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【 ORIGINAL ARTICLE 】

Screening for Gaucher Disease Using Dried Blood Spot Tests: A Japanese Multicenter, Cross-sectional Survey

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Abstract:

Objective For patients with Gaucher disease (GD), a rare, inherited lysosomal storage disease, obtaining a definitive diagnosis is currently time-consuming and costly. A simplified screening method to measure the glucocerebrosidase (GBA) activity using dried blood spots (DBS) on filter paper has recently been developed. Using this newly developed screening method, we evaluated real-world GD screening in patients suspected of having GD.

Methods This multicenter, cross-sectional, observational study with a diagnostic intervention component evaluated real-world screening in patients suspected of having GD based on their clinical symptoms and a platelet count <120,000/μL. The endpoint was the number of patients with low GBA activity determined using DBS.

Results In 994 patients who underwent initial DBS screening, 77 had low GBA activity. The assay was not repeated in 1 patient who was diagnosed as having a high possibility of GD due to clinical symptoms, and a further 21 patients completed the study without undergoing the second assay. Of the remaining 55 patients who had 2 DBS assays performed, 11 had a low GBA activity in both assays. Overall, DBS screening identified 12 (1.2%) patients with a low GBA activity, a proportion consistent with prior screening studies.

Conclusion These results suggest that the simplified DBS method was less burdensome to patients, was easily utilized by many physicians, and could be a useful first-tier screening assay for GD prior to initiating burdensome genetic testing.

Key words: dried blood spot test, Gaucher disease, glucocerebrosidase, lysosomal storage disorder, screening

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Introduction

Inherited genetic defects that affect metabolism often result in insufficiency or complete lack of a specific enzyme required either for energy production or to break down toxic metabolites. Under normal conditions, glycosphingolipids, vital components of the cell membrane, are continuously recycled via enzymatic degradation within intracellular lysosomes (1). However, many inherited defects in lysosomal enzymes have now been documented, and a large number of lysosomal storage diseases have been identified and characterized (2, 3). Although rare, these diseases are generally progressive and can be fatal due to ongoing toxic gly-

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cosphingolipid accumulation if left untreated (1, 2).

One of the breakdown products of glycosphingolipids is glucocerebroside, and the enzyme responsible for this degradation step is glucocerebrosidase (GBA) (1). In Gaucher disease (GD), a rare, inherited lysosomal storage disease caused by an autosomal recessive mutation in the *GBA* gene (4, 5), patients display a decrease in the activity of lysosomal GBA enzyme. This, in turn, leads to an accumulation of glucocerebroside in macrophages. Affected macrophages transform into Gaucher cells, which infiltrate the bone marrow, liver, spleen, and other organs, resulting in tissue damage, which may ultimately lead to life-altering or life-threatening complications (6, 7). Numerous mutations in the *GBA* gene have been identified, which result in variations in the disease course (1). Patients commonly present with an enlarged spleen and liver, hematologic disorders (such as thrombocytopenia, anemia, and leukopenia, which may be associated with an increased risk of bleeding and infection), and skeletal abnormalities (including pain, osteonecrosis, reduced bone density, and growth defects) (2).

According to published estimates, the incidence of GD varies widely between ethnic groups, affecting 1 in 450 to 4,000 Ashkenazi Jews (7, 8), 1.16 in 100,000 people in the US and Europe, (7, 9, 10) and 1 in 330,000 people in Japan (11, 12). GD is steadily progressive if left untreated, so immediate medical attention to obtain a diagnosis and treatment by a specialist is important (13). At present, enzyme replacement therapy (ERT) is the standard treatment in medical practice both in Japan and in other countries (14, 15). With ERT, symptoms, including hepatosplenomegaly, anemia, thrombocytopenia, and bone symptoms (pain and bone crises), are improved, enhancing the quality of life of affected patients (5, 15-17).

The rarity of GD, together with the overlap in similar symptoms and presentations found in many other diseases, makes it difficult to achieve an accurate diagnosis (18, 19). Furthermore, there may be many patients with unrecognized GD who have not received appropriate treatment, partly because a definitive diagnosis can only be established by medical institutions with limited resources (13, 20). A definitive diagnosis of GD requires either a measurement of the GBA activity or a genetic analysis to identify specific mutations in the *GBA* gene. Owing to the large number of unique gene mutations identified in the *GBA* gene (21), genetic testing can be a time-consuming and costly burden for patients. Thus, there is significant interest among clinicians in the development of a rapid and simple assay to evaluate the GBA activity as a first-tier test for patients suspected of having GD (22-25).

