Fabry disease screening in high-risk populations in Japan: A nationwide study

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Abstract
Background: Fabry disease (FD) is a rare, X-linked inherited disorder caused by mutations in the GLA gene, which results in deficiency of α-galactosidase A (α-Gal A). This leads to the progressive accumulation of metabolites, which can cause malfunctions in systemic organs. A recent screening study in newborns demonstrated that the incidence of FD was more frequent than previously estimated and that there are still many undiagnosed or misdiagnosed Fabry patients. Therefore, the purpose of this study was to identify Fabry patients by performing high-risk screening in 18,171 individuals, enrolled from October 2006 to March 2019, with renal, cardiac, or neurological manifestations from all of the prefectures in Japan. A total of 601 hospitals from all the prefectures in Japan participated in this study.

Results: From October 2006 to March 2019, 18,171 individuals with renal, cardiac, or neurological manifestations were enrolled. Low α-Gal A activity was detected in 846 individuals, with 224 of them diagnosed with FD by GLA sequencing. Cases with a family history of FD (n = 64) were also subjected to sequencing, without α-Gal A assay, as per individual request and 12 of them were diagnosed with a variant of FD. A total of 236 Fabry patients (97 males and 139 females) were detected from the 18,235 participants. There were 101 GLA variants, including 26 novel variants, detected in the 236 Fabry patients from 143 families, with 39 amenable variants (39%) and 79 of the 236 patients (33%) suitable for migalastat treatment.

Conclusions: From the 18,235 participants, 101 GLA variants, including 26 novel variants, were identified in the 236 Fabry patients from 143 families. Migalastat was identified as a suitable treatment option in 33% of the Fabry patients and 39% of the GLA variants were detected as amenable. Therefore, the simple screening protocol, using dried blood spots, that was performed in this study could be useful for early diagnosis and selection of appropriate treatments for FD in high-risk and undiagnosed patients suffering from various renal, cardiac, or neurological manifestations.

Background
Fabry disease (FD; OMIM 301500) is an inherited X-linked disorder caused by mutations in the GLA
gene, which encodes the lysosomal enzyme α-galactosidase A (α-Gal A; EC 3.2.1.22). To date, 516 GLA variants have been incorporated into the Fabry-database (Fabry-database.org, ver.3.2.2, last updated on February 15, 2019) [1]. The functional deficiency of α-Gal A results in the progressive accumulation of metabolites, such as globotriaosylceramide in lysosomes, biological fluids, and the vascular endothelium, which can cause the escalation of malfunctions in systemic organs, such as the skin, eyes, kidneys, ears, lungs, heart, and brain [2–4]. Fabry patients who have less, or very low, α-Gal A activity exhibit the classic phenotype and are generally asymptomatic in early childhood [5, 6]. In contrast, Fabry patients with residual α-Gal A activity exhibit milder clinical manifestations and onset occurs later than in those with the classic phenotype. Heterozygous females with pathogenic GLA variants are not only carriers but also express wide manifestation spectra, ranging from asymptomatic to as severe as those of the classic phenotype, depending on random X-chromosomal inactivation [7].

Clinical manifestations are multisystemic, including limb pain, acroparesthesia, angiokeratoma, anhidrosis, and corneal opacity in childhood, with a progression to major organ involvement in adulthood, such as proteinuria, impaired renal function, cardiomyopathy, and stroke. Because these manifestations are frequently observed in individuals with diabetes, hypertension, and arteriosclerosis, which are nonspecific, this might lead to delayed or mistaken diagnosis [8, 9]. Recent newborn screening (NBS) studies, including our previous study [10], demonstrated that the incidence of FD was as high as 1:1,600 to 1:8,485 [11, 12]. Therefore, the prevalence of FD had been underestimated, with evidence suggesting that there are many undiagnosed or misdiagnosed Fabry patients.

