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Increased diagnosis of autoimmune childhood-onset Japanese type 1 diabetes using a new glutamic acid decarboxylase antibody enzyme-linked immunosorbent assay kit, compared with a previously used glutamic acid decarboxylase antibody radioimmunoassay kit

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Keywords
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ABSTRACT

Aims/Introduction: We compared the results of testing for glutamic acid decarboxylase antibodies (GADAb) using a radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA) in individuals with childhood-onset type 1 diabetes mellitus.

Materials and Methods: Serum specimens were collected from 1,024 Japanese children (426 boys and 598 girls) in 2013. The median age at diagnosis was 7 years (0–18 years). The blood specimens were obtained at a median age of 13 years (2–22 years).

Results: Among the 628 children whose serum specimens were collected within 5 years after diagnosis, the rate of GADAb positivity was 47.9% using RIA and 69.4% using ELISA. The participants were divided into four groups according to their RIA and ELISA results for GADAb as follows: group I (RIA+/ELISA+), group II (RIA+/ELISA–), group III (RIA–/ELISA+) and group IV (RIA–/ELISA–). The clinical and genetic characteristics of group I and group III were quite similar in terms of age at diagnosis, male/female ratio, relatively high positive rates for both autoantibody to protein tyrosine phosphatase IA-2 and autoantibody to the cation efflux transporter zinc transporter 8, and human leukocyte antigen genotype. Group II contained just five patients, and was characterized by a younger age at diagnosis, low positive rates for both autoantibody to protein tyrosine phosphatase IA-2 and autoantibody to the cation efflux transporter zinc transporter 8, and a unique human leukocyte antigen genotype. If the positive rates of either autoantibody to protein tyrosine phosphatase IA-2 or autoantibody to the cation efflux transporter zinc transporter 8 or both were added to the GADAb results using RIA, the percentage of autoimmune type 1 diabetes increased from 47.9% to 78.5%.

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Conclusions: The diagnosis of autoimmune childhood-onset Japanese type 1 diabetes increased when GADAb results were obtained using a new ELISA method, compared with a previously utilized RIA method.

INTRODUCTION

Type 1 diabetes mellitus is caused by the autoimmune destruction of pancreatic islet β-cells. Autoantibodies to glutamic acid decarboxylase (GADAb) are some of the major anti-islet autoantibodies, and a high prevalence of GADAb has been shown in patients with type 1 diabetes[1–4]. The risk of developing type 1 diabetes in the population varies remarkably according to the country of residence and ethnicity. Japan has one of the lowest incidences of type 1 diabetes in the world[1], and the incidence of type 1 diabetes in children aged 0–14 years in Japan has been reported to have remained constant over the past decade, averaging 2.25 cases per 100,000 persons[5].

Until 2016, a radioimmunoassay (RIA) using the RIA kit GADAb (Cosmic, Tokyo, Japan), which has been described in detail elsewhere[6–8], was used for the measurement of GADAb; however, since 2016, the method for measuring GADAb has been completely changed in Japan to an enzyme-linked immunosorbent assay (ELISA) using the GADAb ELISA (Cosmic)[9]. Recently, studies have reported that sera from 8% to 15% of GADAb-positive patients with type 1 diabetes showed discrepant results when compared using the two assays[10–12]. Also, 25–30% of GADAb-positive, slowly progressive type 1 diabetes adult-onset patients who had been originally diagnosed using RIA were later found to be negative using ELISA[11,12]. Therefore, the Japan Diabetes Society has proposed that caution be exercised and made some recommendations[13].

The evaluation of RIA and ELISA measurements of GADAb has been limited to Japanese patients with childhood-onset type 1 diabetes. We carried out the present study to examine the clinical and genetic characteristics of Japanese children and adolescents with type 1 diabetes who showed discrepant GADAb measurements using RIA and ELISA.