A simplified method for screening newborn babies using dried blood spots (DBS) on filter paper has recently been developed (26). In this simplified assay, the GBA activity can be easily measured by mailing the filter paper that has absorbed the blood to a specialized laboratory. This method allows for the easy storage of the sample, so ordering the assay from a distance is feasible (26). In addition, this assay only requires 1 mL of blood, which means that it is relatively non-invasive and decreases the patient burden. This method is robust, sensitive, and suitable for high-throughput analysis of hundreds of samples (27).

The objectives of this study were to evaluate real-world GD screening by measuring the GBA activity in patients suspected of having GD based on their clinical symptoms using the DBS assay. The secondary objectives were to investigate the demographic characteristics and medical histories of patients who were/were not diagnosed with GD and evaluate any differences between the groups.

Materials and Methods

Study design and patients

This was a multicenter, cross-sectional, observational study, with a diagnostic intervention component. The study was conducted at 120 sites in Japan between April 25, 2016, and December 31, 2018.

Patients suspected of having GD with a platelet count <120,000/μL were eligible for inclusion in the study and were enrolled in a consecutive series. Since the study aimed to clarify the morbidity of GD, and GD is a rare disease with a small population of affected patients, as many eligible patients as possible were included. However, patients who had been formally diagnosed with idiopathic thrombocytopenic purpura (ITP) or aplastic anemia (AA) were excluded from participation, as were any patients who were not otherwise deemed eligible for participation by the study investigator.

Ethics

This study was conducted in compliance with the 1964 Declaration of Helsinki and its later amendments (28), "Ethical Guidelines on Medical Research for Humans" (Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare) (29), and in accordance with the International Conference on Harmonization E6 guideline for Good Clinical Practice (30).

The director of Kyushu University Hospital and the research ethics committee (REC) of Kyushu University reviewed and approved the study protocol and informed consent form; items were then reviewed, and the study was approved by the REC or institutional review board at each facility (UMIN000021839). All patients or their proxy provided their voluntary, written, informed consent prior to study participation.

Measurements and endpoints

At registration, the following information was collected: baseline demographic data and medical history, clinical symptoms (anemia, bleeding disposition, hepatosplenomegaly, bone symptoms, and neurological symptoms), and clinical laboratory test results [platelet count, hemoglo-

bin (Hb), serum ferritin, angiotensin-converting enzyme (ACE), and acid phosphatase (ACP)].

The study endpoint was the number of patients with a low GBA activity, with the level of GBA activity determined using the DBS assay.

Although a genetic analysis for GD was outside the scope of this study protocol, we reported on the patients who had been definitively diagnosed with GD by genetic testing.

GBA activity assay

The screening protocol for GBA was performed on DBS samples using the method described previously (26), with minor modification. In brief, the study investigator collected blood samples (approximately 1 mL) from each registered patient and spotted it onto a piece of filter paper. The sample was air-dried for at least 5 hours at room temperature and was then sent to the diagnostic laboratory (Mass Screening Team, Department of Pediatrics, Kumamoto University, Graduate School of Medical Sciences, Kumamoto, Japan) by mail within 1 week of collection. A 3.2-mm diameter disk was punched from each DBS filter paper sample, and GBA was extracted into 100 μL of extraction buffer (pH 6.0; 0.1% Triton X-100, 5 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.05% NaN_3 in 25 mM citric acid-potassium phosphate buffer). The DBS sample extract (20 μL) was transferred into a black 96-well assay plate. Substrate solution (40 μL), comprising 3 mM 4-methylumbelliferyl-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, USA) and 0.3% sodium taurodeoxycholate in 100 mM citrate-phosphate buffer (pH 5.0) was added to each well, and the reaction mixture was incubated for 3 h at 37°C. In order to stop the reaction, 200 μL of 300 mM glycine-NaOH buffer (pH 10.6) were added to each well. A fluorometer was used to analyze the assay plates at 370-nm excitation and 465-nm emission wavelengths. Molar product quantities in the assay wells were calculated by linear regression from the standard curve. The enzyme activity was expressed as picomoles of 4-methylumbelliferyl-β-D-glucopyranoside released per hour per disk (pmol/h/disk).