Enzyme replacement therapy (ERT) is now available in Japan and 3 products are on the market, namely Fabrazyme® (Sanofi Genzyme), Replagal® (Shire), Agalsidase beta BS (JCR). Moreover, an oral pharmacological chaperone, migalastat (Galafold®; Amicus Therapeutics), has become available for specific pathogenic GLA variants, i.e., migalastat-amenable GLA variants [13]. ERT can slow renal deterioration and progression of cardiomyopathy, thereby delaying morbidity and death [14]. Migalastat has the same effects on renal function as ERT [15]. Early treatment is essential to preserve
organ function and prevent progression of the disease. The high-risk screening for FD is considered a practical strategy for early treatment.

Nakagawa et al. [16] reported partial results regarding high-risk Japanese patients in the Hokkaido prefecture with cardiac, renal, or neurological manifestations. In the current study, we aimed to identify undiagnosed patients with FD by performing high-risk screening in 18,171 individuals with renal, cardiac, or neurological manifestations, from all of the prefectures in Japan, as well as to assess the usefulness of our simple screening protocol for the definite diagnosis of FD in the aforementioned high-risk groups.

Results
High-risk screening for Fabry disease
The demographic characteristics of the enrolled individuals are shown in Table 1. Of the 18,171 individuals that were screened, 8,872 had renal manifestations (5,537 males, 3,107 females, and 228 did not provide information on gender), 4,058 showed signs of cardiac manifestations (2,854 males, 1,176 females, and 28 did not provide information on gender), 3,076 evidenced central neurological manifestations (1,927 males, 968 females, and 181 did not provide information on gender), 894 experienced peripheral neurological manifestations (522 males, 371 females, and 1 did not provide information on gender), 715 had a family history of FD (333 males, 378 females, and 4 did not provide information on gender), and 571 were classified as ‘other’ (350 males, 206 females, and 15 did not provide information on gender). The flow chart and results of the high-risk screening program for FD are shown in Fig. 1. The 2,553 individuals, who had low α-Gal A activity, were recalled and 1,965 of them proceeded to the second α-Gal A assay. Figure 2 shows the distribution of α-Gal A activity in the first assay. About 4% and 28% of male and female patients, respectively, fell below the cutoff value. The median α-Gal A activity obtained using Method I was 24.47, 24.50 and 24.06 (AgalU) in total, males and females, respectively (Fig. 2a, b and c). The median α-Gal A activity obtained using Method II was 57.50, 62.54 and 45.53 (AgalU) in total, males and females, respectively. From Method I and II, 846 individuals were detected and 224 of them were diagnosed with FD by GLA sequencing. No GLA gene variants were identified in 650 individuals, while 52 individuals presented with a
possible functional polymorphism allele, p.E66Q. Of the 224 individuals that presented with GLA gene variants, 28 (19 males, 9 females), 29 (17 males, 12 females), 5 (3 males, 2 females), 30 (20 males, 10 females), 128 (32 males, 96 females), and 4 (3 males, 1 female) variants were identified in the renal, cardiac, central neurological, peripheral neurological, family history, and ‘other’ groups, respectively (Table 1). The prevalence of FD was 0.42% (male: 0.45%, female: 0.38%), 0.94% (male: 0.77%, female: 1.33%), 0.22% (male: 0.20%, female: 0.27%), 4.37% (male: 4.98%, female: 3.50%), and 23.40% (male: 12.49%, female: 33.02%) in the renal, cardiac, central neurological, peripheral neurological, family history, and ‘other’ groups, respectively. The median age was 49 years for males and 58 years for females, 47 years for males and 47.5 years for females, 13 years for both males and females, and 20 years for males and 38 years for females in the renal, cardiac, central neurological, peripheral neurological, family history, and ‘other’ groups, respectively. Individuals with a family history of FD (n = 64) were also sequenced, without α-Gal A assay, as per individual request. GLA gene variants were detected in 12 (3 males and 9 females) of the 64 individuals. Therefore, a total of 236 Fabry patients were detected from 18,235 individuals in this high-risk screening study.

