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ORIGINAL ARTICLE

Detection of novel Fabry disease-associated pathogenic variants in Japanese patients by newborn and high-risk screening

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Abstract

Background: In Japan, newborn and high-risk screening for Fabry disease (FD), an inherited X-linked disorder caused by *GLA* mutations, using dried blood spots was initiated in 2006. In newborn screening, 599,711 newborns were screened by December 2018, and 57 newborns from 54 families with 26 FD-associated variants were detected. In high-risk screening, 18,235 individuals who had symptoms and/or a family history of FD were screened by March 2019, and 236 individuals from 143 families with 101 FD-associated variants were detected. Totally 3, 116 variants were detected; 41 of these were not registered in Fabry-database.org or ClinVar and 33 were definitely novel. Herein, we report the clinical outcomes and discuss the pathogenicity of the 41 variants.

Methods: We traced nine newborns and 46 individuals with the 33 novel variants, and nine newborns and 10 individuals with eight other variants not registered in the FD database, and analyzed the information on symptoms, treatments, and outcomes.

Results: Thirty-eight of the 46 individuals with the 33 novel variants showed symptoms and received enzyme-replacement therapy and/or chaperone treatment.

Conclusion: Delayed diagnosis should be avoided in patients with FD. Our results will help clinicians diagnose FD and determine the appropriate treatment for patients with these variants.

KEYWORDS

Fabry disease, high-risk screening, novel variant, pathogenicity, α-galactosidase A

1 | INTRODUCTION

Fabry disease (FD; OMIM 301500) is an inherited X-linked disorder caused by mutations in *GLA*, which encodes the lysosomal enzyme α-galactosidase A (α-Gal A; EC 3.2.1.22). To date, 500–600 variants have been registered in various databases, including Fabry-database.org (Saito et al., 2011) and ClinVar (Landrum et al., 2020). In Japan, newborn screening

(NBS) and high-risk screening for FD were initiated in 2006. From August 2006 to December 2018, 599,711 newborns were screened, and 57 newborns from 54 families with 26 FD-associated variants were detected (Sawada et al., 2020). Moreover, 18,235 individuals who had renal, cardiac, or neurological manifestations and a family history of FD were screened from October 2006 to March 2019, and 236 individuals from 143 families with 101 FD-associated variants were detected. Eleven variants overlapped between the two studies, and thus, 116 variants were detected (Supplementary Table S1).

Of these 116 variants, 41 were not registered in Fabry-database.org or ClinVar, and 33 were definitely novel because their pathogenicity has not been reported. Information on the pathogenicity of these variants is important for clinicians to diagnose FD and determine the appropriate treatment for patients with these variants. PolyPhen-2 (Adzhubei et al., 2013), which is a bioinformatic tool to identify missense variants, and Human Splicing Finder (Desmet et al., 2009), which is used to detect variants based on splicing signals, are useful to estimate pathogenicity. However, these tools are not useful for diagnosis and evaluation of pathogenicity. Moreover, there is a lack of appropriate bioinformatic tools to evaluate frame-shift or nonsense mutations. Information about the presented symptoms, treatments, and treatment outcomes for each patient is essential to evaluate the disease status.

In this study, we traced nine of the 57 newborns and 46 of the 236 individuals with the 33 novel variants and followed their clinical treatment outcomes. Additionally, we traced nine newborns and 10 individuals to evaluate eight other variants not registered in the FD database. Herein, we report the clinical outcomes and discuss the pathogenicity of the 41 variants. This information will help clinicians to diagnose patients with FD and determine the appropriate treatment for patients with a variant of unknown significance (VOUS). Moreover, these results will help avoid delayed diagnosis and increase the quality of life and life expectancy of patients who require specific treatments.

2 | MATERIALS AND METHODS

2.1 | Study design

In Japan, NBS for FD was initiated in August 2006. Overall, 599,711 newborns from six prefectures (Kumamoto, Fukuoka, Miyazaki, Saga, Hiroshima, and Kagawa) and two hospitals (Palmore Hospital in Hyogo Prefecture and University of the Ryukyus Hospital in Okinawa Prefecture) were screened until December 2018. As a result, 57 newborns from 54 families with 26 FD-associated variants were detected (Sawada et al., 2020). High-risk screening for FD was initiated in December 2006. From all prefectures in Japan, 601 hospitals participated, and 18,235 individuals who showed cardiac, renal, or neurological manifestations and had a family history of FD were screened until March 2019. The manifestations in these individuals included at least one of the following: (a) cardiac manifestations (e.g., left ventricular hypertrophy in electrocardiography or echocardiography); (b) renal manifestations (e.g., proteinuria, chronic kidney disease anhidrosis, diabetic nephropathy, mulberries in urine, and receiving dialysis); (c) history of cerebral infarction and neurological manifestations (e.g., parkinsonism and hearing loss); (d) acroparesthesia, clustered angiokeratoma, corneal opacity, and hypohidrosis; (e) other manifestations (e.g., liver failure); and (f) a family history of FD. Among 236 individuals from 143 families, 101 FD-associated variants were identified. The preparation of DBSs and the flowchart for NBS have been described previously (Sawada et al., 2020). This study was approved by the Kumamoto University Ethics Committee. Written informed consent was obtained from the patients or their parents (in cases where the patients were not of legal age).

