Human Genomics

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Long-read sequencing enables comprehensive molecular genetic diagnosis of Fabry disease

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Abstract

Background The clinical diagnosis of Fabry Disease (FD) can be challenging due to the clinical heterogeneity, especially in females. Patients with FD often experience a prolonged interval between the onset of symptoms and receiving a diagnosis. Genetic testing is the gold standard for precise diagnosis of FD, however conventional genetic testing could miss deep intronic variants and large deletions or duplications. Although next-generation sequencing, which analyzes numerous genes, has been successfully used for FD diagnosis and can detect complex variants, an effective and rapid tool for identifying a wide range of variants is imminent, contributing to decrease the diagnostic delay.

Methods The comprehensive Analysis of FD (CAFD) assay was developed for FD genetic diagnosis, employing long-range PCR coupled with long-read sequencing to target the full-length *GLA* gene and its flanking regions. Its clinical performance was assessed through a comparative analysis with Sanger sequencing.

Results Genetic testing was performed on 82 individuals, including 48 probands and 34 relatives. The CAFD assay additionally identified variants in two probands: one had a novel and de novo pathogenic variant with a 1715 bp insertion in intron 4, and the other carried two deep intronic VUS variants in cis-configuration also in intron 4. In total, CAFD identified 47 different variants among 48 probands. Of these, 42 (89.36%, 42/47) were pathogenic, while 5 (10.64%, 5/47) were VUS. Sixteen (34.04%, 16/47) of the variants were novel, including 15 SNV/Indels and one large intronic insertion. Pedigree analysis of 21 probands identified four de novo disease-causing variants. Hence, FD exhibits not only variable clinical presentations but also a wide spectrum of variants. Utilizing a comprehensive testing algorithm for diagnosing FD, which includes enzyme activity, clinical features, and genetic testing, the diagnostic yield of CAFD is 97.92% (47/48), which is higher than that of conventional Sanger sequencing, at 95.83% (46/48).

Conclusion The duration between initial clinical presentation and diagnosis remains long and winding. CAFD provides precise diagnosis for a wide spectrum of *GLA* variants, promoting timely diagnosis and appropriate treatment for FD patients.

Keywords Fabry disease, Long-read sequencing, Genetic diagnosis, *GLA*, Novel variant

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Introduction

Fabry disease (FD, OMIM #301,500) is an X-linked lysosomal storage disorder caused by pathogenic variants in the *GLA* gene, which encodes the enzyme α-galactosidase A (α-Gal A) [1]. Deficiency of α-Gal A leads to progressive accumulation of globotriaosylceramide (Gb3) and its deacylated derivative, lyso-Gb3, triggering irreversible damage in multiple organs, including the kidneys, heart, blood vessels, and peripheral nervous system [2, 3]. In males, FD is classified into severe classic type and late-onset types based on clinical symptoms and residual enzyme activity. The classic form present with absent or severely reduced α-Gal A activity, leading to prominent clinical presentations in childhood, such as neuropathic pain, cornea verticillata, acroparesthaesia, and angiokeratomas. As FD progresses with age, the eventual development of disease includes hypertrophic cardiomyopathy, arrhythmias, strokes, and renal failure, particularly in untreated patients [4]. Patients with the later-onset form of FD typically have residual α-Gal A activity and exhibit clinical manifestations primarily involving a single organ, usually the heart, in adulthood. Symptoms of the classic form are generally absent, although some patients may show variable degrees of renal, peripheral nervous system, and gastrointestinal involvement. In females, random X-inactivation complicates the phenotype. The classic and later-onset phenotypes can range from as severe as those seen in affected male relatives to asymptomatic. However, they generally present with a later onset of organ damage [3–6].

FD is more frequent than initially detected, especially with the increased recognition of the later-onset type [5, 7–10]. Initially, the frequency of FD was estimated to be between 1 in 117,000 [7] and 1 in 40,000 [9] live births. However, newborn screening has shown that the incidence of the later-onset type of FD is at least 1 in 4000 live male births [11], which may still be an underestimate due to the increasing numbers of attenuated later-onset cases. Screening studies of high-risk populations have revealed that up to 1% of patients with hypertrophic cardiomyopathy (HCM), cryptogenic stroke, and end-stage renal failure indeed have previously undiagnosed FD [12, 13].