GLA variants detected in the Fabry patients

In this study, 101 GLA variants were detected in 236 patients from 143 families. Of the 101 variants, 39 (39%) were amenable and 79 of the 236 patients (33%) were considered suitable for migalastat treatment (Table 2). The individual variant detected in each patient and family is shown in Table S1. Regarding mutation type of the 101 variants, 64 were identified as missense mutations, 18 were frameshift mutations, 10 were nonsense mutations, 3 were in-frame deletions, 3 were intronic mutations, 2 were silent mutations (which potentially alters splicing), and 1 was a large deletion mutation of exon 3 and 4. Of the 101 variants, 68 were registered in ClinVar or Fabry-database.org. Two variants, c.218C > A [17] and c.908_928del21 [16], were described in our previous report which included detailed information on each patient. The variant c.625T > C was detailed in our previous report regarding NBS for FD [10]. Four variants, namely c.725T > C, c.801 + 1G > A, c.1124G > A, and c.1165C > G, were reported by Tsukimura et al. [18], Li et al. [19], Iwafuchi et al. [20], and van der Tol et al. [21], respectively. The remaining 26 variants were considered as novel variants. The most
The second most common variant was c.334C > T/p.R112C (2.8%, 4/143). The third most common variants were c.335G > A/p.R112H (2.1%, 3/143), c.658C > T/p.R220* (2.1%, 3/143), c.679C > T/p.R227* (2.1%, 3/143), c.718_719delAA/p.K240Efs*8 (2.1%, 3/143), c.902G > A/p.R301Q (2.1%, 3/143), c.1033_1034delTC/p.S345Rfs*28 (2.1%, 3/143), c.1124G > A/p.G375E (2.1%, 3/143), and c.1235_1236delCT/p.T412Sfs*37 (2.1%, 3/143). The geographic distribution of the variants is shown in Figure S1.

**Discussion**

The median α-Gal A activity in the high-risk screening populations was 24.47, 24.50, and 24.06 (AgalU) in total, males, and females, respectively. Figure 2d presents a histogram of α-Gal A activity in an NBS study [10]. The median α-Gal A activity in newborns was 42.58 (AgalU), which is approximately two times more than that of the current high-risk screening populations. FD is associated with a significantly reduced life expectancy compared to the general population [22]. Although the detailed mechanism for lowered α-Gal A activity in adults is unknown, lowered α-Gal A activity may be associated with a premature ageing process through the dysfunction of blood vessels. Therefore, ageing and lowered α-Gal A activity are closely related. The cutoff values in the high-risk screening populations were 12 (AgalU) for males and 20 (AgalU) for females, which is representative of the cutoff values for the 0.5 percentile in the NBS population. This is because α-Gal A activity in adults is lower than that of newborns.

The high-risk screening for FD in selected patient cohorts has been reported. Doheny et al. [23] reanalyzed studies related to hemodialysis (27 reports, 23,954 males, and 12,866 females), left ventricular hypertrophy (LVH) and/or hypertrophic cardiomyopathy (HCM) (17 reports, 4,054 males, and 1,437 females), and ischemic or cryptogenic strokes (16 studies, 3,904 males, and 2,074 females). The revised prevalence was estimated as 0.21% for male and 0.15% for female hemodialysis patients, 0.94% for male and 0.90% for female cardiac patients, and 0.13% for male and 0.14% for female stroke patients. In the current study, the prevalence was estimated as 0.42% (male: 0.45%, female: 0.38%) in the renal manifestation group (a), 0.94% (male: 0.77%, female: 1.33%) in
the cardiac manifestation group (b), and 0.22% (male: 0.20%, female: 0.27%) in the central neurological manifestation group (c), which are comparable to those of previous reports.