2.2 | α-Gal A assay

α-Gal A activity was determined using a fluorescent substrate, as previously described (Chamoles et al., 2001) (Method I). To achieve multiple screening of Fabry, Pompe, and Gaucher diseases simultaneously, Method I was improved to Method II and used practically from November 2016. Here, 483,026 and 116,685 newborns were screened using Methods I and II, respectively (Sawada et al., 2020). In high-risk screening, 16,061 and 2074 individuals were screened using Methods I and II, respectively.

2.2.1 | Method I

A single disk of diameter 3.2 mm punched from DBSs was incubated in each well of a 96-well clear microwell plate (Corning, NY, USA) containing 40 μl of McIlvaine buffer (100 mM citrate, 200 mM NaH_2PO_4, 36.8:63.2; pH 6.0). The samples were then processed for extraction by incubation for 2 h at room temperature. Aliquots of blood extracts (30 μl) were transferred to a fresh 96-microwell plate. Subsequently, 100 μl of the reaction mixture (3.5 mM 4-methylumbelliferyl-α-D-galactopyranoside [4MU-αGal], 100 mM citrate, 200 mM K_2HPO_4, and 100 mM N-acetyl-D-galactosamine; pH 4.4) was added to each well of the microwell plate and incubated for 24 h at 37°C. The reaction was then terminated by adding 150 μl of termination solution (300 mM glycine/NaOH; pH 10.6), and the fluorescence intensity of 4-methylumbelliferone was measured at 450 nm using a fluorescence plate reader (BIO-TEK, Winooski, VT,

USA). One unit (1 Agal U) of enzymatic activity was equal to 0.34 pmol of 4MU-αGal cleaved/h per disc.

2.2.2 | Method II

A single disk of diameter 3.2 mm punched from DBSs was incubated in each well of a 96-well clear microwell plate (AS ONE Corporation, Osaka, Japan) with 100 µl of 25 mM citrate/potassium phosphate buffer (pH 6.0) containing 5 mM of $MgCl_2$, 0.5 mM of dithiothreitol, 0.05% of NaN_3, and 0.1% of triton X-100 for 1 h at room temperature by gentle mixing. Subsequently, a 20-µl aliquot of this solution was added to 40 µl of the reaction mixture (3.0 mM 4MU-αGal and 100 mM N-acetyl-D-galactosamine in 100 mM citrate/200 mM potassium phosphate buffer; pH 4.4) in a 96-well black microwell plate (Thermo Fisher Scientific Inc., MA, USA), and the plate was incubated for 3 h at 38°C. The reaction was stopped by adding 200 µL of 300 mM glycine/NaOH buffer (pH 10.6) containing 10 mM of ethylenediaminetetraacetic acid to facilitate the measurement of fluorescence intensity. The residual extract was also used for the analysis of acid α-glucosidase (Pompe disease) and glucocerebrosidase (Gaucher disease).

In NBS, the cutoff value in Method I was 20 (Agal U) in the first assay and 15 (Agal U; men) and 20 (Agal U; women) in the second assay. The cutoff value in Method II was 30 (Agal U) in the first assay and 20 (Agal U; men) and 30 (Agal U; women) in the second assay. These cutoff values accounted for 36% of the median α-Gal A activity in men and 47% of the median α-Gal A activity in women. In the high-risk screening, the cutoff value in Method I was 12 (Agal U; men) and 20 (Agal U; women), and the cutoff value in Method II was 20 (Agal U; men) and 30 (Agal U; women). These cutoff values represented 49% of the median α-Gal A activity in men and 83% of the median α-Gal A activity in women.

2.3 | Sequencing of *GLA*

2.3.1 | Sanger method

The Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from total blood, and the samples were stored at −80°C until use. All seven exons and the flanking intronic sequences of *GLA* were amplified by polymerase chain reaction (PCR). Additionally, a specific region of intron 4 was amplified to evaluate the variant c.639+919G>A. The PCR products were sequenced on an ABI3500xl autosequencer (Applied Biosystems, Foster City, CA, USA) and evaluated using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

2.3.2 | Next-generation sequencing (NGS)

Next-generation sequencing has been used to sequence *GLA* for high-throughput analysis, since September 2017. Briefly, the 13.3-kbp region, which includes *GLA*, was amplified by long-range PCR. Library preparation and sequencing were carried out using the Nextera XT Kit (Illumina, San Diego, CA, USA) and MiSeq sequencer (Illumina). After sequencing, the data were aligned to the human reference genome sequence (NC_000023.10) using MiSeq Reporter software (Illumina). MiSeq Reported v2 (Illumina) was used for sequence data analysis, mapping, and variant calling. The sequenced reads were visualized using IGV_2.3.10 (Broad Institute). The variants detected in *GLA* by NGS were re-sequenced using the Sanger method.