The *GLA* gene is located on the long arm of the X chromosome (band q22), spans 12 kb, and contains seven exons [14]. Over 1,000 variants have been identified in *GLA*, including single nucleotide variants and small insertions/deletions (SNVs/indels), as well as large deletions and duplications caused by gene rearrangements [6, 15, 16]. Previous studies have shown that the *GLA* gene is rich in Alu repeat elements, and Alu-Alu recombination can lead to large gene rearrangements [15, 17], which are challenging to detect using conventional genetic testing techniques, especially in FD heterozygotes.

FD patients often experience a prolonged interval between initial clinical manifestations and receiving diagnosis and appropriate treatment due to clinical heterogeneity. Timely and accurate diagnosis can expedite this process, helping to prevent tissue damage and organ failure. In male patients with classic FD, diagnosis can typically be established based on extremely low or undetectable α-Gal A activity. However, in some female patients, α-Gal A activity overlaps with normal values, posing significant diagnostic challenges [18]. Genetic testing combined with the assay of α-Gal A enzyme activity is performed in FD screening studies [10, 13, 19]. Lyso-Gb3 has been shown to be a sensitive biomarker that can improve the diagnosis of FD and serves as a promising tool in screening studies [20–22]. However, genetic testing remains the gold standard for the diagnosis of FD, especially in females [2]. Genetic testing of FD is frequently performed by conventional Sanger sequencing or next-generation sequencing (NGS) including whole exome sequencing (WES), Whole genome sequencing (WGS) and targeted NGS. Conventional Sanger sequencing can only detect variants in all exons and exon boundaries of the *GLA* gene, as same as WES. Deep intronic variants cannot be diagnosed with this method [23, 24]. While nested PCR or long-range PCR followed by Sanger sequencing can address this shortcoming by detecting deep intronic and intragenic variants, these strategies are not high-throughput and are labor-intensive [25]. Although WGS or targeted NGS can identify complex *GLA* variants, including SNVs/indels and large deletions/duplications resulting from gene rearrangements, these methods analyze a large number of genes, which significantly slows down the analysis process [26, 27]. Long-read sequencing (LRS), primarily using Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (ONT), is a promising technology for identifying complex variants [24, 28]. Recently, Nanopore-based sequencing has been used to diagnose FD in a small cohort of twelve patients [29], but, LRS has not yet been employed in FD diagnosis, particularly in large cohorts.

In this study, the comprehensive Analysis of FD (CAFD) assay was developed for FD genetic diagnosis in a larger cohort., employing long-range PCR coupled with long-read sequencing to target the full-length *GLA* gene and its flanking region, The clinical utility of CAFD was further accessed by comparing its performance with Sanger sequencing. CAFD accurately identified three variants that Sanger sequencing missed in two probands. This study presents that the CAFD could be a promising alternative for Sanger sequencing in FD diagnosis, which

is crucial for improving treatment protocols and achieving good outcomes of patients.

Methods

Patient cohort

This retrospective cohort included 48 unrelated patients suspected of having FD and 34 of their relatives, evaluated at Peking Union Medical College Hospital from 2004 to 2023.

Patients in this study were diagnosed as FD if they met the following criteria: (i) Males with a pathogenic or likely pathogenic *GLA* variant combined with low leukocyte α-Gal A activity. (ii) Males with a *GLA* variant of unknown significance (VUS), combined with low leukocyte α-Gal A activity and at least one of the following symptoms: acroparesthesias, neuropathic pain, cornea verticillata or angiokeratomas. (iii) Females with a pathogenic or likely pathogenic *GLA* variant combined with either deficient or normal leukocyte α-Gal A activity. (iv) Females with a *GLA* VUS, combined with at least one of the following criteria: acroparesthesias, neuropathic pain, cornea verticillata, angiokeratomas or an affected family member with a confirmed diagnosis according to the above criteria [2, 8, 23, 30].

The patients with a *GLA* VUS presenting with non-specific signs were determined an uncertain diagnosis of FD. Non-specific signs of FD included left ventricular hypertrophy (LVH), stroke at young age, proteinuria, hypohidrosis and gastrointestinal involvement [23].

Patients were determined to be unaffected with FD if they do not fulfill the above criteria [2, 8, 23, 30]. Males and females diagnosed with FD who exhibited an attenuated phenotype were defined as having non-classic FD.

The α-Gal A activity was determined by a fluorometric test using leukocytes from blood samples. The α-Gal A values in this study were lower than the minimum values of normal ranges (29.0–64.4 nmol/ml). Additionally, all individuals in the cohort underwent conventional Sanger sequencing to detect variants in exons and exon boundaries of the *GLA* gene (National Center for Biotechnology Information ref-seq: NM_000169). Patient data were obtained through retrospective clinical chart review. The demographic and clinical characteristics of the study groups are shown in Table 1.