The prevalence of FD in the peripheral neurological manifestation group (d) was second highest at 4.37% (male: 4.98%, female: 3.50%), whereas the patients’ age (male: median 13 [IQR: 11-18.5] years old, female: median 13 [IQR: 9-25] years old) was younger than the other groups. Therefore, manifestations such as limb pain, acroparesthesias, clustered angiokeratoma, cornea verticillata, and hypo-anhidrosis, could be useful in the detection of Fabry patients. Politei et al. [24] has recommended that the cause of pain should be diagnosed early in unrecognized or newly diagnosed Fabry patients in order to improve treatment possibilities. FD experts consider that, regardless of gender or age, pain related to FD could be an early indication to commence ERT before potentially irreversible organ damage, to the kidneys, heart, or brain, prevails. However, a study conducted in Russia by Namazova-Baranova et al. [25] reported that no Fabry patients were detected from 214 patients (110 males and 104 females) with chronic limb pain. Moreover, the genetic, epidemiological, and ethnical information related to Russian Fabry patients are insufficient and future studies and information related to FD in Russia are required.

The prevalence of FD in individuals with a family history (e) was the highest at 23.40% (male: 12.49%, female: 33.02%). The GLA sequencing for individuals who had a family history of FD demonstrated its usefulness in detecting undiagnosed or pre-symptomatic Fabry patients. Therefore, when patients experience FD-related symptoms, clinicians should confirm the presence of a family history of FD and, if applicable, whether similar symptoms developed.

The variant spectra of GLA in Japanese patients have been reported [26, 27]. GLA gene analysis was performed for 207 Fabry patients [26]. The most common variant was c.888G > A/p.M296I (allele frequency: 5.8%, 12/207). The second most common variants were c.639 + 919G > A (4.3%, 9/207) and c.679C > T/p.R227* (4.3%, 9/207), followed by c.334C > T/p.R112C (3.9%, 8/207), c.335G > A/p.R112H (3.9%, 8/207), and c.902G > A/p.R301Q (3.9%, 8/207). In another study, 73 pathogenic variants were detected in 176 patients from 115 families [27] and the most common variant was c.334C > T/p.R112C (allele frequency: 2.65%). The second most common variant was c.888G >
A/p.M296I (1.89%), followed by c.658C > T/p.R220* (1.52%), c.718_719delAA/p.K240Efs*8 (1.52%), and c.1025G > A/p.R342Q (1.52%). The common variants identified in these studies, as well as those of the current study, overlap.

We previously reported the first large-scale NBS program for FD in the western region of Japan [10]. A total of 599,711 newborns were screened and 26 GLA variants, including 8 novel variants, were detected in 57 newborns from 54 families. Of the 26 variants, 10 were also detected in the current study and most of them were detected in patients from the western region of Japan (Fig. S1).

In the current study, 4 pedigrees (4/68, 5.9%) were perceived as de novo mutations (Table S2). The frequency might be comparable with those of previous reports from Japan (6.8% (5/74); Kobayashi et al. [28]), Italy (2.8% (3/108); Romani et al. [29]; and 14.3% (2/14); Morrone et al. [30]), Spain (4.5% (1/22); Rodriguez-Mari et al. [31]), and the United Kingdom (UK) (6.3% (1/16); Davies et al. [32]). A high frequency of de novo mutations have been reported in X-linked disorders, such as Duchenne muscular dystrophy (DMD) and hemophilia A (F8), and de novo mutations account for approximately one-third of the mutations in these two disorders [33, 34]. The size and structure of the gene and its position within the genome may contribute to the high frequency of de novo mutations. The high rate of de novo mutations in the two abovementioned diseases is thought to be related to the large size of the genes (DMD: 2,400 kb, F8: 186.9 kb). The presence of CpG dinucleotides is also reported to increase mutational frequency [35]. The relatively low frequency of de novo mutations in the GLA gene may be owing to its smaller gene size (10.2 kb) and relatively high CpG dinucleotide content, for example, GLA contains 19 CpG sites (1/68 bases) in the coding region compared to F8 which has 68 CpG sites (1/104 bases). Of these 19 potential mutation sites in GLA, 13 variants were identified in the current study, namely c.146G > C, c.334C > T, c.335G > A, c.427G > A, c.658C > T, c.659G > C, c.679C > T, c.901C > T, c.902G > A, c.902G > C, c.1024C > T, c.1025G > A, and c.1066C > T. Twenty-three kinds of variants were reported as de novo mutational hotspots (Table S2). However, no particular sites, responsible for these de novo mutations, were identified.