2.3.3 | Significance analysis of the variants

The mRNA reference sequence (RefSeq, NM_000169.2) was utilized, whereby the "A" nucleotide in the ATG codon at nucleotide position 111 of RefSeq constituted +1 numbering of the cDNA sequence. The ATG codon also represented +1 for amino acid numbering established by α-Gal A preprotein sequence NP_000160.1. Variant nomenclature followed the guidelines established by the Human Genome Variation Society (http://varnomen.hgvs.org/). Public databases, including Fabry-database.org (Saito et al., 2011) (http://fabry-database.org/, updated at February 15, 2019) and ClinVar (Landrum et al., 2020) (http://www.ncbi.nlm.nih.gov/clinvar) were used for the classification of each variant. PolyPhen-2 software (Adzhubei et al., 2013) was used to predict the potential effect of missense mutations and the resulting amino acid alterations on the function of α-Gal A. Human Splicing Finder (Desmet et al., 2009) was also used for the variants based on splicing signals.

2.4 | Tracing studies

In our previous NBS study (Sawada et al., 2020), 57 newborns from 54 families with 26 FD-associated variants were identified. Among them, 10 were not registered in the Fabry-database.org or ClinVar database and seven variants were definitely novel. In this study, we selected nine newborns from nine families with seven novel variants and nine newborns from eight families with three variants not registered in the databases (Table 1). In our screening study, 236 high-risk individuals from 146 families with 101 FD-associated variants were detected. Among them, 33 variants were not registered in the Fabry-database.org or ClinVar database and 27 variants were definitely novel. In this study, we selected

TABLE 1 Patients selected for tracing studies who were identified in newborn screening for Fabry disease

Subject ID	Sex	α-Gal A activity	Age at 2020/June	Variants		Clinical symptoms at 2020/June	Amenability[b]	PolyPhen-2 (score)	Classification	Reference
				Nucleic acid	Amino acid					
1	F	21.7	5 years 8 months	**c.428C>T**	**p.A143V**	None	+	probably damaging	VOUS	—
2	F	17.8	6 years 6 months	**c.493G>A**	**p.D165N**		+	probably damaging	VOUS	—
3	M	8.8	13 years	**c.538T>G**	**p.L180V**		+	possibly damaging (0.470)	VOUS	—
4	M	<1[a]	NA	**c.605G>T**	**p.C202F**		−	probably damaging	VOUS	—
5	F	18.1	5 years 6 months	**c.625T>C**	**p.W209R**		+	benign (0.000)	nonpathogenic	—
6	M	16.5	11 years 7 months							
7	M	16.2	6 years 7 months							
8	M	11.9	NA	**c.714T>A**	**p.S238R**		−	probably damaging	VOUS	—
9	M	2.0[a]	2 years 1 months	**c.1231G>A**	**p.G411S**		+	probably damaging	VOUS	—
10	F	15.9	NA	c.685T>G	p.F229V		−	probably damaging	pathogenic (later-onset)	Turkmen et al. (2016)
11	F	16.8	8 years 2 months	c.725T>C	p.I242T		+	benign (0.079)	pathogenic (later-onset)	Tsukimura et al. (2014)
12	F	17.6	5 years 4 months							
13	M	7.6	8 years 10 months							
14-1	M	6.4	6 years 9 months							
14-2	M	7.5	5 years 1 months							
15	F	5.5[a]	2 years 8 months							
16	M	6.2	NA	c.1171A>G	p.K391E		+	benign (0.266)	benign (0.266)	Wakakuri et al. (2016)
17	M	<1[a]	2 years 7 months							

Bold: novel variants.

[a]Method II, NA: not available, Subject ID 14-1 and 14-2 are siblings.

[b]+: amenable, −: not amenable, http://www.galafoldamenabilitytable.jp/ (accessed at 2020/03/06).

TABLE 2 Patients selected for tracing studies who were identified in high-risk screening for Fabry disease