In this analysis, a low GBA activity level (study endpoint) was defined as a GBA activity <3 pmol/h/disk, and any patient with two consecutive results of GBA activity below this level was deemed, in principle, to have a low GBA activity. Additional measurements to exclude sample defects or to determine the impact of pancytopenia on the GBA activity included α-galactosidase and α-glucosidase activity.

The molecular analysis of the GBA gene

The analysis method of the *GBA* gene has been previously reported (31). A Puregene Blood Core Kit B (Qiagen, Hilden, Germany) was used to extract genomic deoxyribonucleic acid from peripheral blood, and a long-range polymerase chain reaction was performed to amplify the *GBA* gene plus its flanking regions. Sequencing was conducted using a MiSeq sequencer (Illumina, San Diego, USA), alongside a reference sequence comprising the region from 155,203,938 to 155,217,562 of chromosome 1 (NC_000001.10). Processing of the sequencing data, analysis, mapping, and variant identification was conducted using MiSeq Reporter v2 (Illumina).

Statistical analyses

For this analysis, the target number of patients was 1,200 nationwide, with the aim of enrolling 10 patients per site (120 sites). However, the study would finish at the end of the specified analysis period, irrespective of whether or not the number of enrolled patients had reached 1,200.

In accordance with the intention-to-treat (ITT) principle, all patients who provided their consent and were enrolled in the study were included in the analyses. No imputations for missing data were performed. Patient background factors were described descriptively, using numbers and percentages for categorical variables and means and standard deviations (SD) for continuous variables. The total number and proportion of patients with a low GBA activity according to the DBS assay were calculated. The demographic and clinicopathologic factors of patients were recorded and compared between the ITT population and the group of patients with a low GBA activity. Since there were few patients with a low GBA activity, no formal statistical comparisons were performed. All statistical calculations were performed using the SAS software program, version 9.4 (SAS, Cary, USA).

Results

Patient characteristics

Between April 25, 2016, and December 31, 2018, 1,005 patients were registered in the study, and 994 patients were included in the ITT population (Figure). Eleven patients were excluded from the analysis, either because they were found not to have met the selection criteria (n=2) after registration or due to the patient's or physician's decision not to continue the study (n=9).

Patients' clinicodemographic factors are shown in Table. The mean age was 67 years old, and approximately 85% of patients were ≥50 years old. The most common initial diagnoses were as follows: suspected ITP in 228 (22.9%), myelodysplastic syndrome (MDS; including suspected MDS) in 188 (18.9%), and possible AA in 99 (10.0%). The main clinical symptoms reported were anemia (n=267; 26.9%), bleeding disposition, (n=246; 24.7%), and splenomegaly (n=215; 21.6%). The platelet count was $\leq 12\times10^4$/μL in all patients, and the mean value was $7.45\pm3.18\times10^4$/μL.

The GBA activity

The results of GD screening by measuring the GBA activity using the simplified DBS assay are shown in Figure. Following the initial DBS assay, 77 patients were recorded as having a low GBA activity. One patient was diagnosed with a high possibility of GD due to clinical symptoms; the GBA activity measurement was thus not repeated. Therefore,