METHODS

Participants

The participants of the present study were 1,024 Japanese children and adolescents (426 boys and 598 girls) diagnosed as having type 1 diabetes when they were aged ≤16 years and who had been enrolled in the 2013 cohort (March 2013) for the Japanese Study Group of Insulin Therapy for Childhood and Adolescent Diabetes (JSGIT) from 75 institutions across Japan[14]. Thus, the participants were a part of the fourth cohort for the JSGIT, which is a multicenter collaborative nationwide cohort study in Japan. Type 1 diabetes was diagnosed according to the criteria of the Japan Diabetes Society and the American Diabetes Association[15,16]. The participants were diagnosed as having type 1 diabetes at a median age of 7 years (range 0–18 years); they were registered in this study, and their blood specimens were collected when they were a median age of 13 years (range 2–22 years). Of the participants, 628 (252 boys and 376 girls) were recruited within 5 years of being diagnosed as having type 1 diabetes, and human leukocyte antigen (HLA) typing data were obtained from 649 participants (263 boys and 386 girls) who provided additional informed consent for genetic analysis out of the total of 1,024 type 1 diabetes patients (Table 1).

Most of the 1,024 type 1 diabetes patients in the present study were diagnosed after an acute onset. Just 14 patients (7 boys and 7 girls, aged 1–14 years at diagnosis) were considered to have fulminant-type type 1 diabetes based on the following criteria: (i) ketoacidosis within a week after the onset of hyperglycemic symptoms; and (ii) a plasma glucose level ≥288 mg/dL and glycated hemoglobin <8.9% at the first visit[17]. A total of 10 of the 14 patients with fulminant type 1 diabetes tested positive for GADAb at the time of their diagnosis. Just seven patients (2 boys and 5 girls, aged 7–14 years at diagnosis) of the total of 1,024, and four out of 628 patients within 5 years after diagnosis were diagnosed as having slowly progressive type 1 diabetes

Table 1 | Positivity rates of radioimmunoassay and enzyme-linked immunosorbent assay for glutamic acid decarboxylase antibodies in children and adolescents with type 1 diabetes

T1D patients	*n*	Positivity for GADAb		Concordance rate (%)
		RIA (%)	ELISA (%)	
All recruited patients	1,024	41.4	62.1	76.6
Within 5 years of diagnosis	628	47.9	69.4	76.9
6~17 years after diagnosis	396	31.1	50.5	76.0
HLA haplotype	649	41.7	64.7	75.5

ELISA, enzyme-linked immunosorbent assay; GADAb, glutamic acid decarboxylase antibodies; RIA, radioimmunoassay; T1D, type 1 diabetes.

based on the following criteria: (i) the presence of GADAb at some time point during the patient's clinical course; (ii) the absence of ketosis or ketoacidosis at the onset (or diagnosis) of diabetes without the need for insulin treatment to correct hyperglycemia immediately after diagnosis; and (iii) no requirement for insulin treatment for at least 6 months after diagnosis[18].

Autoantibody assay

The serum GADAb titers were measured using a commercial RIA and ^{125}I-labeled recombinant human GAD65 as the tracer reagent (Cosmic); patients with GADAb titers of ≥1.5 U/mL (mean in Japanese controls + 3 SD) were judged as being positive, in accordance with previous reports[6–8,19]. The GADAb titers in the same serum specimens were then measured by ELISA using the GADAb ELISA kit (Cosmic), and patients with GADAb titers of ≥5.0 U/mL (99th percentile of the 300 normal controls) were judged as being positive, in accordance with previous reports[9,10,19].

The autoantibody to protein tyrosine phosphatase IA-2 (IA-2Ab) level was measured by RIA using the IA-2Ab kit (Cosmic), and patients with IA-2Ab titers of ≥0.4 U/mL (mean in Japanese normal controls + 3 SD) were judged as being positive, in accordance with a previous report[20].

The autoantibody to the cation efflux transporter zinc transporter 8 (ZnT8Ab) level was determined by a radioligand binding assay using a dimeric complementary deoxyribonucleic acid construct of the carboxy-terminal domains (aa268–369) carrying 325Trp and 325Arg (CW-CR)[21]. The cut-off value for ZnT8A-CW-CR was an index of 0.007, which was based on the 99th percentile in sera obtained from 139 healthy control individuals[21].

HLA typing

Genomic DNA was extracted from whole blood specimens. HLA class II typing was carried out using a Luminex Multi-Analyte Profiling system with a WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan)[22]. Briefly, highly polymorphic exon 2 of the HLA-DRB1 and -DQB1 genes were amplified using the primer pairs included in the kit. Each polymerase chain reaction product was hybridized using sequence-specific oligonucleotide probes that were complementary to the allele-specific sequences[22].