Patient ID	Sex	Age 1	α-Gal A	Variants		Age 2	Symptoms						Others	Tissue examination	Lyso-Gb3 (ng/mL)	Treatment	Outcome
				Nucleic acid	Amino acid		Ac	Hy	An	Co	Ot	Ga					
1	F	NA	14.2	**c.33C>G**	**p.G11G**[a]	NA	Untraceable										
2	F	26	5.7	**c.35_41del**	**p.C12Lfs*107**	31	Untraceable										
3	M	38	1.1	**c.97G>C**	**p.D33H**	39	−	−	−	+	+	−	Chronic renal failure	Focal segmental glomerulosclerosis and myeloid bodies in renal biopsy	17.7	ERT	Stable
4	F	54	6.7	**c.157A>T**	**p.N53Y**	56	−	−	−	−	−	−	Cardiac hypertrophy	Interstitial fibrosis and fat infiltration in myocardial tissue	1.17	Migalastat	Stable
5-1	M	17	4.8	**c.184dupT**	**p.S62Ffs*18**	28	+	+	−	+	−	−	Cardiac hypertrophy	—	174	ERT	Stable
5-2	M	20	5.3			31	+	+	−	+	−	−	Cardiac hypertrophy	—	181	ERT	Stable
5-3	F	43	23.4			54	+	−	−	+	−	−	Cardiac hypertrophy	—	15.5	ERT	Stable
6	M	47	2.0	**c.205T>C**	p.F69L	49	+	−	−	−	+	−	Left ventricular hypertrophy, Arrhythmia	—	40.2	Migalastat	Stable
7-1	F	10	19.0	**c.207del**	**p.F69Lfs*52**	21	+	−	+	+	+	+	—	—	7.2	ERT	Stable
7-2	M	40	5.1			Dead	NA						Renal failure	—	NA	ERT	Dead by stroke at 40 years old
8-1	F	13	7.7	**c.264C>G**	**p.Y88***	24	+	+	−	+	−	−	—	—	35.7	ERT, CBZ	Improved
8-2	M	36	5.3			47	+	−	−	−	−	−	Proteinuria, Renal dysfunction	—	NA	ERT	Stable
8-3	F	13	11.5			23	+	+	−	+	−	−	—	—	8.5	ERT, CBZ	Improved
9-1	F	18	12.9	**c.329del**	**p.P110Lfs*11**	27	+	+	−	+	−	+	—	—	21.6	ERT	Improved
9-2	F	21	9.2			30	+	+	−	+	−	−	—	—	7.4	ERT	Improved
9-3	F	49	8.6			58	+	+	+	+	−	−	—	—	7	ERT	Improved
10-1	M	47	2.9	**c.369+806_c.640-416del**	**-**	50	+	+	−	−	−	+	Renal failure, Heart failure	—	NA	ERT	Stable
10-2	F	17	12.6			20	−	−	−	−	−	−	Mulberries in urine	—	NA	Under follow-up	
11-1	M	15	NA	**c.386_389dupTGAA**	**p.L131Efs*9**	26	+	+	+	−	−	+	—	Angiokeratoma in skin biopsy	NA	ERT, CBZ	Improved
11-2	F	43	NA			54	+	+	−	+	−	−	Cardiac hypertrophy	Zebra bodies in renal biopsy	15.4	ERT	Stable
12-1	M	45	1.0	**c.440G>T**	**p.G147V**	49	+	+	+	−	+	−	Cerebral infarction, Renal dysfunction	—	NA	ERT	Stable
12-2	F	73	NA			75	−	−	−	+	−	−	Heart failure, Renal dysfunction	—	13.9	ERT	Stable

Continues

TABLE 2 Continued

Patient ID	Sex	Age 1	α-Gal A	Variants		Age 2	Symptoms						Others	Tissue examination	Lyso-Gb3 (ng/mL)	Treatment	Outcome
				Nucleic acid	Amino acid		Ac	Hy	An	Co	Ot	Ga					
13	M	7	NA	**c.563delC**	**p.Y188Sfs*4**	17	+	−	−	+	−	−	—	—	193	ERT	Stable
14-1	M	45	3.5	**c.610T>G**	**p.W204G**	47	+	+	+	+	+	+	Cardiac hypertrophy, Left ventricular hypertrophy, Proteinuria	—	170.7	ERT	Exacerbated owing to poor adherence → change to Migalastat
14-2	F	67	3.8			69	+	+	+	+	+	−	Hypertrophic cardiomyopathy, Cerebrovascular disease	—	26.7	ERT	Stable → change to Migalastat
15-1	F	67	16.4	**c.625T>C**	**p.W209R**	69	−	−	−	−	−	−	—	—	NA	Under follow-up	
15-2	F	6	23.8			7	−	−	−	−	−	−	—	—	NA	Under follow-up	
16	F	68	10.8	**c.691_693** GAC>TAT	**p.D231Y**	75	+	−	−	−	−	−	Heart failure	Myocardial biopsy findings suggestive of FD (details unknown), Ceramide deposition in skin biopsy	NA	ERT	Stable
17-1	M	45	2.7	**c.758_760delTTG**	**p.V254del**	49	+	+	−	−	+	−	Heart failure, Lacunar infarction	Vacuolization of myocardial cells	NA	ERT	Stable
17-2	F	67	18.3			78	−	−	−	−	−	−	Heart failure	—	NA	ERT	Stable
18	F	59	NA	**c.825delC**	**p.S276Afs*6**	64	NA						Heart failure	-	NA	ERT	Stable
19-1	M	12	6.9	**c.827G>C**	**p.S276T**	15	+	+	+	−	−	−	—	—	NA	ERT	Stable
19-2	F	44	10.2			47	+	+	−	+	−	−	—	—	NA	ERT	Stable →change to Migalastat
20	M	10	2.4	**c.848A>G**	**p.Q283R**	13	+	+	+	−	−	+	—	—	20.8	ERT, CBZ	Improved
21-1	F	56	2.8	**c.908T>C**	**p.I303T**	59	+	−	−	+	−	−	Heart failure	—	NA	ERT	Stable
21-2	F	30	14.1			32	−	−	−	+	+	−	—	—	NA	ERT	Stable
22-1	F	53	2.6	**c.987C>A**	**p.Y329***	57	+	−	−	−	+	−	Heart failure, Lacunar infarction	—	NA	ERT	Stable
22-2	F	24	12.1			27	+	−	−	−	−	−	Heart failure	—	NA	ERT	Stable
23	M	55	4.4	**c.1019delG**	**p.E341Nfs*57**	Dead	+	+	+	−	−	−	Heart failure, Proteinuria	—	NA	ERT	Dead
24	M	13	1.0	**c.1054G>C**	**p.A352P**	25	+	+	−	−	−	−	—	—	NA	ERT	Improved
25	F	NA	NA	**c.1067_1082del16insT ACTCTTAT**	**p.R356Lfs*33**	65	−	−	−	−	−	−	—	—	NA	Under follow-up	