A few case reports regarding the homozygous or compound heterozygous female Fabry patient have been reported [30, 36]. However, homozygous or compound heterozygous female Fabry patients
were not identified in the current study, or our previous NBS study [10]. Generally, in female Fabry patients, only those with heterozygous mutations in the GLA gene are detected. The reason for this is not fully understood and should be further investigated. Interestingly, a male patient who possessed two GLA variants, c.70T > A/p.W24R and c.1255A > G/p.N419D, was detected in the current study (Table S1). Unfortunately, genetic information regarding his family was not available. Of 100 GLA variants, 70 were detected only in single pedigrees while 20 were identified in two pedigrees. Because bias was introduced in the distribution of variants in these pedigrees, it was difficult to discuss the correlation between genotype and phenotype, especially organ specific pathogenicity. In the follow-up study of each patient, 21 of the 26 novel variants were indicated as pathogenic, namely c.97G > C/p.D33H, c.157A > T/p.N53Y, c.184dupT/p.S62Ffs*18, c.205T > C/p.F69L, c.207del/p.F69Lfs*52, c.264C > G/p.Y88*, c.329del/p.P110Lfs*11, c.386_389dupTGAA/p.L131Efs*9, c.440G > T/p.G147V, c.563delC/p.Y188Sfs*4, c.610T > G/p.W204G, c.691_693GAC > TAT/p.D231Y, c.825delC/p.S276Afs*6, c.827G > C/p.S276T, c.848A > G/p.Q283R, c.908T > C/p.I303T, c.987C > A/p.Y329*, c.1019delG/p.E341Nfs*57, c.1054G > C/p.A352P, c.1085_1088dupCTCG/p.Y365Lfs*11, and c.1100dupT/p.A368Rfs*7. There were also 5 variants identified which were not registered in ClinVar or Fabry-database.org, specifically c.725T > C/p.I242T, c.801 + 1G > A/p.L268I, c.908_923del21/p.S304_310Ldel, c.1124G > A/p.G375E, and c.1165C > G/p.P389A. Patients who possess the abovementioned variants developed FD-related symptoms, and some had even passed away from a stroke or heart failure. The current high-risk screening program identified individuals who are considered suitable candidates for migalastat treatment. Some patients were already receiving migalastat treatment. Moreover, gene therapy holds promise in the effective treatment of a wide variety of diseases, and the clinical trials for gene therapy for FD are ongoing in Canada and USA (https://fabrydiseasenews.com/gene-therapy-for-fabry-disease/). In the future, the development of new treatment methods for FD, other than ERT, is expected. **Conclusions** In the current study, we performed high-risk screening for FD in individuals from all the prefectures in
Japan. A total of 18,235 individuals were screened, using DBSs, and 101 GLA variants, including 26 novel variants, were identified in 236 patients from 143 families. The distribution of variants is diverse for each region of Japan, and de novo mutations in the GLA gene is detected in a significant proportion of these variants. Therefore, further novel mutations will likely be identified in the future. With regards to treatment, 33% of the Fabry patients were identified as suitable candidates for migalastat therapy and 39% of the GLA variants were identified as amenable. Therefore, the simple screening protocol, using DBSs, could be useful in the early diagnosis, and selection of appropriate treatments, of FD in high-risk and undiagnosed patients suffering from various renal, cardiac, or neurological manifestations. FD screening is essential in individuals who present with peripheral neuropathy or a family history of FD as the latter and former conditions have been identified as strong predictive factors in FD development.