Statistical analysis

The results are expressed as the mean ± standard deviation or median (range). Clinical data among the four groups were compared using the Kruskal–Wallis test. Fisher's exact test was applied to a two-by-two contingency table, and the corrected *P*-values, equivalent to the *P*-values multiplied by the number of comparisons for each locus or haplotype of HLA DRB1 and DQB1, were determined. *P*-values or corrected *P*-values of <0.05 were considered to denote statistical significance. All the statistical analyses were carried out using IBM SPSS, version 24.0 (Armonk, NY, USA).

Ethical approval

This study was approved by the review board of Tokyo Women's Medical University and each of the participating institutions, and was carried out in accordance with the ethical guidelines and regulations laid out in the Declaration of Helsinki. Written consent was obtained from each of the patients or their parents.

RESULTS

Among the 1,024 patients with type 1 diabetes who were enrolled in the present study, a positive result for GADAb was obtained using RIA in 424 patients (41.4%), and using ELISA in 636 patients (62.1%; Table 1). Among the 628 patients in whom the assays were carried out within 5 years after diagnosis, a positive result for GADAb was obtained using RIA in 301 patients (47.9%), and by ELISA in 436 patients (69.4%). The concordance rate was as low as 77%. The positivity rate for GADAb was significantly lower (31.1% using RIA and 50.5% using ELISA) in patients with long-standing type 1 diabetes (tested between 6 and 17 years after the diagnosis) than in those who were tested within 5 years of the diagnosis. Of note, the positivity rate using RIA to test for GADAb was approximately 20% lower than that using ELISA (Table 1).

Figure 1 shows the correlation between the GADAb titers determined using RIA and ELISA in the 628 type 1 diabetes patients who were assayed within 5 years after diagnosis. A significant positive correlation between the GADAb titers determined using RIA (0.6–100 U/mL) and ELISA (2.0–2,000 U/mL) was observed in a linear range in 387 of the 628 patients in whom the testing was carried out within 5 years after diagnosis:

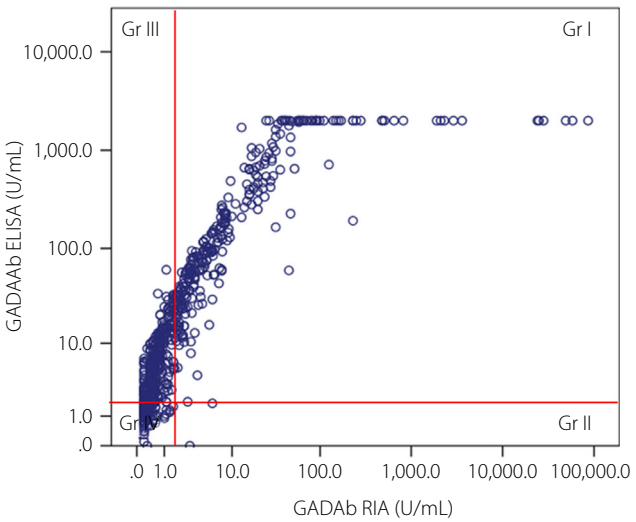

Figure 1 | Correlation between the glutamic acid decarboxylase antibodies (GADAb) titers measured using radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) in 628 type 1 diabetes patients within 5 years after diagnosis. Gr 1, group 1; G II, group II; Gr III, group III; Gr IV, group IV.

Table 2 | Positivity rates of radioimmunoassay and enzyme-linked immunosorbent assay for glutamic acid decarboxylase antibodies in 628 patients with type 1 diabetes assayed within 5 years after diagnosis

Patient number (%)		GADAb RIA		
		Positive (%)	Negative (%)	Total (%)
GADAb ELISA	Positive	296 (47.1)	140 (22.3)	436 (69.4)
	Negative	5 (0.8)	187 (29.8)	192 (30.6)
	Total	301 (47.9)	297 (47.3)	628 (100)

ELISA, enzyme-linked immunosorbent assay; GADAb, glutamic acid decarboxylase antibodies; RIA, radioimmunoassay.

$y = 27.807 \quad x - 19.526 \quad (r = 0.845, \quad P < 0.001, \quad y =$ GADAb [U/mL] using ELISA, and $x =$ GADAb [U/mL] using RIA).

Table 2 shows the correlation between the positivity rates of RIA and ELISA for GADAb in the 628 patients with type 1 diabetes assayed within 5 years after diagnosis. These patients were divided into four groups according to their RIA/ELISA results for GADAb (Figure 1; Table 2), as follows: group (Gr) I (RIA+/ELISA+), $n = 296$ patients (47.1%); Gr II (RIA+/ELISA−), $n = 5$ patients (0.8%); Gr III (RIA−/ELISA+), $n = 140$ patients (22.3%); and Gr IV (RIA−/ELISA−), $n = 187$ patients (29.8%; Tables 2, 3).