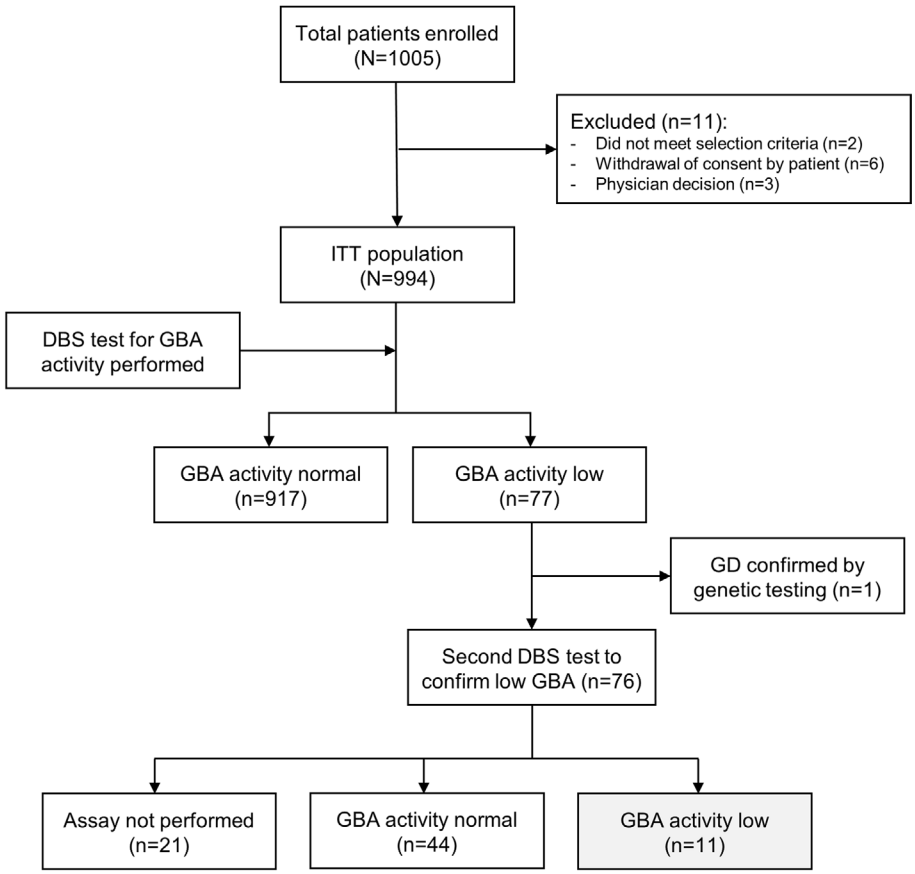

Figure. Results of GBA activity measurement using DBS. DBS: dried blood spots, GBA: glucocerebrosidase, GD: Gaucher disease, ITT: intention-to-treat

the number of patients requiring a second assay for confirmation of a low GBA activity was 76. Of these, 21 patients completed the study without undergoing the second assay. In total, 11 patients were diagnosed with a low GBA activity at both the first and second assays. When combined with the patient who had a low GBA count after the first assay and was subsequently diagnosed with GD, we identified a total of 12 (1.2%) patients with a low GBA activity (primary outcome) in this study.

Patient factors correlated with a low GBA activity

During the screening process to assess the GBA activity, the background factors in the patients with low values were evaluated and compared with those in the ITT population (Table). We found no notable differences in the age, sex, height, weight, or body mass index (BMI) between the groups. There were also no meaningful differences in clinical symptoms, nor were any notable changes in laboratory test values recorded, despite the disparity in the numbers of patients between groups, with the exception of serum ferritin. In patients with a low GBA activity, the mean serum ferritin level was 383.28 ng/mL, which was higher than in the ITT population (243.29 ng/mL), both at the initial assay and second assay.

The genetic analysis

We evaluated one patient who was definitively diagnosed with GD and another with confirmed GD-related gene mutations (heterozygous) who was nevertheless not definitively diagnosed with GD.

The first patient was a 75-year old woman who presented for a general checkup. She had suffered a compression bone fracture in T4 at 72 years-old. Her current blood laboratory results were normal except for pancytopenia. The platelet count was very low (6.3×10^4/μL), as was the Hb level (5.3 g/dL). The white blood cell count was 2,800/μL, and her BMI was 16.0 kg/m^2. On a physical examination, her abdomen appeared normal, without bulging or masses. The liver and spleen were not palpable below the costal margins. She had kyphosis, but she had no signs of oculomotor problems or other neurological abnormalities. Computed tomography showed mild hepatosplenomegaly. Gaucher cells were observed in 6.4% of the bone marrow obtained by bone marrow aspiration. To confirm the diagnosis of GD, two additional tests were conducted. The first test, for enzyme activity, showed that her glucocerebrosidase (filter paper method) had deteriorated. The second test, for genotype modification, showed that her glucocerebrosidase genotype was R353W (homozygosis). Based on these results, she was diagnosed with GD Type I. ERT was initiated, but the serum level of

Table. Comparison of Patient Clinicodemographic Characteristics.

