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. In 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 (Sawada et al., 2020). 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 | 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 NaH2PO4, 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 K2HPO4, 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, and Human Splicing Finder (Desmet et al., 2009), 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). 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).
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 MgCl₂, 0.5 mM of dithiothreitol, 0.05% of NaN₃, 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 (Illunina, 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 | Nucleic acid | Amino acid | α-Gal A activity | Clinical symptoms at 2020/June | Amenabilityb | PolyPhen-2 (score) | Classification | Reference |
|-----------|-----|------------------|------------------|--------------|-------------|------------------|-------------------------------|--------------|-------------------|---------------|-----------|
| 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<sup>a</sup>  | 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<sup>a</sup>  | 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<sup>a</sup>  | 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<sup>a</sup>  | 2 years 7 months |              |             |                  |                               |              |                   |               |           |

**Bold:** novel variants.

<sup>a</sup>Method II, NA: not available, Subject ID 14-1 and 14-2 are siblings.

<sup>b</sup>+: amenable, −: not amenable, [http://www.galafolmamenabilitytable.jp/](http://www.galafolmamenabilitytable.jp/) (accessed at 2020/03/06).
| Patient ID | Sex | Age 1 | α-Gal A | Variants | Nucleic acid | Amino acid | Age 2 | Ac | Hy | An | Co | Ot | Ga | Others | Tissue examination | Lyso-Gb3 (ng/mL) | Treatment | Outcome |
|------------|-----|-------|---------|----------|-------------|------------|-------|----|----|----|----|----|----|--------|-------------------|----------------|-----------|---------|
| 1          | F   | NA    | 14.2    | c.33C>G  | p.G11G<sup>a</sup> | NA         | Untraceable |     |    |    |    |    |    |        |                   |                |           |         |
| 2          | F   | 26    | 5.7     | c.35_41del | p.C121Fs*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 | Intersitial fibrosis and fat infiltration in myocardial tissue | 1.17 | Migalastat | Stable |
| 5-1        | M   | 17    | 4.8     | c.184dupT | p.S620Fs*18 | 28         | Cardiac hypertrophy | — | 174 | ERT | Stable |
| 5-2        | M   | 20    | 5.3     | c.184dupT | p.S620Fs*18 | 31         | Cardiac hypertrophy | — | 181 | ERT | Stable |
| 5-3        | F   | 43    | 23.4    | c.184dupT | p.S620Fs*18 | 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.F6901Fs*52 | 21         | — | — | 7.2 | ERT | Stable |
| 7-2        | M   | 40    | 5.1     | c.207del  | p.F6901Fs*52 | 24         | Dead | NA | Renal failure | — | NA | ERT | Stable |
| 7-3        | F   | 13    | 7.7     | c.264C>G  | p.Y88*      | 23         | + | + | 8.5 | ERT, CBZ | Improved |
| 8-1        | M   | 36    | 5.3     | c.264C>G  | p.Y88*      | 47         | + | − | 35.7 | ERT, CBZ | Improved |
| 8-2        | F   | 13    | 11.5    | c.329del  | p.P1100Fs*11 | 27         | + | + | 8.5 | ERT, CBZ | Improved |
| 9-1        | F   | 18    | 12.9    | c.329del  | p.P1100Fs*11 | 30         | + | + | 21.6 | ERT | Improved |
| 9-2        | F   | 21    | 9.2     | c.329del  | p.P1100Fs*11 | 58         | + | + | 7.4 | ERT | Improved |
| 9-3        | F   | 49    | 8.6     | c.329del  | p.P1100Fs*11 | 50         | + | + | 7 | ERT | Improved |
| 10-1       | M   | 47    | 2.9     | c.360+806_c.640- | -           | 20         | − | − | NA | ERT | Stable |
| 10-2       | F   | 17    | 12.6    | c.360+806_c.640- | -           | 26         | + | + | — | NA | Under follow-up |
| 11-1       | M   | 15    | NA      | c.386_389dupTGAA | p.L131Efs*9 | 29         | + | + | — | NA | ERT, CBZ | Improved |
| 11-2       | F   | 43    | NA      | c.386_389dupTGAA | p.L131Efs*9 | 54         | + | + | — | NA | ERT | Stable |
| 12-1       | M   | 45    | 1.0     | c.440G>T  | p.G147V     | 49         | Cerebral infarction, Renal dysfunction | Zebra bodies in renal biopsy | 15.4 | ERT | Stable |
| 12-2       | F   | 73    | NA      | c.440G>T  | p.G147V     | 75         | Heart failure, Renal dysfunction | — | 13.9 | ERT | Stable |