Continues

TABLE 2 Continued

Patient ID	Sex	Age 1	α-Gal A	Variants		Age 2	Symptoms						Others	Tissue examination	Lyso-Gb3 (ng/mL)	Treatment	Outcome
				Nucleic acid	Amino acid		Ac	Hy	An	Co	Ot	Ga					
26-1	M	33	4.3	**c.1085_1088dupCTCG**	**p.Y365Lfs*11**	40	+	+	+	+	−	+	Renal failure, Cardiac hypertrophy	—	NA	ERT	Stable
26-2	F	7	5.4			14	−	−	−	+	−	−	—	—	NA	Under follow-up	
26-3	F	8	10.5			15	−	−	−	+	−	−	—	—	NA	Under follow-up	
27-1	M	19	NA	**c.1100dupT**	**p.A368Rfs*7**	22	+	−	+	−	−	−	—		NA	ERT	Remission tendency
27-2	F	55	NA			58	−		−	−	−	−	Cardiac hypertrophy	—	NA	ERT	Stable
28	F	58	7.9	c.218C>A	p.A73E	70	NA						Chronic renal failure, Blindness, Cardiac hypertrophy, Cerebral infarction	—	NA	none	Exacerbated, initiation of hemodialysis at 60 years old
29-1	M	47	6.5	c.725T>C	p.I242T	Dead	+	−	−	−	−	−	Heart failure, renal failure	Findings of diabetic nephropathy	NA	ERT	Dead at 55 years old
29-2	F	15	9.3			23	−	−	−	−	−	−	—	—	NA	Under follow-up	
30-1	M	51	NA	c.801+1G>A	p.L268Ifs*3	55	+	+	+	+	+	+	Cardiac hypertrophy, Proteinuria	Zebra bodies in renal biopsy	NA	ERT	Stable
30-2	F	19	NA			22	+	−	−	+	−	+	Proteinuria	Zebra bodies in renal biopsy	18.3	ERT	Stable
30-3	F	17	NA			19	+	−	−	+	−	−	—	—	NA	Under follow-up	
31	M	55	2.9	c.908_928 del21	p.S304_310L del	Dead	+	+	−	−	+	+	Hemodialysis for renal failure, Cardiac hypertrophy	—	NA	ERT	Stable, Dead at 57 years old
32	M	55	3.6	c.1124G>A	p.G375E	57	−	−	−		−	−	Proteinuria, Renal dysfunction	—	NA	ERT	Exacerbated (Cr 2 → 3 mg/dl)
33	M	66	4.5			Dead	−	−	−	−	−	−	Cardiac hypertrophy, Heart failure	Myocardial biopsy findings suggestive of FD (details unknown)	NA	none	Dead at 66 years old
34	M	61	3.5	c.1165C>G	p.P389A	67	+	+	−	+	+	−	Heart failure, Renal failure	—	NA	ERT	Stable

Abbreviations: Ac, Acroparesthesia; Age 1, Age at diagnosis; Age 2, Age in June 2020; An, Angiokeratoma; CBZ, Carbamazepine; Co, Corneal opacity; Cr, creatinine; Ga, Gastrointestinal symptoms; Hy, Hypohidrosis; NA, not available; Ot, Otological symptoms.

Bold: Novel variants

[a]Potential alteration of splicing predicted by Human Splicing Finder.

TABLE 3 Classification of variants detected in NBS and high-risk screening for Fabry disease