Among the 396 patients with long-standing type 1 diabetes (6–17 years after diagnosis), 114 patients (28.8%) were categorized in Gr I, nine patients (2.3%) were categorized in Gr II; 86 patients (21.7%) were categorized in Gr III; and 187 patients (47.2%) were categorized in Gr IV. There were no significant differences in the percentages of Gr II (0.8% vs 2.3%) or Gr III (29.8% vs 21.7%) according to the years after diagnosis (within 5 years vs 6–17 years).

Table 3 shows the clinical characteristics of the 628 patients who were assayed within 5 years after diagnosis and were divided into four groups according to their RIA/ELISA results for GADAb. Of note, the clinical characteristics of Gr I and Gr III were quite similar in terms of the age at diagnosis (Gr I: mean of 9.2 years, Gr III: mean of 7.9 years), the male/female ratio (Gr I: 115/181, Gr III: 47/93) and the relatively high positive rates for both IA-2Ab (Gr I: 70.6%, Gr III: 62.9%) and ZnT8Ab (Gr I: 36.8%, Gr III: 62.9%). However, the GADAb titer in Gr III was lower (5.1–60.3 U/mL) than that in Gr I. Gr II had just five (out of the 628) patients and had unique clinical features. The age at diagnosis in this group (3.2 ± 2.5 years) was significantly lower than that in Gr I (9.2 ± 6.9 years), and the male/female ratio (3/2) in this group was higher than that in Gr I. The prevalences of both IA-2Ab (20.0%) and ZnT8Ab (0%) were significantly lower than those in both Gr I (IA-2Ab: 70.6%, ZnT8Ab: 36.8%) and Gr III (IA-2Ab: 62.9%, ZnT8Ab: 32.9%; $P < 0.001$ for IA-2Ab and $P < 0.01$ for ZnT8Ab; Table 3).

Although the positivity rate for GADAb using RIA was lower than that using ELISA by approximately 20% (Table 1), when the results for IA-2Ab and ZnT8Ab were added to the RIA results, the total prevalence of autoantibody-positive (type 1A) type 1 diabetes became nearly equal to that determined using ELISA in the 628 patients with type 1 diabetes assayed within 5 years after diagnosis (Figure 2). Thus, the percentage positivity for antibodies in the type 1A patients increased significantly with the addition of the IA-2Ab and ZnT8Ab results to the RIA/ELISA results for GADAb as follows: 47.9% using RIA for GADAb alone, 76.0% using RIA for GADAb + IA-2Ab, and 78.5% using RIA for GADAb + IA-2Ab + ZnT8Ab; 69.4% using ELISA for GADAb alone, 83.6% using ELISA for GADAb + IA-2Ab, and 84.9% using ELISA for GADAb + IA-2Ab + ZnT8Ab (Figure 2). These results

Table 3 | Clinical characteristics of the four groups divided according to the results of radioimmunoassay/enzyme-linked immunosorbent assay for glutamic acid decarboxylase antibodies among subjects assayed within 5 years after diagnosis

Gr	Group I RIA+/ELISA+	Group II RIA+/ELISA-	Group III RIA-/ELISA+	Group IV RIA-/EISA-	*P*
GADAb RIA range (U/mL)	1.5–86,000	1.5–5.7	<1.5	<1.5	
GADAb ELISA range (U/mL)	5.0->2,000	<5.0	5.1–60.3	<5.0	
n	296	5	140	187	
%	47.1%	0.8%	22.3%	29.8%	
Mean age (SD) at diagnosis	9.2 (6.9)	3.2 (2.5)	7.9 (3.5)	7.9 (7.9)	$P < 0.01$: I vs II, I vs IV
Years after diagnosis mean (SD)	2.8 (1.6)	3.3 (1.7)	3.0 (1.7)	3.3 (1.6)	$P < 0.05$: I vs IV
Male/female	115/181	3/2	47/93	87/100	NS
Mean HbA1c (SD) %	8.0 (1.3)	8.9 (1.0)	8.2 (1.4)	8.3 (1.2)	NS
Positivity for IA-2Ab (%)	70.6	20.0	62.9	47.1	$P < 0.001$
Positivity for ZnT8Ab (%)	36.8	0.0	32.9	23.5	$P < 0.01$

ELISA, enzyme-linked immunosorbent assay; GADAb, glutamic acid decarboxylase antibodies; HbA1c, glycated hemoglobin; IA-2Ab, autoantibody to protein tyrosine phosphatase IA-2; NS, not significant; RIA, radioimmunoassay; SD, standard deviation; ZnT8Ab, autoantibody to the cation efflux transporter zinc transporter 8.