Continues
| Patient ID | Sex | Age | α-Gal A | Nucleic acid | Amino acid | Symptoms | Tissue examination | Lyso-Gb3 (ng/mL) | Treatment | Outcome |
|------------|-----|-----|---------|-------------|------------|----------|-------------------|----------------|-----------|---------|
| 13         | M   | 7   | NA      | c.563delC   | p.Y188fs*4 | Age 17   | + + + + + +      | 193            | ERT       | Stable  |
| 14-1       | M   | 45  | 3.5     | c.610T>G    | p.W204G    | Age 47   | + + + + + +      | 170.7          | ERT       | Exacerbated owing to poor adherence → change to Migalastat |
| 14-2       | F   | 67  | 3.8     |             |            |          | 69 + + + + + +   | 26.7            | ERT       | Stable → change to Migalastat |
| 15-1       | F   | 67  | 16.4    | c.625T>C    | p.W209R    | Age 69   | + + + + + +      | NA             | ERT       | Under follow-up |
| 15-2       | F   | 6   | 23.8    |             |            |          | 7 + + + + + +    | NA             | ERT       | Under follow-up |
| 16         | F   | 68  | 10.8    | c.691_693GAC>TAT | p.D231Y | Age 75   | + + + + + +      | NA             | ERT       | Stable  |
| 17-1       | M   | 45  | 2.7     | c.758_760delTTG | p.V254del | Age 49   | + + + + + +      | NA             | ERT       | Stable  |
| 17-2       | F   | 67  | 18.3    | c.825delC   | p.S276As*6 | Age 78   | + + + + + +      | NA             | ERT       | Stable  |
| 18         | F   | 59  | NA      | c.825delC   | p.S276T    | Age 64   | + + + + + +      | NA             | ERT       | Stable  |
| 19-1       | M   | 12  | 6.9     | c.827G>C    | p.S276T    | Age 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    | Age 13   | + + + + + +      | 20.8           | ERT, CBZ | Improved |
| 21-1       | F   | 56  | 2.8     | c.908T>C    | p.I303T    | Age 59   | + + + + + +      | NA             | ERT       | Stable  |
| 21-2       | F   | 30  | 14.1    |             |            |          | 32 + + + + + +   | NA             | ERT       | Stable  |
| 22-1       | F   | 53  | 2.6     | c.987C>A    | p.Y329*    | Age 57   | + + + + + +      | NA             | ERT       | Stable  |
| 22-2       | F   | 24  | 12.1    |             |            |          | 27 + + + + + +   | NA             | ERT       | Stable  |
| 23         | M   | 55  | 4.4     | c.1019delG  | p.E341Nfs*57 | Age 27   | + + + + + +      | NA             | ERT       | Dead    |
| 24         | M   | 13  | 1.0     | c.1054G>C   | p.A352P    | Age 25   | + + + + + +      | NA             | ERT       | Improved |
| 25         | F   | NA  | NA      | c.1067_1082del16insTACTCTTAT | p.R356fs*33 | Age 65   | + + + + + +      | NA             | ERT       | Under follow-up |