Long-range PCR and LRS

Long-range PCR was performed with two sets of primers, the location of which were shown in Fig. 1A. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). All samples met quality standards, with a mean sequencing depth of 2,717 (ranges from 30 to 11,960). Long-range PCR was conducted using KOD FX Neo (Toyobo Co. Ltd., Osaka, Japan) in 50-μL reactions containing 10–100 ng of genomic DNA, 1×PCR buffer for KOD FX Neo, 0.4 mM of each dNTP, 1 μM of primer mixture, and 1 μL of KOD FX Neo (TOYOBO). The amplicons generated were sequenced using LRS. The prelibraries of single-molecule real-time (SMRT) sequencing were prepared though a one-step end repair. A barcoded adaptor was added through a ligation reaction. The SMRTbell library was constructed using the Sequel Binding Kit 2.0 (PacBio) and sequenced on the Sequel II platform (PacBio).

PacBio data analysis and variant calling

The raw data were processed to obtain highly accurate circular consensus sequencing (CCS) reads, and then the filtered and split CCS reads were aligned to human GRCh38/hg38 genome sequences using pbmm2 of the SMRT Link analysis software (PacBio). SNVs/Indels were identified using FreeBayes 1.3.4. Deletions in the *GLA* gene were analyzed through alignment with the reference genome GRCh38/hg38. The exact breakpoints of deletions were determined and displayed using the Integrative Genomics Viewer (IGV).

Results

The design of molecular genetic diagnosis for GLA gene

LRS was introduced for the accurate molecular diagnosis of FD, called comprehensive analysis of FD (CAFD). Two sets of primers were designed to identify the variants in the *GLA* gene (Fig. 1A). Primer set 1 included GLA-F and GLA-R, located at both ends of the *GLA* gene, to detect single-nucleotide variants (SNVs), small insertions/deletions (Indels), and intragenic large deletions or duplications. Primer set 2 consisted of three gap primer pairs to detect intergenic large deletions or duplications (Fig. 1B). The flowchart of molecular diagnosis is shown in Fig. 1C. High-quality DNA fragments were generated through long-range PCR for subsequent library construction and sequencing, from which Qualified data were obtained.

Study population

A total of 82 individuals, including 48 probands and 34 relatives, were enrolled in this study. The clinical characteristics and genetic testing results of all 82 individuals are shown in Table 1. Among the 48 probands, there are 44 males and 4 females (Fig. 2A). The age at diagnosis is illustrated in Fig. 2B, with no significant difference ($p=0.1426$) between male and female groups. The age at diagnosis for males ranged from 8 to 60 old years, highlighting that some individuals were diagnosed much later in life.

The results of α-Gal A activity assay were shown in Fig. 2C. Enzyme activity in the male group was significantly lower than in the female group, with an extremely

Table 1 The baseline characteristics and molecular diagnosis results in 82 individuals