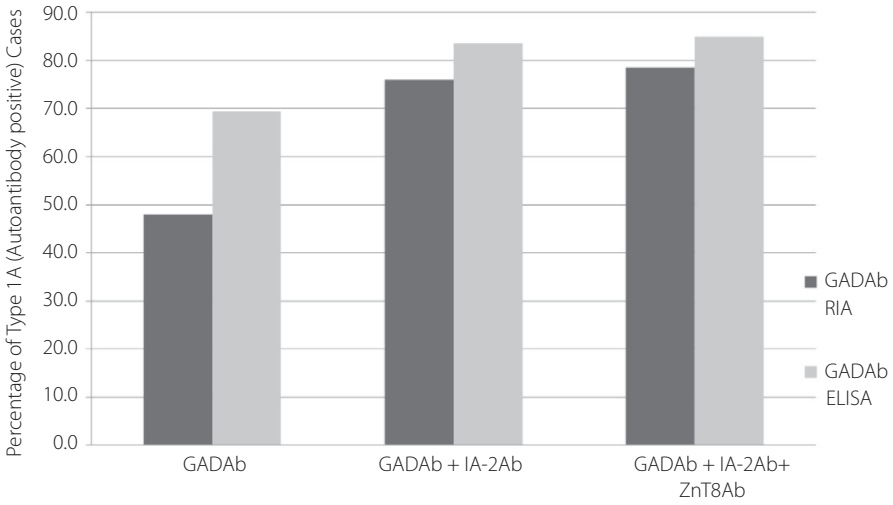

Figure 2 | Ratio of type 1A (autoantibody-positive) diabetes patients based on the positive detection of either autoantibody to protein tyrosine phosphatase IA-2 (IA-2Ab) or autoantibody to the cation efflux transporter zinc transporter 8 (ZnT8Ab) or both in addition to glutamic acid decarboxylase antibodies (GADAb) in 628 type 1 diabetes patients within 5 years after diagnosis. The percentage of type 1A patients diagnosed increased significantly with the addition of the IA-2Ab and ZnT8Ab results to the GADAb results using radioimmunoassay (RIA): from 47.9% using the RIA results for GADAb alone to 76.0% using the RIA + IA-2Ab results and 78.5% using the RIA + IA-2 Ab + ZnT8 Ab results, and from 69.4% using the ELISA results for GADAb alone to 83.6% using the ELISA + IA-2 Ab results and 84.9% using the ELISA + IA-2Ab + ZnT8Ab results.

suggest that many type 1A (autoimmune) patients were present (false negative cases) among the patients that were determined to be GADAb-negative using RIA.

Table 4 shows the HLA-DRB1, DQB1 allele frequencies in the four groups divided according to the positivity profiles for GADAb RIA and ELISA in 649 children with type 1 diabetes. In Table 4, significant differences were seen between Gr I versus the control, between Gr III versus the control, and between Gr IV versus the control in terms of the frequencies of susceptible and protective DRB1 and DQB1 alleles. In contrast, significant differences in DRB1*09:01 ($P < 0.01$) and DQB1*03:03 ($P < 0.05$) were only observed for Gr II versus the control. The other allele numbers in Gr II might have been too small to be analyzed statistically. Gr I and Gr III only had different frequencies of the protective allele DQB1*03:01 ($P < 10^{-5}$), and Gr I and Gr IV had different frequencies of the susceptible allele DQB1*04:01 ($P < 0.05$), as well as the protective allele DQB1*03:01 ($P < 0.01$).

Table 5 shows the HLA-DRB1-DQB1 haplotype frequency in the four groups. In Table 5, Gr I, Gr III and Gr IV differed significantly from the control in terms of the major three susceptible haplotypes and three protective haplotypes, but Gr II only had a significant difference for the DRB1*09:01-DQB1*03:03 haplotype ($P < 0.01$) relative to the control. Gr I and Gr IV had a significant difference in the susceptible DRB1*04:05-DQB1*04:01 haplotype, but Gr I and Gr III did not have any differences in any of the DRB1-DQB1 haplotypes.