Methods

Study design

In order to meet the study aim; in identifying patients with FD by performing high-risk screening in 18,171 individuals, enrolled from October 2006 to March 2019, with renal, cardiac, or neurological manifestations from all the prefectures in Japan; the following study design was implemented. A total of 601 hospitals, from all the prefectures in Japan, participated in this study. From October 2006 to March 2019, the DBSs of 18,171 patients with various cardiac, renal, or neurological manifestations were analyzed. Written informed consent was obtained from the patients or their parents (in cases where the patients were not of legal age). The individuals were enrolled in the study if they developed at least one of the following manifestations: (a) renal manifestations, such as proteinuria, chronic kidney disease anhidrosis, diabetic nephropathy, mulberries in the urine, and the need for dialysis; (b) cardiac manifestations, such as left ventricular hypertrophy detected using electrocardiography or echocardiography; (c) central neurological manifestations, such as parkinsonism, hearing loss, and history of stroke; (d) peripheral neurological manifestations, including limb pain, acroparesthesias, clustered angiokeratoma, cornea verticillata, and hypo-anhidrosis; (e) family history of FD; or (f) other reasons, such as liver failure and unavailable information.
The preparation of DBS specimens was detailed in our previous report [16]. Briefly, after dropping blood spots onto filter papers (Toyo Roshi Kaisha, Ltd., Tokyo, Japan), the DBSs were dried for at least 4 h at room temperature, sent to Kumamoto University by mail within 1 week of preparation, and if necessary, stored at -20 °C until use. The high-risk screening for FD using α-Gal A assays with DBSs was performed in two steps. In the first step, individuals with α-Gal A activity under the cutoff value (In Method I: <12 [Agal U] for males and <20 [Agal U] for females; and Method II: <15 [Agal U] for males and <20 [Agal U] for females) were recalled and their DBSs re-prepared. In the second step, individuals with α-Gal A activity under the cutoff value were assessed clinically, and GLA gene sequencing was performed after informed consent was obtained from the patients or their parents (in cases where the patients were not of legal age).

α-Gal A assay

Method I

The procedures for α-Gal A assays using DBSs were described in our previous report [10]. Briefly, a single 3.2 mm diameter disk, punched from DBSs, was incubated in a well of a 96-well clear microwell-plate (Corning, NY, USA) with 40 μL of McIlvaine buffer (100 mM citrate; 200 mM NaH₂PO₄; 36.8:63.2; pH 6.0) and processed for extraction at room temperature for 2 h. Aliquots of 30 μL blood extract were transferred to fresh 96-microwell plates. An aliquot of 100 μL of the reaction mixture (3.5 mM 4-methylumbelliferyl-α-D-galactopyranoside (4MU-αGal); 100 mM citrate; 200 mM K₂HPO₄; 100 mM N-acetyl-D-galactosamine) was added to each well of the microwell plates and incubated at 37°C for 24 h. The reaction was terminated using 150 μL of termination solution (300 mM glycine/NaOH; pH 10.6) immediately after the reaction occurred. The fluorescence intensity, from the 4-methylumbelliferones in the wells, 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.
Method II

The method II for multiple assays was developed in collaboration with KM Biologics Co., Ltd. (see details at JP6360848B) and practically implemented from November 2016. Briefly, a single 3.2 mm diameter disk, punched from DBSs, was incubated in a 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 MgCl₂, 0.5 mM DTT, 0.05% NaN₃, and 0.1% Triton X-100 for 1 h at room temperature with gentle mixing. A 20 μL aliquot of the extract was then added to 40 μL of the reaction mixture (3.0 mM 4MU-αGal; 100 mM N-acetyl-D-galactosamine in 100 mM citrate/200 mM potassium phosphate buffer; pH 4.4) in a black 96-well microwell plate (Thermo Fisher Scientific Inc., MA, USA). The reaction mixture was incubated at 38°C for 3 h, and the reaction was stopped by adding 200 μL of 300 mM glycine/NaOH buffer (pH 10.6) containing 10 mM ethylenediaminetetraacetic acid (EDTA) to measure fluorescence intensity. The residual extract could be used for the assay of acid α-glucosidase (Pompe disease) and glucocerebrosidase (Gaucher disease) activity.