		GBA activity measurement			
		Total (ITT)	Initial assay GBA activity low level	Second assay Genetic diagnosis performed	Initial assay Genetic diagnosis performed
Age (years)	N	994	77	11	1
	Mean±SD	66.7±15.8	67.4±15.1	65.5±15.0	75
	<10	0 (0.0)	0 (0.0)	0 (0.0)	-
	10–19	11 (1.1)	1 (1.3)	0 (0.0)	-
	20–29	21 (2.1)	1 (1.3)	0 (0.0)	-
	30–39	50 (5.0)	5 (6.5)	1 (9.1)	-
	40–49	55 (5.5)	2 (2.6)	0 (0.0)	-
	50–59	116 (11.7)	7 (9.1)	2 (18.2)	-
	60–69	247 (24.8)	20 (26.0)	3 (27.3)	-
	70–79	289 (29.1)	27 (35.1)	4 (36.4)	1 (100.0)
	≥80	205 (20.6)	14 (18.2)	1 (9.1)	-
Sex	Male	524 (52.7)	34 (44.2)	6 (54.5)	-
Height (cm)	N	987	76	11	1
	Mean±SD	159.41±9.41	159.17±9.16	161.24±10.69	153.00
Weight (kg)	N	988	76	11	1
	Mean±SD	57.85±12.69	56.24±13.09	58.08±17.12	37.50
BMI (kg/m^2)	N	987	76	11	1
	Mean±SD	22.65±3.92	22.08±4.22	21.97±4.26	16.02
	<18.5	133 (13.4)	15 (19.5)	2 (18.2)	1 (100.0)
	18.5-<25	632 (63.6)	47 (61.0)	7 (63.6)	-
	≥25	222 (22.3)	14 (18.2)	2 (18.2)	-
Comorbidity	Yes	434 (43.7)	24 (31.2)	5 (45.5%)	1 (100.0)
Complications	Yes	686 (69.0)	45 (58.4)	9 (81.8)	1 (100.0)
Treatment for complications	Yes	598 (60.2)	39 (50.6)	7 (63.6)	-
Initial diagnosis[a,b]					
	ITP	228 (22.9)	6 (7.8)	1 (9.1)	-
	MF	16 (1.6)	5 (6.5)	0 (0.0)	-
	AA	99 (10.0)	10 (13.0)	2 (18.2)	-
	MDS	188 (18.9)	24 (31.2)	1 (9.1)	-
	Others	415 (41.8)	26 (33.8)	6 (54.5)	1 (100.0)
Clinical symptoms[a]	Bleeding disposition	246 (24.7)	24 (31.2)	3 (27.3)	1 (100.0)
	Anemia	267 (26.9)	30 (39.0)	4 (36.4)	1 (100.0)
	Hepatomegaly	85 (8.6)	7 (9.1%)	0 (0.0)	-
	Splenomegaly	215 (21.6)	20 (26.0)	2 (18.2)	1 (100.0)
	Fracture	16 (1.6)	1 (1.3)	1 (9.1)	-
	Neurological symptoms	19 (1.9)	0 (0.0)	0 (0.0)	-
	Others	3 (0.3)	1 (1.3)	0 (0.0)	-
Platelet count (10^4/μL)	N	994	77	11	1
	Mean±SD	7.45±3.18	6.91±3.64	7.41±4.52	4.50
Hb (g/dL)	N	994	77	11	1
	Mean±SD	11.98±2.55	11.56±2.66	11.42±3.21	9.30
Serum ferritin (ng/mL)	N	542	49	4	1
	Mean±SD	243.29±469.34	383.28±563.54	1,253.55±1276.85	66.70
ACE (U/L)	N	58	7	0	0
	Mean±SD	14.43±6.21	14.30±6.70	-	-
ACP (U/L)	N	7	0	0	0
	Mean±SD	217.49±195.76	-	-	-

Values are shown as n (%) except where stated. [a]Multiple answers, [b]Including suspicion.

AA: aplastic anemia, ACE: angiotensin-converting enzyme, ACP: acid phosphatase, BMI: body mass index, GBA: glucocerebrosidase, Hb: hemoglobin, ITP: idiopathic thrombocytopenic purpura, ITT: intention-to-treat population, MDS: myelodysplastic syndrome, MF: myelofibrosis, SD: standard deviation

angiotensin-converting enzyme did not increase.