Classification	Variants		Symptoms	Amenability[b]	PolyPhen-2
	Nucleic acid	Amino acid			
Pathogenic (classic)	**c.97G>C**	**p.D33H**	Corneal opacity, Otological symptoms, Chronic renal failure	+	probably damaging
	c.184dupT	**p.S62Ffs*18**	Acroparesthesia, Hypohidrosis, Corneal opacity, Cardiac hypertrophy	NA	NA
	c.205T>C	p.F69L	Acroparesthesia, Otological symptoms, Left ventricular hypertrophy, Arrhythmia	+	probably damaging
	c.207del	**p.F69Lfs*52**	Acroparesthesia, Angiokeratoma, Corneal opacity, Otological symptoms, Gastrointestinal symptoms, Renal failure	NA	NA
	c.264C>G	**p.Y88***	Acroparesthesia, Hypohidrosis, Corneal opacity, Proteinuria, Renal dysfunction	−	NA
	c.329del	**p.P110Lfs*11**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Gastrointestinal symptoms	NA	NA
	c.369+806_c.640-416del	**—**	Acroparesthesia, Hypohidrosis, Gastrointestinal symptoms, Renal failure, Heart failure, Mulberries in urine	NA	NA
	c.386_389dupTGAA	**p.L131Efs*9**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Gastrointestinal symptoms, Cardiac hypertrophy	NA	NA
	c.440G>T	**p.G147 V**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Otological symptoms, Cerebral infarction, Heart failure, Renal dysfunction	−	probably damaging
	c.563delC	**p.Y188Sfs*4**	Acroparesthesia, Corneal opacity	NA	NA
	c.610T>G	**p.W204G**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Otological symptoms, Gastrointestinal symptoms, Cardiac hypertrophy, Left ventricular hypertrophy, Proteinuria, Hypertrophic cardiomyopathy, Cerebrovascular disease	+	probably damaging
	c.691_693GAC>TAT	**p.D231Y**	Acroparesthesia, Heart failure	−	probably damaging
	c.758_760delTTG	**p.V254del**	Acroparesthesia, Hypohidrosis, Otological symptoms, Heart failure, Lacunar infarction	NA	NA
	c.827G>C	**p.S276T**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity	+	possibly damaging (0.923)
	c.848A>G	**p.Q283R**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Gastrointestinal symptoms	−	probably damaging
	c.908T>C	**p.I303T**	Acroparesthesia, Corneal opacity, Otological symptoms, Heart failure	+	probably damaging
	c.987C>A	**p.Y329***	Acroparesthesia, Otological symptoms, Heart failure, Lacunar infarction	−	NA
	c.1019delG	**p.E341Nfs*57**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Heart failure, Proteinuria	NA	NA
	c.1054G>C	**p.A352P**	Acroparesthesia, Hypohidrosis	−	probably damaging
	c.1085_1088dupCTCG	**p.Y365Lfs*11**	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Gastrointestinal symptoms, Renal failure, Cardiac hypertrophy	NA	NA
	c.1100dupT	**p.A368Rfs*7**	Acroparesthesia, Angiokeratoma, Cardiac hypertrophy	NA	NA
	c.725T>C	p.I242T	Acroparesthesia, Heart failure, Renal failure	+	benign (0.079)
	c.801+1G>A	p.L268Ifs*3	Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Otological symptoms, Gastrointestinal symptoms, Cardiac hypertrophy, Proteinuria	NA	NA

Continues

TABLE 3 Continued

Classification	Variants		Symptoms	Amenability[b]	PolyPhen-2
	Nucleic acid	Amino acid			
	c.908_928del21	p.S304_310Ldel	Acroparesthesia, Hypohidrosis, Otological symptoms, Gastrointestinal symptoms, Hemodialysis for renal failure, Cardiac hypertrophy	NA	NA
	c.1165C>G	p.P389A	Acroparesthesia, Hypohidrosis, Corneal opacity, Otological symptoms, Heart failure, Renal failure	−	probably damaging
Pathogenic (classic or LO)	**c.157A>T**	**p.N53Y**	Cardiac hypertrophy	+	probably damaging
	c.825delC	**p.S276Afs*6**	Heart failure	NA	NA
	c.218C>A	p.A73E	Chronic renal failure, Blindness, Cardiac hypertrophy, Cerebral infarction	−	probably damaging
Pathogenic (LO)	c.1124G>A	p.G375E	Proteinuria, Renal dysfunction, Cardiac hypertrophy, Heart failure	+	possibly damaging (0.893)
VOUS	**c.33C>G**	**p.G11G**[a]	Untraceable	NA	NA
	c.35_41del	**p.C12Lfs*107**	Untraceable	NA	NA
	c.1067_1082del16insTACTCTTAT	**p.R356Lfs*33**	Untraceable	NA	NA
Nonpathogenic	**c.625T>C**	**p.W209R**	None	+	benign (0.000)

Abbreviations: LO: later-onset; NA: not available.

Bold: novel variants.

References; c.725T>C: Tsukimura et al. (2014), c.801+1G>A: Li et al. (2019), c.908_928del21: Nakagawa et al. (2019), c.1165C>G: Arends, et al. (2017)-1), c.218C>A: Nishino et al. (2012), c.1124G>A: Iwafuchi et al. (2017).

[a]Potential alteration of splicing predicted by Human Splicing Finder.

[b]+: amenable, −: not amenable, http://www.galafoldamenabilitytable.jp/reference (last update: 2019/04/08).

46 individuals from 27 families with 27 novel variants and 10 individuals from seven families with six variants not registered in the databases (Table 2). The clinical phenotypes, symptoms, treatments, and outcomes were obtained from the physicians attending the newborns or patients by e-mail or telephone correspondence.

3 | RESULTS

In this study, 18 newborns and 56 individuals were selected for tracing studies. The individuals had 34 definitely novel pathogenic variants and seven variants not registered in the Fabry-database.org or ClinVar database. Eight variants, namely, c.218C>A/p.A73E, c.685T>G/p.F229V, c.725T>C/p.I242T, c.801+1G>A/p.L268Ifs*3, c.908_928del21/p.S304_310Ldel, c.1124G>A/p.G375E, c.1165C>G/p.P389A, and c.1171A>G/p.K391E, have been reported by Nishino et al. (2012), Turkmen et al. (2016), Tsukimura et al. (2014), Li et al. (2019), Nakagawa et al. (2019), Iwafuchi et al. (2017), Arends, et al. (2017), and Wakakuri et al. (2016), respectively. Of the 33 novel variants, 17 were missense, two were nonsense, two were deletion, one was splicing, and 11 were frame-shift variants. Sixteen of the 17 missense variants were predicted using PolyPhen-2 as "probably damaging" or "possibly damaging." Another variant, c.625T>C/p.W209R, was predicted using PolyPhen-2 as "benign" (Table S1).