DISCUSSION

We showed that the positivity rate for GADAb increased by approximately 20%, from 50% to 70%, by changing the assay method from RIA to ELISA in Japanese children and adolescents with type 1 diabetes. The better sensitivity and specificity of the GADAb ELISA have also been shown in the Diabetes Antibody Standardization Program[23].

In previous reports, Oikawa *et al.* showed that in 165 Japanese patients with type 1 diabetes, just 10 patients (6.1%) were RIA-negative and ELISA-positive for GADAb (Gr III), and 14 patients (22.2%) were RIA-positive and ELISA-negative (Gr II) among the 63 patients with slowly progressive type 1 diabetes[10]. Also, 25–30% of GADAb-positive slowly progressive type 1 diabetes adult-onset patients originally diagnosed using RIA were later found to be negative when tested using ELISA[11,12]. In contrast to previous reports, the number of patients that were RIA-negative and ELISA-positive for GADAb (Gr III) was as high as 140 (22.3%) among the 628 patients with type 1 diabetes in the present study who were assayed within 5 years after diagnosis, and just five patients (0.8%) were RIA-positive and ELISA-negative for GADAb (Gr II; Tables 2, 3). Recently, Kawasaki *et al.* showed that the RSR-RIA kit (which is the same as the RIA kit from Cosmic) identifies both high- and low-affinity GADAb, whereas the RSR-ELISA kit (which is the same as the ELISA kit from Cosmic) identifies only high-affinity GADAb[19]. Thus, the patients in Gr II who were RIA-positive and ELISA-negative for GADAb might have only low-affinity GADAb, and not high-affinity GADAb.

Table 4 | Human leukocyte antigen DRB1 and DQB1 allele frequencies in the four groups divided according to the positivity profiles for glutamic acid decarboxylase antibodies radioimmunoassay and enzyme-linked immunosorbent assay in 649 children with type 1 diabetes

HLA allele	Group I		Group II		Group III		Group IV		Control		I vs Control	II vs Control	III vs Control	IV vs Control	I vs II	I vs III	I vs IV	II vs III	II vs IV	III vs IV
	n = 532	%	*n* = 10	%	*n* = 308	%	*n* = 448	%	*n* = 2432	%										
DRB1																				
Susceptible																				
*04:05	126	23.68	2	20.00	90	29.22	142	31.70	322	13.26	$<10^{-6}$	NS	$<10^{-9}$	$<10^{-16}$	NS	NS	NS	NS	NS	NS
*08:02	48	9.02	0	0.00	32	10.39	29	6.47	102	4.18	$<10^{-3}$	NS	$<10^{-3}$	NS	NS	NS	NS	NS	NS	NS
*09:01	169	31.77	6	60.00	102	33.12	132	29.46	342	14.08	$<10^{-17}$	<0.01	$<10^{-12}$	$<10^{-11}$	NS	NS	NS	NS	NS	NS
Protective																				
*08:03	6	1.13	0	0.00	3	0.97	7	1.56	202	8.29	$<10^{-9}$	NS	$<10^{-5}$	$<10^{-6}$	NS	NS	NS	NS	NS	NS
*15:01	3	0.56	0	0.00	0	0.00	7	1.56	173	7.11	$<10^{-9}$	NS	$<10^{-7}$	$<10^{-4}$	NS	NS	NS	NS	NS	NS
*15:02	13	2.44	0	0.00	6	1.95	14	3.13	246	10.13	$<10^{-7}$	NS	$<10^{-5}$	$<10^{-4}$	NS	NS	NS	NS	NS	NS
Neutral																				
13:02	36	6.77	0	0.00	21	6.82	30	6.70	166	6.83	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
*01:01	38	7.14	2	20.00	11	3.57	37	8.26	141	5.81	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Others	93	17.48	0	0.00	43	13.96	50	11.16	738	30.35	$<10^{-7}$	NS	$<10^{-7}$	$<10^{-16}$	NS	NS	NS	NS	NS	NS
DQB1																				
Susceptible																				
*03:02	97	18.23	0	0.00	59	19.16	62	13.84	227	9.32	$<10^{-5}$	NS	$<10^{-4}$	<0.05	NS	NS	NS	NS	NS	NS
*03:03	172	32.33	6	60.00	103	33.44	134	29.91	361	14.86	$<10^{-16}$	<0.05	$<10^{-11}$	$<10^{-10}$	NS	NS	NS	NS	NS	NS
*04:01	111	20.86	2	20.00	85	27.60	133	29.69	317	13.03	$<10^{-3}$	NS	$<10^{-7}$	$<10^{-14}$	NS	NS	<0.05	NS	NS	NS
Protective																				
*03:01	35	6.58	0	0.00	0	0.00	9	2.01	282	11.61	$<10^{-2}$	NS	$<10^{-13}$	$<10^{-9}$	NS	$<10^{-5}$	$<10^{-2}$	NS	NS	NS
*06:01	18	3.38	0	0.00	6	1.95	19	4.24	440	18.11	$<10^{-19}$	NS	$<10^{-14}$	$<10^{-13}$	NS	NS	NS	NS	NS	NS
*06:02	1	0.19	0	0.00	0	0.00	7	1.56	151	6.22	$<10^{-9}$	NS	$<10^{-5}$	$<10^{-3}$	NS	NS	NS	NS	NS	NS
Neutral																				
*04:02	6	1.13	0	0.00	6	1.95	7	1.56	97	3.98	$<10^{-2}$	NS	NS	NS	NS	NS	NS	NS	NS	NS
*06:04	36	6.77	0	0.00	21	6.82	29	6.47	167	6.88	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
*05:01	39	7.33	2	20.00	12	3.90	37	8.26	159	6.53	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Others	17	3.20	0	0.00	16	5.19	11	2.46	231	9.50	$<10^{-4}$	NS	NS	$<10^{-5}$	NS	NS	NS	NS	NS	NS