The second patient was a 76-year old woman with clinical signs of bleeding and anemia. Additionally, she presented with pancytopenia and emaciation. She was thought to have AA. Her BMI was 15.8 kg/m^2, and her platelet, Hb, and serum ferritin measurements were 0.6×10^4/μL, 7.2 g/dL, and 210 ng/mL, respectively. This patient underwent two DBS assays and was found to have a low GBA activity in both. On genetic testing, the mutation found was 1582A>G (heterozygote), which had not yet been reported. As it was similar to a previously reported mutation, I528T, a possible diagnosis of GD was considered. The patient had no hepatosplenomegaly or nerve or bone lesions. Although her bone marrow was very hypoplastic, no Gaucher cells were observed. Regarding her familial history, the patient's daughter and grandchild had an autoimmune disease. As a result, the patient was not definitively diagnosed with GD.

Discussion

This large, observational, Japanese study was conducted to evaluate real-world GD screening in clinical practice by measuring the GBA activity with a simplified DBS method in patients who had a blood platelet count <12×10^4/μL and who were suspected of having GD based on clinical symptoms. At present, making the diagnosis of GD is costly and time-consuming (13, 20); the clinical symptoms can be difficult to differentiate from other more common disorders (18, 19), and genetic screening requires specialized laboratories and trained personnel. However, if left untreated, GD will continue to progress, causing an increase in morbidity and potential mortality in affected patients (6, 7). As such, there is a clear need for a first-tier test to better screen for patients with potential GD in order to focus subsequent resources on those who truly need more in-depth evaluations. While DBS testing has been used in clinical analyses for decades, recent technical advances have opened up new clinical applications for this routine procedure (32, 33). DBS testing has emerged as a key screening method to identify newborns with lysosomal storage diseases (34), but the widespread acceptance of a simple DBS test for GBA activity (26) could also facilitate the rapid identification of previously undiagnosed adult patients with GD.

In the current analysis, after measuring the GBA activity in the 994 enrolled patients using the simplified DBS method, the number of patients with a low GBA activity was determined to be 12 (1.2%). This proportion of affected patients is in line with data reported from a previous study using a very similar test to screen for GD with neurological symptoms in high-risk Japanese patients (N=102, aged 0 to 57 years), in which 2 patients (2.0%) were found to have a low GBA activity (26). The utility of the first-tier DBS screen in that study was subsequently confirmed by genetic testing, by which both patients with low activity were found to have GD-related mutations (26). Similarly, a recent Chinese pilot analysis evaluated the utility of a DBS test for GBA activity in children (18 days to 14 years old) at a high risk of GD (35). Of the 73 children tested, 4 were diagnosed with GD (prevalence rate of 5.5%), and all 4 were subsequently found to have gene mutations (35). In another Chinese study, DBS was used during newborn screening for GD in Shanghai (36); of 80,855 babies tested, 3 had a low GBA activity on the first test, and 1 was found to have a low activity on the second test, yielding a prevalence of 1.24/100,000, which is similar to the prevalence of 1.16/100,000 reported in the US and Europe (7, 9, 10).

Although confirmation of the DBS screen by genetic testing was not prespecified in our analysis, we nevertheless feel that the results obtained were of sufficient diagnostic importance; clinicians could have confidence in moving forward with initiating ERT or other treatments intended to reduce disease progression and improve patient health. The simplified DBS method is cost-effective and would help improve the time until the diagnosis and the accuracy of screening in patients with GD if used as an initial screening tool for patients with GD symptomatology, together with other diagnostic procedures (such as biochemical, bone marrow, and imaging tests). Only patients meeting test criteria (prespecified GBA levels) would need to undergo the more accurate but costly and time-consuming genetic analysis to confirm GD.

However, it must be noted that, in the present study, the criterion for a low GBA activity was set at <3 pmol/h/disk. This cut-off has been used previously in screening for GD (37), and we feel that setting a low value can help reduce the false-positive rate. Conversely, lowering the false-positive rate may not be the optimal route for detecting the incidence of a rare disease. Nevertheless, whichever cut-off is chosen, only a tentative identification of GD can be made based on DBS testing and clinical manifestations; genetic testing is still required to confirm a definitive diagnosis.