Eighteen newborns were traced, and all showed no symptoms of FD by June 2020 (Table 1). The plasma Lyso-Gb3 level was not measured in them. The results of tracing in the individuals selected from high-risk screening are shown in Table 2. The clinical phenotypes, symptoms, and outcomes were based on the physicians' judgment. The plasma maximum Lyso-Gb3 level before or during treatment was measured in 19 individuals, and the median Lyso-Gb3 level was 18.3 (interquartile range: 11.2–38.0) ng/ml. Forty-four of the 56 individuals showed symptoms and received enzyme-replacement therapy (ERT) and/or chaperone treatment. Nine of the 44 individuals with acroparesthesia showed improved symptoms after treatment. The outcomes in the other 35 individuals were stable. One female patient, patient ID No. 28, refused to receive ERT (Nishino et al., 2012). She has started receiving a regular hemodialysis treatment because of renal failure.

Five male patients died during the follow-up period. One patient, patient ID No. 7-2, died of stroke at 40 years of age. One patient, patient ID No. 29-1, died at 55 years of age after diagnosis. The patient had already developed heart and renal failure at diagnosis. One patient, patient ID No. 31, died at 57 years of age, 2 years after diagnosis. He had received hemodialysis and exhibited renal failure and cardiac hypertrophy at diagnosis. The outcomes of ERT were stable. One patient, patient ID No. 23, died of heart failure and had received ERT. One patient, patient ID No. 33, died at 66 years of age immediately after diagnosis. He exhibited heart failure and cardiac hypertrophy at diagnosis. Three patients from three families with the c.33C>G, c.35_41del and c.1067_1082del16insTACTCTTAT variants were untraceable.

4 | DISCUSSION

NBS for FD is performed in several nations, including Taiwan, Japan, and the USA. Determining whether patients diagnosed with FD are considered to have classic or later-onset type FD is essential to evaluate long-term outcomes in patients (Arends, et al., 2017). Here, we followed up patients with FD or potential FD presenting novel variants, identified by high-risk screening or NBS, and then, evaluated their clinical conditions including symptoms, plasma Lyso-Gb3 level, tissue manifestations, treatments, and outcomes after screening. Three newborns with c.625T>C (p.W209R), patient ID Nos. 23 (female, 5 years old), 24 (male, 11 years old), and 25 (male, 6 years old), did not show symptoms of FD. Furthermore, two individuals with c.625T>C (p.W209R), patient ID Nos. 15-1 (grandmother, 69 years old) and 15-2 (granddaughter, 6 years old), did not show symptoms. Their α-Gal A activity was not exceedingly low (16.2–23.8 Agal U). The prediction by PolyPhen-2 for this variant was benign (score: 0.000). Thus, c.625T>C might be a nonpathogenic variant. One patient, patient ID No. 29-2, harboring c.725T>C (p.I242T), was a 23-year-old female who showed no symptoms. However, her father (patient ID No. 29-1), who harbored the same variant, presented with acroparesthesia and heart/renal failure and died at 55 years of age. Although the PolyPhen-2 estimate for this variant was benign (score: 0.079), it was regarded as pathogenic (classic type). Although two individuals, patient ID Nos. 26-2 and 26-3, 14- and 15-year-old sisters who harbored c.1085_1088dupCTCG (p.Y365Lfs*11), did not develop classical FD symptoms except for corneal opacities, their father exhibited some classical FD symptoms and was diagnosed with FD; he received ERT. The variant was regarded as pathogenic (classic type).

We classified the disease type (classic or later-onset) according to the patients' clinical course (Arends, et al., 2017), including their disease condition and severity (Table 3). Twenty-five variants, namely, c.97G>C, c.184dupT, c.205T>C, c.207del, c.264C>G, c.329del, c.369+806_c.640-416del, c.386_389depTGAA, c.440G>T, c.563delC, c.610T>G, c.691_693GAC>TAT, c.758_760delTTG, c.827G>C, c.848A>G, c.908T>C, c.987C>A, c.1019delG, c.1054G>C, c.1085_1088dupCTCG, c.1100depT, c.725T>C, c.801+1G>A, c.908_928del21, and c.1165C>G, were identified in patients presenting with one or more classic type symptoms (i.e., acroparesthesia, angiokeratoma,

and/or corneal opacity). They were regarded as classic type pathogenic variants. Additionally, c.1124G>A was identified in male patients without classic type symptoms and was considered a late-onset pathogenic variant. The pathogenic variants c.157A>T, c.825delC, and c.218C>A could not be classified as classic or late-onset types because all patients with these variants were females developing cardiac or renal manifestations without classic type symptoms. However, we assumed that c.157A>T and c.825delC were close to late-onset types and that c.218C>A was close to the classic type, based on the clinical phenotypes, Lyso-Gb3 level, tissue findings, and onset age in the FD family harboring these variants.