**TABLE 2 Continued**
| Patient ID | Sex | Age 1 | Age 2 | α-Gal A | Nucleic acid | Amino acid | Variants | Symptoms | Tissue examination | Lyso-Gb3 (ng/mL) | Treatment | Outcome |
|------------|-----|-------|-------|---------|--------------|------------|----------|----------|-------------------|----------------|-----------|---------|
| 26-1 M     | 33  | 4.3   | 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    | −       | −           | −          | −        | −        | Cardiac hypertrophy | —             | NA        | Under follow-up |
| 27-1 M     | 19  | NA    | 22    | +       | +           | +          | +        | −        | NA                | ERT           | Stable    |
| 27-2 F     | 55  | NA    | 58    | −       | −           | −          | −        | −        | Cardiac hypertrophy | NA            | NA        | ERT     | Stable |
| 28 F       | 58  | 7.9   | 70    | NA      | Chronic renal failure, Blindness, Cardiac hypertrophy, Cerebral infarction | — | NA | none | Exacerbated, initiation of hemodialysis at 60 years old |
| 29-1 M     | 47  | 7.5   | 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    | 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    | +       | −           | +          | −        | −        | Hemodialysis for renal failure, Cardiac hypertrophy | — | NA | ERT | Stable, Dead at 57 years old |
| 31 M       | 55  | 2.9   | Dead  | +       | +           | −          | −        | +        | Proteinuria, Renal dysfunction | —             | NA | ERT | Exacerbated (Cr 2 → 3 mg/dl) |
| 32 M       | 55  | 3.6   | 57    | −       | −           | −          | −        | −        | Cardiac hypertrophy, Heart failure | Myocardial biopsy findings suggestive of FD (details unknown) | NA | none | Dead at 66 years old |
| 33 M       | 66  | 4.5   | Dead  | −       | −           | −          | −        | −        | —                 | NA            | ERT | Stable |
| 34 M       | 61  | 3.5   | 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, Otolological symptoms.

**Bold:** Novel variants

*Potential alteration of splicing predicted by Human Splicing Finder.*
| Classification | Variants | Amino acid | Symptoms | Amenability<sup>b</sup> | PolyPhen-2 |
|----------------|----------|------------|----------|-------------------------|------------|
| Pathogenic (classic) | c.97G>C | p.D33H | Corneal opacity, Otolaryngological 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, Otolaryngological symptoms, Left ventricular hypertrophy, Arrhythmia | + | probably damaging |
|                  | c.207del | p.F69Lfs*52 | Acroparesthesia, Angiokeratoma, Corneal opacity, Otolaryngological 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.G147V | Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Otolaryngological 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, Otolaryngological 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, Otolaryngological 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, Otolaryngological symptoms, Heart failure | + | probably damaging |
|                  | c.987C>A | p.Y329* | Acroparesthesia, Otolaryngological 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.L242T | Acroparesthesia, Heart failure, Renal failure | + | benign (0.079) |
|                  | c.801+1G>A | p.L268Ifs*3 | Acroparesthesia, Hypohidrosis, Angiokeratoma, Corneal opacity, Otolaryngological symptoms, Gastrointestinal symptoms, Cardiac hypertrophy, Proteinuria | NA | NA |

Continues
| Classification | Variants | Amino acid | Symptoms | Amenability<sup>b</sup> | PolyPhen-2 |
|----------------|----------|------------|----------|--------------------------|------------|
| **Pathogenic** (classic or LO) | 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 |
| | c.157A>T | p.N53Y | Cardiac hypertrophy | + | probably damaging |
| | c.1124G>A | p.G375E | Proteinuria, Renal dysfunction, Cardiac hypertrophy, Heart failure | + | possibly damaging (0.893) |
| **VOUS** | c.33C>G | p.G11G<sup>a</sup> | Untraceable | NA | NA |
| | c.35_41del | p.C12Lfs*107 | Untraceable | NA | NA |
| | c.1067_1082del16insTA | 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).

<sup>a</sup>Potential alteration of splicing predicted by Human Splicing Finder.

<sup>b</sup+: 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_389deptTGAA, 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-Mari 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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REFERENCES