Disappointingly, while our study results were able to record and categorize the GBA activity in GD patients using the simplified DBS method, they were not able to demonstrate any clinicodemographic factors affecting the screening results using this method. Unfortunately, owing to the small number of patients with GD, the proportion of patients with a low GBA activity was extremely small (1.2%) in our analysis; it is possible that the amount of information was simply insufficient to indicate any marked differences between patients with low and normal GBA levels. However, we have included the clinical features of two cases, including one patient who was definitively diagnosed with GD and another with confirmed GD-related gene mutations (heterozygous) who was not definitively diagnosed with GD. In the first case, the main clinical features were a history of bone involvement (T4 compression fracture), thrombocytopenia, anemia, and mild hepatosplenomegaly, and her glucocerebrosidase genotype was R353W (homozygosis). These clinical features are consistent with those previously reported in five other cases of GD Type I identified recently

in Montenegro (38), in which the most common presentations included a variable degree of hepatosplenomegaly and thrombocytopenia. Notably, in those five cases, there was one case of homozygosity as in our case but with a different gene involvement (N370S mutation) (38). To our knowledge, in the second case, the mutation identified [1582A>G (heterozygote)] in the patient with confirmed GD-related gene mutations (heterozygous) but without a definitive diagnosis of GD has not been previously reported. The main clinical features of her case were bleeding, anemia, pancytopenia, and emaciation. Other cases of unique mutations have been reported in Japan (39). Based on the current reports, the mutation patterns and clinical phenotype of Japanese GD patients seem to differ considerably, with a much lower morbidity in Japanese than in Netherlands patients (40, 41). However, the better characterization of the genotypes, as well as phenotypes, can lead to a higher index of suspicion; prompt investigations, particularly in cases of splenomegaly, may result in earlier treatment and the prevention of complications.

Overall, the results from this study indicate that the simplified DBS method is capable of measuring the GBA activity quickly and inexpensively (26, 42). Another strength of this technique was that, in addition to GBA, the activities of α-galactosidase and α-glucosidase were also measured in order to ascertain whether the effect of pancytopenia alone was responsible for the enzymatic reduction. We consider that our data sufficiently eliminate the likelihood that a low GBA activity was an effect of the abnormal reduction in white blood cells. Furthermore, this technique was not burdensome to patients or physicians, and the simple sample preparation and lack of complex storage requirements allow physicians to order the test remotely.

However, we acknowledge that some improvements to the DBS methodology are needed. In our study, the assay results were not always consistent between the first and second DBS tests, with some patients who had a low GBA activity during the first test being found to have a normal GBA activity during the second test. By conducting two tests, as was also the procedure in the Chinese newborn study (36), the risk of false-positives and false-negatives owing to possible mishandling during the process of preparation or transport of the blood samples is somewhat reduced. It was noted that precise testing could not be performed in cases of sample defects resulting from inadequate drying of the filter at the time of blood collection and spotting. Furthermore, maintaining the samples at ambient temperature (>4°C and <24°C) is also necessary to retain enzyme activity; storage for prolonged periods outside this range has been shown to result in enzyme degradation and false-positive test results (43). Thus, there is a need for further education regarding the assay method, and research into identification and validation of improved methods to prepare and transport samples for the assay are warranted. It is also possible that pediatric doctors are more skilled at preparing and preserving the filter papers than clinicians who routinely see only adult patients and consequently are less familiar with the technique; additional training may then be needed in such cases.

In addition to highlighting the possible need for improvements in sample preparation, we acknowledge that our study has several limitations. First, clinical symptoms and complications in patients were reported by study investigators but were not confirmed by a third party (central review). Second, in some patients, insufficient test data were collected to be able to definitively diagnose GD. It is worth noting that ACP testing was not included in insurance coverage when this study was initiated, so only seven patients had measured ACP values. Furthermore, information on bone marrow tests, which are commonly used to support a GD diagnosis, was not collected. In addition, the number of patients with available ACE values was also small (only 58 patients in the ITT population). Third, there were 21 patients with low values after the initial measurement of GBA activity who did not undergo a second assay; their final diagnostic results are unknown, and this lack of information may introduce a degree of bias into the results obtained from the already small population with a low GBA activity who were available for analysis. Finally, verification of the final definitive diagnosis of GD and its accuracy was not performed. As genetic testing was outside the scope of our study, we cannot definitively link the DBS GBA activity with subsequent genetic data.

In conclusion, in this multicenter, cross-sectional, observational Japanese study, use of the simplified DBS method in patients suspected of having GD revealed that a small proportion of patients (1.2%) had a low GBA activity. Although our study revealed some issues in the preparation and handling of blood samples, the results suggested that the simplified DBS method was less burdensome to patients, was easily utilized by many physicians, and could be a useful screening assay for GD prior to costly and time-consuming genetic testing.

Author's disclosure of potential Conflicts of Interest (COI).
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