Sequencing of the GLA gene

Sanger method

The genomic DNA was extracted from total blood using a Gentra Puregene Blood Kit (Qiagen, Hilden, Germany), or equivalent product, and stored at -80°C until use. All seven exons as well as the flanking intronic sequences of the GLA gene were amplified by polymerase chain reaction (PCR) [37, 38]. The region of intron 4 was also amplified to evaluate the variant, c.639+919G>A [39]. The PCR products were sequenced on an ABI3500xl autosequencer (Applied Biosystems) and analyzed using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

Next-generation sequencing (NGS) method

The sequencing method for high-throughput assay of the GLA gene by NGS was developed in collaboration with KM Biologics Co., Ltd. and practically implemented from September 2017. The procedures were described in our previous report [10]. Briefly, the 13.3 kbp region, including the GLA
gene, was amplified by long-range PCR. Library preparation and sequencing were performed using a Nextera XT Kit (Illumina, San Diego, CA, USA) and MiSeq sequencer (Illumina). After sequencing runs were completed, the data were aligned to the human reference genome sequence (NC_000023.10) using MiSeq Reporter software (Illumina). Sequence data analysis, mapping, and variant calling were streamlined using MiSeq Reporter v2 (Illumina). Visualization of sequencing reads was performed using IGV_2.3.10 (Broad Institute). Variants detected in the GLA gene by NGS were resequenced using the Sanger method.

**Prediction and statistical tools**

**Significance analysis for the variants**

The GLA mRNA reference sequence (RefSeq; NM_000169.2) was used in this study, whereby the “A” nucleotide of the ATG codon at nucleotide position 111 of RefSeq constituted +1 numbering of the cDNA sequence. The ATG codon also represented +1 for the amino acid numbering as set forth by the 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 [1] (http://fabry-database.org/, updated on February 15, 2019), and ClinVar [40] (http://www.ncbi.nlm.nih.gov/clinvar) were used for the classification of each variant. The software PolyPhen-2 [41] (http://genetics.bwh.harvard.edu/pph2) was used for missense mutations to predict the potential impact of an amino acid alteration on α-Gal A function.

**Abbreviations**

DBSs
dried blood spots; DMD: Duchenne muscular dystrophy; ERT: enzyme replacement therapy; FD: Fabry disease; HCM: hypertrophic cardiomyopathy; IQR: interquartile range; LVH: left ventricular hypertrophy; NBS: newborn screening; NGS: next-generation sequencing; α-Gal A: α-Galactosidase A; 4MU-αGal: 4-Methylumbelliferyl-α-D-Galactopyranoside

**Declarations**

**Ethics approval and consent to participate**
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).

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and/or analyzed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions SY, JK, KS, and KM were responsible for the design of the research. SY, JK, TS, KM, KS FE contributed to measurements and data collection. SY, JK, TS, FE and KM checked and analyzed data. JK and SM wrote the manuscript. JK and KM 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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Tables
Due to technical limitations, tables are only available as a download in the supplemental files section.

Figures

Figure 1

Flowchart of the high-risk screening for Fabry disease.
Figure 2

Histograms of α-Gal A activity in the high-risk and newborn screening groups. Histograms of α-Gal A activity are shown for (a) the total population (N = 14,653), (b) males (N = 7,078), and (c) females (N = 5,156) in the high-risk screening group, as well as (d) the newborn screening group (N = 483,026). Dashed line: cutoff level.

Supplementary Files
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Table 2.xlsx
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