Adzhubei, I., Jordan, D. M., & Sunyaev, S. R. (2013). Predicting functional effect of human missense mutations using PolyPhen-2. *Current Protocols in Human Genetics, Chapter 7(Unit 7)*, 20. https://doi.org/10.1002/0471142905.hg0720s76
Arends, M., Biegstraaten, M., Hughes, D. A., Mehta, A., Elliott, P. M., Oder, D., … Hollak, C. E. M. (2017). Retrospective study of long-term outcomes of enzyme replacement therapy in Fabry disease: Analysis of prognostic factors. *PLoS One*, 12(8), e0182379. https://doi.org/10.1371/journal.pone.0182379
Arends, M., Wanner, C., Hughes, D., Mehta, A., Oder, D., Watkinson, O. T., … Hollak, C. E. (2017). Characterization of classical and nonclassical Fabry disease: A multicenter study. *Journal of the American Society of Nephrology*, 28(5), 1631–1641. https://doi.org/10.1681/ASN.2016090964
Chamoles, N., Blanco, M., & Gaggioli, D. (2001). Fabry disease: Enzymatic diagnosis in dried blood spots on filter paper. *Clinica Chimica Acta*, 308(1–2), 195–196. https://doi.org/10.1016/S0009-8981(01)00478-8
Desmet, F.-O., Hamroun, D., Lalande, M., Collod-Béroud, G., Claustres, M., & Béroud, C. (2009). Human Splicing Finder: An online bioinformatics tool to predict splicing signals. *Nucleic Acids Research*, 37(9), e67. https://doi.org/10.1093/nar/gkp215
Germain, D. P., Foulhoux, A., Decramer, S., Tardieu, M., Pillet, P., Fila, M., … Lacombe, D. (2019). Consensus recommendations for diagnosis, management and treatment of Fabry disease in paediatric patients. *Clinical Genetics*, 96(2), 107–117. https://doi.org/10.1111/cge.13546
Germain, D. P., Oliveira, J. P., Bichet, D. G., Yoo, H.-W., Hopkin, R. J., Lemay, R., … Warnock, D. G. (2020). Use of a rare disease registry for establishing phenotypic classification of previously unassigned GLA variants: A consensus classification system by a multispecialty Fabry disease genotype–phenotype workgroup. *Journal of Medical Genetics*, 57(8), 542–551. https://doi.org/10.1136/jmedgenet-2019-106467
Iwafuchi, Y., Maruyama, H., Morioka, T., Noda, S., Nagata, H., Oyama, Y., & Narita, I. (2017). Enzyme replacement therapy in a patient of heterozygous Fabry disease: Clinical and pathological evaluations by repeat kidney biopsy and a successful pregnancy. *CEN Case Reports*, 6(2), 210–214. https://doi.org/10.1007/s13730-017-0277-y
Kobayashi, M., Ohashi, T., Iizuka, S., Kaneshiro, E., Higuchi, T., Eto, Y., & Ida, H. (2014). Frequency of de novo mutations in Japanese patients with Fabry disease. *Molecular Genetics and Metabolism Reports*, 1(1), 283–287. https://doi.org/10.1016/j.mrgmr.2014.07.001
Landrum, M. J., Chitipiralla, S., Brown, G. R., Chen, C., Gu, B., Hart, J., … Kattman, B. L. (2020). ClinVar: improvements to accessing data. *Nucleic Acids Research*, 48(D1), D835–D844. https://doi.org/10.1093/nar/gkz972
Li, P., Zhang, L., Zhao, N., Xiong, Q., Zhou, Y.-A., Wu, C., & Xiao, H. (2019). A novel α-galactosidase a splicing mutation predisposes to Fabry disease. *Frontiers in Genetics*, 10, 60. https://doi.org/10.3389/fgene.2019.00060
Liao, H.-C., Hsu, T.-R., Young, L., Chang, C.-C., Huang, C.-K., Liu, H.-C., … Chen, Y.-J. (2018). Functional and biological studies of α-galactosidase A variants with uncertain significance from newborn screening in Taiwan. *American Society of Nephrology*, 28(2), 140–147. https://doi.org/10.1016/j.asn.2017.06.002
Maruyama, H., Miyata, K., Mikame, M., Taguchi, A., Guili, C., Shimura, M., … Ishii, S. (2019). Effectiveness of plasma lyso-Gb3 as a biomarker for selecting high-risk patients with Fabry disease from multispecialty clinics for genetic analysis. *Genetics in Medicine*, 21(1), 44–52. https://doi.org/10.1038/gim.2018.31
Morrone, A., Cavicchi, C., Bardelli, T., Antuzzi, D., Parini, R., Di Rocco, M., … Zammarchi, E. (2003). Fabry disease: Molecular studies in Italian patients and X inactivation analysis in manifesting carriers.
