± 3.9	24.6 ± 6.6	NS
HbA1c (%)	8.8 ± 3.4	8.2 ± 1.7	NS
Fasting C-peptide (ng/mL)	1.50 ± 0.92	1.43 ± 0.86	NS
GADA titer (U/mL)[†]	5.9 (5.3–6.3)	12.0 (6.6–152.0)	0.0009
Interval between the first GADA determination and retest (days)[†]	9 (5–390)	72 (9–418)	NS
IA-2A positive (yes/no)	0/7	0/8	NS
IAA-positive[‡] (yes/no)	0/4	0/5	NS
Glucose-lowering agents			
Insulin use (yes/no)	3/4	2/7	NS
Insulin secretagogues[§] use (yes/no)	6/1	9/0	NS
Others[¶] use (yes/no)	3/4	8/1	NS
None (yes/no)	0/7	1/8	NS

Data are shown as the mean ± standard deviation or *n* (%) at initial glutamic acid decarboxylase autoantibody (GADA) screening unless otherwise indicated. Statistical analysis was carried out using the Mann–Whitney *U*-test, χ^2-test or Fisher's exact test. BMI, body mass index; HbA1c, glycated hemoglobin; IA-2A, insulinoma-associated antigen-2 autoantibodies; IAA, insulin autoantibodies; NS, not significant. [†]Median (range). [‡]Determined in subjects who had no history of insulin treatment. [§]Insulin secretagogues include sulfonylureas, glinides, dipeptidyl peptidase-4 inhibitors, and glucagon-like peptide-1 receptor agonists. [¶]Others include biguanides, thiazolidines, α-glucosidase inhibitors and sodium–glucose cotransporter 2 inhibitors.

that plasma contains proteins that could interfere with the GADA assay. As the disappearance of autoantibodies occurred within several weeks in most cases, pre-analytical errors, including insufficient mixing of collection tubes, sample handling or processing, could be responsible for the false positive results. In a patient whose interval of two GADA measurements was >1 year, we cannot exclude the possibility of the natural course of GADA disappearance.

There were several limitations to the present study, to begin with, the number of GADA-ELISA positive patients was relatively small, and in some patients, we were unable to retest for GADA positivity, which might raise some concerns in generalizing the data. Therefore, further investigation using a larger cohort is required. Additionally, we only measured the autoantibodies in a single sample collection, and so cannot rule out the possibility that the disappearance of GADA is due to intra- or interassay variations. Furthermore, GADA-negative patients were not retested, which is important to evaluate false negatives of this kit. Finally, as the antigen specificity in GADA-positive sera is yet unknown, competitive binding experiments with unlabeled recombinant GAD65 using sera that have been incidentally identified as GADA-positive during routine screening will be required to determine this specificity.

In summary, the current study showed that a portion of phenotypic type 2 diabetic patients who were incidentally identified as GADA-positive by bridging-type GADA-ELISA kits might be false positives. Thus, GADA-positive patients with very low titers should receive careful follow up to determine whether they are truly negative, transient positive or truly positive to ascertain an accurate classification of diabetes.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Murata T, Tsuzaki K, Nirengi S, et al. Diagnostic accuracy of the anti-glutamic acid decarboxylase antibody in type 1 diabetes mellitus: Comparison between radioimmunoassay and enzyme-linked immunosorbent assay. *J Diabetes Investig* 2017; 8: 475–479.
2. Oikawa Y, Tanaka M, Horie I, et al. A study on the correlation between GAD antibody titers measured by ELISA kit and RIA kit. *Jpn J Med Pharm Sci* 2015; 72: 1551–1560 (Japanese).
3. Lundgren VM, Isomaa B, Lyssenko V, et al. GAD antibody positivity predicts type 2 diabetes in an adult population. *Diabetes* 2010; 59: 416–422.
4. Gale EA. Latent autoimmune diabetes in adults: a guide for the perplexed. *Diabetologia* 2005; 48: 2195–2199.
5. Brooking H, Ananieva-Jordanova R, Arnold C, et al. A sensitive non-isotopic assay for GAD65 autoantibodies. *Clin Chim Acta* 2003; 331: 55–59.
6. Kawasaki E, Okada A, Uchida A, et al. Discrepancy of glutamic acid decarboxylase 65 autoantibody results between RSR radioimmunoassay and enzyme-linked immunosorbent assay in patients with type 1 diabetes is related to autoantibody affinity. *J Diabetes Investig* 2019; 10: 990–996.
7. Oikawa Y, Kondo T, Shimada A, et al. Actual condition survey regarding mismatch of measurements between radioimmunoassay and enzyme-linked immunosorbent assay tests for anti-glutamic acid decarboxylase antibody in real-world clinical practice. *J Diabetes Investig* 2019; 10: 685–689.
8. Katahira M, Ogata H, Ito T, et al. Association of autoimmune thyroid disease with anti-GAD antibody ELISA test positivity and risk for insulin deficiency in slowly progressive type 1 diabetes. *J Diabetes Res* 2018; 2018: 1847430.
9. Nilson E, Ekholm B, Rees Smith B, et al. Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA. *Clin Chim Acta* 2008; 388: 130–134.