Others of DRB1: *03:01, *04:01, *04:03, *04:04, *04:06, *04:07, *04:10, *07:01, *10:01, *11:01, *11:05, *11:06, *12:01, *12:02, *14:01, *14:03, *14:06, *16:02 Others of DQB1: *02:01, *05:02, *06:09 Control: from ref. 26. HLA, human leukocyte antigen; NS, not significant.

Table 5 | Human leukocyte andtigen DRB1-DQB1 haplotype frequencies in the four groups divided according to the positivity profiles for glutamic acid decarboxylase antibodies radioimmunoassay and enzyme-linked immunosorbent assay in 649 children with type 1 diabetes

HLA haplotype	Group I		Group II		Group III		Group IV		Control		Pc									
DRB1-DQB1	*n* = 532	%	*n* = 10	%	*n* = 308	%	*n* = 448	%	*n* = 1032	%	I vs Control	II vs Control	III vs Control	IV vs Control	I vs II	I vs III	I vs IV	II vs III	II vs IV	III vs IV
Susceptible																				
*09:01-*03:03	164	30.8	6	60.0	100	32.5	128	28.6	138	13.37	$<10^{-13}$	<0.01	$<10^{-10}$	$<10^{-8}$	NS	NS	NS	NS	NS	NS
*04:05-*04:01	112	21.1	2	20.0	87	28.2	133	29.7	134	12.98	$<10^{-3}$	NS	$<10^{-6}$	$<10^{-10}$	NS	NS	<0.05	NS	NS	NS
*08:02-*03:02	48	9.0	0	0.0	31	10.1	25	5.6	20	1.94	$<10^{-7}$	NS	$<10^{-6}$	<0.01	NS	NS	NS	NS	NS	NS
*04:05-*03:02	11	2.1	0	0.0	3	1.0	8	1.8	0	0.00	$<10^{-3}$	NS	NS	$<10^{-3}$	NS	NS	NS	NS	NS	NS
Protective																				
*08:03-*06:01	5	0.9	0	0.0	1	0.3	7	1.6	62	6.01	$<10^{-4}$	NS	$<10^{-3}$	$<10^{-3}$	NS	NS	NS	NS	NS	NS
*15:02-*06:01	12	2.3	0	0.0	5	1.6	12	2.7	92	8.91	$<10^{-4}$	NS	$<10^{-3}$	$<10^{-3}$	NS	NS	NS	NS	NS	NS
*15:01-*06:02	1	0.2	0	0.0	0	0.0	7	1.6	118	11.43	$<10^{-18}$	NS	$<10^{-11}$	$<10^{-9}$	NS	NS	NS	NS	NS	NS
Neutral																				
*04:07-*03:02	8	1.5	0	0.0	6	1.9	7	1.6	4	0.39	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
*01:01-*05:01	38	7.1	2	20.0	11	3.6	37	8.3	40	3.88	NS	NS	NS	<0.01	NS	NS	NS	NS	NS	NS
*13:02-*06:04	36	6.8	0	0.0	21	6.8	29	6.5	56	5.43	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
*15:01-*03:01	2	0.4	0	0.0	0	0.0	0	0.0	2	0.19	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Others	95	17.9	0	0.0	43	14.0	55	12.3	366	35.47	$<10^{-10}$	NS	$<10^{-11}$	$<10^{-18}$	NS	NS	NS	NS	NS	NS