Family	Sample	Family member	Gender	Years at diagnosis	Age at diagnosis	α-Gal A activity (nmol/mL)	Sanger sequencing	CAFD	Phenotype	Concordance	De novo
F1	F1-1	Proband	Male	2004	14	0.4	c.796G > A	c.796G > A	Classic	Yes	
	F1-2	Mother	Female	NA	NA	NA	c.796G > A	c.796G > A	NA	Yes	
F2	F2-1	Proband	Male	2022	10	0.1	c.1213_1217del AGTCA	c.1213_1217del AGTCA	Classic	Yes	
F3	F3-1	Proband	Female	2022	13	21.4	c.769G > C	c.769G > C	Later-onset	Yes	De novo
	F3-2	Mother	Female	NA	NA	NA	NV	NV	NA	Yes	
	F3-3	Father	Male	NA	NA	NA	NV	NV	NA	Yes	
F4	F4-1	Proband	Male	2023	60	1.1	c.886A > G	c.886A > G	Later-onset	Yes	
	F4-2	Daughter	Female	NA	NA	NA	c.886A > G	c.886A > G	NA	Yes	
F5	F5-1	Proband	Male	2021	10	0.1	c.305C > A	c.305C > A	Classic	Yes	
F6	F6-1	Proband	Male	2020	27	0.3	c.1095dup	c.1095dup	Classic	Yes	
F7	F7-1	Proband	Male	2019	25	3.9	c.1067G > A	c.1067G > A	Classic	Yes	
F8	F8-1	Proband	Male	2019	18	0.3	c.130 T > G (VUS)	c.130 T > G (VUS)	Classic	Yes[VUS]	
F9	F9-1	Proband	Male	2019	30	0.1	c.779G > C	c.779G > C	Classic	Yes	
F10	F10-1	Proband	Male	2018	16	0.1	c.77dup	c.77dup	Classic	Yes	
F11	F11-1	Proband	Male	2018	33	0.1	c.1094dup	c.1094dup	Classic	Yes	
	F11-2	Mother	Female	NA	NA	NA	c.1094dup	c.1094dup	NA	Yes	
F12	F12-1	Proband	Male	2018	33	0.1	c.408 T > G	c.408 T > G	Classic	Yes	
F13	F13-1	Proband	Male	2018	8	0.1	c.548-1G > A	c.548-1G > A	Classic	Yes	De novo
	F13-2	Mother	Female	NA	NA	NA	NV	NV	NA	Yes	
F14	F14-1	Proband	Male	2018	21	0.1	c.425G > T	c.425G > T	Classic	Yes	
	F14-2	Mother	Female	NA	NA	NA	c.425G > T	c.425G > T	NA	Yes	
F15	F15-1	Proband	Male	2018	17	0.2	c.782G > A	c.782G > A	Classic	Yes	
	F15-2	Mother	Female	NA	NA	NA	c.782G > A	c.782G > A	NA	Yes	
	F15-4	Sister	Female	NA	NA	NA	c.782G > A	c.782G > A	NA	Yes	
F16	F16-1	Proband	Male	2018	31	0.2	c.1021G > A	c.1021G > A	Classic	Yes	
F17	F17-1	Proband	Male	2018	42	0.2	c.266 T > C	c.266 T > C	Classic	Yes	
F18	F18-1	Proband	Male	2018	31	2.7	c.644A > G	c.644A > G	Later-onset	Yes	
F19	F19-1	Proband	Male	2017	41	0.3	c.892A > G	c.892A > G	Classic	Yes	
	F19-2	Daughter	Female	NA	NA	NA	c.892A > G	c.892A > G/c.640-363C > T (VUS)	NA	No	
F20	F20-1	Proband	Male	2016	14	0.1	c.613C > A	c.613C > A	Classic	Yes	
	F20-2	Mother	Female	NA	NA	NA	c.613C > A	c.613C > A	NA	Yes	

Table 1 (continued)

Family	Sample	Family member	Gender	Years at diagnosis	Age at diagnosis	α-Gal A activity (nmol/mL)	Sanger sequencing	CAFD	Phenotype	Concordance	De novo
F21	F21-1	Proband	Male	2015	11	0.4	c.424 T > C	c.424 T > C	Classic	Yes	De novo
	F21-2	Mother	Female	NA	NA	NA	NV	NV	NA	Yes	
F22	F22-1	Proband	Male	2015	19	0.2	c.195-1G > A	c.195-1G > A	Classic	Yes	
F23	F23-1	Proband	Male	2015	15	0.1	c.931C > T	c.931C > T	Classic	Yes	
F24	F24-1	Proband	Female	2014	25	10.2	c.1188_1208dup (VUS)	c.1188_1208dup (VUS)	Later-onset	Yes^{VUS}	
	F24-2	Mother	Female	NA	NA	NA	c.1188_1208dup (VUS)	c.1188_1208dup (VUS)	NA	Yes^{VUS}	
	F24-3	Father	Male	NA	NA	NA	NV	NV	NA	Yes	
F25	F25-1	Proband	Male	2014	16	0	c.388_390dup (VUS)	c.388_390dup (VUS)	Classic	Yes^{VUS}	
	F25-2	Mother	Female	NA	NA	NA	c.388_390dup (VUS)	c.388_390dup (VUS)	NA	Yes^{VUS}	
F26	F26-1	Proband	Male	2014	14	0	c.896A > G	c.896A > G	Classic	Yes	
	F26-2	Mother	Female	NA	NA	NA	c.896A > G	c.896A > G	NA	Yes	
	F26-3	Sister	Female	NA	NA	NA	c.896A > G	c.896A > G	NA	Yes	
F27	F27-1	Proband	Male	2014	32	0.2	c.658C > T	c.658C > T	Classic	Yes	
F28	F28-1	Proband	Male	2014	12	0.1	c.1235_1236del	c.1235_1236del	Classic	Yes	
F29	F29-1	Proband	Male	2013	12	0.3	c.658C > T	c.658C > T	Classic	Yes	
	F29-2	Mother	Female	NA	NA	NA	c.658C > T	c.658C > T	NA	Yes	
F30	F30-1	Proband	Male	2012	9.5	0.1	c.644A > G	c.644A > G	Classic	Yes	
F31	F31-1	Proband	Male	2014	19	0.1	c.778G > C	c.778G > C	Classic	Yes	
F32	F32-1	Proband	Male	2012	22	0.1	c.837G > T	c.837G > T	Classic	Yes	
F33	F33-1	Proband	Male	2011	33	0.2	c.334C > T	c.334C > T	Classic	Yes	
	F33-2	Mother	Female	NA	NA	NA	c.334C > T	c.334C > T	NA	Yes	
	F33-3	Auntie	Female	NA	NA	NA	c.334C > T	c.334C > T	NA	Yes	
F34	F34-1	Proband	Male	2021	14	0.2	c.1220 T > A	c.1220 T > A	Classic	Yes	
F35	F35-1	Proband	Male	2011	19	0.2	c.718_719del	c.718_719del	Classic	Yes	
F36	F36-1	Proband	Male	2011	12	0.2	c.719dup	c.719dup	Classic	Yes	
	F36-2	Mother	Female	NA	NA	NA	c.719dup	c.719dup	NA	Yes	
F37	F37-1	Proband	Male	2008	14	0.1	c.678G > A	c.678G > A	Classic	Yes	
	F37-2	Mother	Female	NA	NA	NA	c.678G > A	c.678G > A	NA	Yes	
F38	F38-1	Proband	Male	2005	11	0.3	c.647A > G	c.647A > G	Classic	Yes	
	F38-2	Mother	Female	NA	NA	NA	c.647A > G	c.647A > G	NA	Yes	
	F38-3	Other family member	Unknown	NA	NA	NA	c.647A > G	c.647A > G	NA	Yes	