The diagnostic criteria for classic and late-onset type have not been clearly defined. The type classification of FD is performed according to the appearance of classic type symptoms, such as acroparesthesia and angiokeratoma, as well as using the α-Gal A activity level. However, even in male FD patients with the same pathogenic variant, each patient developed different symptoms, such as classic type symptoms and/or late-onset type symptoms, including renal, myocardial, or neurological symptoms (Pan et al., 2016). It is difficult to assign a type classification of FD when only a few patients with variants have been identified. Recently, the FD Genotype–Phenotype Workgroup suggested a five-stage iterative system based on expert clinical assessment, published literature, and clinical evidence of pathogenicity using a two-point scoring system based on clinical hallmarks of classic disease (Germain et al., 2020). This iterative system requires data from more than five patients presenting with one pathogenic variant. α-Gal A activity alone cannot determine the disease type. Although c.335G>A (p.R112H) was considered a late-onset type variant in a previous study (Sakuraba et al., 2018), the α-Gal A activity in newborns with c.335G>A (p.R112H) was as low as that in newborns with classic type FD (Sawada et al., 2020).

Based on the previous discussion, the assessment of novel variants in this study was insufficient to clarify the genotype–phenotype correlations because of a lack of cases and families with the aforementioned novel variants. Our high-risk screening and NBS could detect families with novel variants of *GLA*. Moreover, 2.8%–14.3% of patients with pathogenic variants of *GLA* were considered to harbor de novo mutations (Kobayashi et al., 2014; Morrone et al., 2003; Rodríguez-Marí et al., 2003; Romani et al., 2015). We aim to identify many new FD patients with novel variants other than the aforementioned novel variants. In Taiwan, 792,247 newborns were screened for FD from 2008 to 2014, and 25 variants, including 13 VOUSs, were detected (Liao et al., 2018). In our previous study, 599,711 newborns were screened from 2006 to 2018, and 26 variants, including 10 VOUSs, were detected (Sawada et al., 2020). In the ClinVar database, 129 of 612 variants (21.0%) are classified as uncertain significance or conflicting interpretations of pathogenicity (i.e., VOUS). Because most families with FD have unique pathogenic variants and there is significant phenotypic variability, even among individuals with the same pathogenic variant, it is difficult to establish a genotype–phenotype correlation. Therefore, follow-up studies for potential FD with VOUSs are important, and tracing studies are needed. During recent years, for the diagnosis of late-onset type FD or potential FD with nonclassical pathogenic variants, the demonstration of glycosphingolipid deposits, using the plasma or urine Lyso-Gb3 level, tissue Gb3 level, ultrastructural analysis of biopsies, tissue Gb3 immunohistochemistry, and magnetic resonance imaging of the heart, is considered necessary because some variants such as p.D313Y and p.R118C, which were considered pathogenic variants, do not demonstrate Gb3 accumulation in the body. The FD-related clinical manifestations are more likely due to other genetic or environmental factors and may or may not be linked to the variant allele (Germain et al., 2019; Schiffmann et al., 2016). The plasma Lyso-Gb3 level was measured in 19 individuals. Only the plasma Lyso-Gb3 level in one patient, patient ID No. 15-1, was less than 2 ng/ml. The reference cutoff level was defined as <2.0 ng/ml by Maruyama et al. (2019) and ≤1.1 ng/ml by Nowak et al. (2017). One patient, patient ID No. 15-1, was considered to have late-onset FD based on cardiac manifestations and cardiac tissue findings. In the future, we will assess the implications of pathogenic variants in *GLA* by detecting and following individuals and families with novel variants and develop a screening system by measuring the α-Gal A activity level, as well as the Lyso-Gb3 level, in the DBS.

The outcomes of treatment in patients with FD in this study were consistent with those previously reported. Acroparesthesia alone is likely to be improved with ERT alone or combination therapy with ERT and carbamazepine (Sasa et al., 2019; Schuller et al., 2016). ERT can slow the progression of FD-related renal or myocardial disorders; however, poor outcomes are often detected in patients with advanced renal or myocardial disorders (Sasa et al., 2019). ERT for Gaucher disease can alleviate hepatosplenomegaly, thrombocytopenia, and bone pain (Stirnemann et al., 2017). However, it is difficult to determine the effects of ERT in patients with novel pathogenic variants because ERT for FD can slow the progression of organ and tissue disorders, but does not alleviate renal and myocardial disorders. In the future, monitoring plasma Lyso-Gb3 level during ERT might be a useful tool to evaluate the effects of ERT (Sakuraba et al., 2018).

In conclusion, we identified patients with FD with novel pathogenic variants through high-risk screening and NBS. The high-risk screening and NBS could identify individuals with FD and potential FD. Because the detection of novel pathogenic variants of *GLA* can increase in the future, the pathogenic significance of FD and the subsequent clinical course should be carefully assessed, and then, appropriate

measures should be employed to treat the resulting medical problems.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTIONS

TS, JK, KS, and KN were responsible for the design of the research. TS, JK, KS, SM, FT, KT, AO, and FE contributed to measurements and data collection. TS, JK, KS, and KM checked and analyzed the data. JK and KS wrote the manuscript. JK and KN supervised this study. All authors read and approved the final manuscript for submission. All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1

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