Control: from Ref. 27. HLA, human leukocyte antigen; NS, not significant.

In the present study, Gr II contained just five patients, and was unique in terms of the age at diagnosis (which was significantly lower in this group than in Gr I), being predominantly male, and showing significantly lower positivity rates for IA-2Ab and ZnT8Ab (Table 3). Gr II was also genetically unique in our study, as four of the five cases in this group had HLA-DRB1*09:01-DQB1*03:03 (Table 5), which is a susceptible genotype for type 1 diabetes among Japanese type 1 diabetes patients, and has been reported to occur at a significantly higher frequency among patients with acute-onset type 1 diabetes aged between 2 and 5 years[22]. In contrast to previous reports on adult-onset type 1 diabetes, Gr II in the present study did not contain any patients with the clinical or genetic characteristics of slowly progressive type 1 diabetes[24]. In the present study, just four of the 628 patients within 5 years after diagnosis had slowly progressive type 1 diabetes. This relatively small number of patients with slowly progressive type 1 diabetes might be the major reason for the discrepancy between the results of the previous study examining adults and those of the present study examining children.

Gr III showed similar characteristics to Gr I in terms of the age at diagnosis, the male/female ratio, and the relatively high positivity rates for both IA-2Ab and ZnT8Ab; however, the GADAb titers in this group were relatively low. Of note, the genetic characteristics in terms of the HLA genotypes were quite similar between Gr I and Gr III (Tables 4, 5). Gr I and Gr III showed no significant difference in DRB1-DQB1 haplotype frequency (Table 5).

We considered it striking that there was a discrepancy in the positivity rates for GADAb between RIA and ELISA in the present study, because the prevalence of type 1A patients among Japanese childhood-onset type 1 diabetes patients would decrease by 20% if RIA alone were used to measure GADAb. However, the percentage of type 1A patients increased significantly when the results of tests for IA-2Ab and ZnT8Ab were added to the results of RIA for GADAb; the prevalence increased from 47.9%, as determined using RIA for GADAb alone, to 78.5% using RIA for GADAb + IA-2Ab + ZnT8Ab. These results strongly suggest that Gr III might have contained type 1A patients who had been misdiagnosed using RIA for GADAb alone (false negative cases; Figure 2). These discordant results between the two assays might be related to the epitope specificity of the two assays, because the GAD65 molecules used in these two kits are different: a truncated GAD65 lacking amino acids 2–45 in the N-terminal region is used in the RIA kit, and a full-length recombinant protein is used in the ELISA kit[19,25]. Another possible reason for the discordant GADAb result might be related to the cut-off values for the two kits. The GADAb titer (y) using an ELISA kit was correlated with the GADA titer (x) using an RIA kit as follows: $y = 27.807x - 19.526$ ($r = 0.845$, $P < 0.001$). The cut-off value for the ELISA kit (5.0 U/mL) was equivalent to 0.88 U/mL using the RIA kit, which is below the cut-off value (1.5 U/mL) for the RIA kit. Therefore, the negative results using the RIA

kit in our 140 ELISA-positive sera samples might have been due to the low GADAb titers, even though they have high-affinity antibodies. In other words, the positivity rate in cases of childhood-onset Japanese type 1 diabetes with lower titers of GADAb might be improved using ELISA.

In conclusion, the positivity rate in Japanese patients with childhood-onset type 1 diabetes and a relatively low GADAb titer was increased using an ELISA; the positivity rate for GADAb increased by approximately 20%, from 50% using RIA alone to 70% using ELISA, when the assay method was switched in cases assayed within 5 years after diagnosis.

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DISCLOSURE

The authors declare no conflict of interest.

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