Table 1 (continued)

Family	Sample	Family member	Gender	Years at diagnosis	Age at diagnosis	α-Gal A activity (nmol/mL)	Sanger sequencing	CAFD	Phenotype	Concordance	De novo
F39	F39-1	Proband	Male	2014	28	0.2	c.927del	c.927del	Classic	Yes	
	F39-2	Mother	Female	NA	NA	NA	c.927del	c.927del	NA	Yes	
F40	F40-1	Proband	Male	2012	13	0.2	c.1196G > A	c.1196G > A	Classic	Yes	
	F40-2	Mother	Female	NA	NA	NA	c.1196G > A	c.1196G > A	NA	Yes	
F41	F41-1	Proband	Male	2019	47	0.1	c.738_775del	c.738_775del	Classic	Yes	
	F41-2	Other family member	Unknown	NA	NA	NA	c.738_775del	c.738_775del	NA	Yes	
	F41-3	Other family member	Unknown	NA	NA	NA	c.738_775del	c.738_775del	NA	Yes	
F42	F42-1	Proband	Male	2008	33	0.2	c.485G > A	c.485G > A	Classic	Yes	
F43	F43-1	Proband	Male	2009	25	0.3	c.773G > T	c.773G > T	Classic	Yes	
	F43-2	Mother	Female	NA	NA	NA	c.773G > T	c.773G > T	NA	Yes	
F44	F44-1	Proband	Female	2008	10	15.7	c.901C > T	c.901C > T	Later-onset	Yes	
F45	F45-1	Proband	Male	2008	13	0.2	c.1022A > G	c.1022A > G	Classic	Yes	
F46	F46-1	Proband	Male	2005	24	0.4	c.119C > A	c.119C > A	Classic	Yes	
	F46-2	Other family member	Unknown	NA	NA	NA	NV	NV	NA	Yes	
	F46-3	Other family member	Unknown	NA	NA	NA	NV	NV	NA	Yes	
F47	F47-1	Proband	Male	2016	12	0.7	NV	Int4 ins 1715 bp (chrX:101399559ins1715bp)	Classic	No	De novo
	F47-2	Mother	Female	NA	NA	NA	NV	NV	NA	Yes	
	F47-3	Sister	Female	NA	NA	NA	NV	NV	NA	Yes	
F48	F48-1	Proband	Female	2008	4	19.1	NV	c.640-363C > T (VUS), c.639 + 761G > A (VUS)	Later-onset	No[VUS]	
	F48-2	Mother	Female	NA	NA	NA	NV	c.639 + 761G > A (VUS)	NA	No[VUS]	

NA was used as an abbreviation for Not applicable, *NV* was used as an abbreviation for no variants

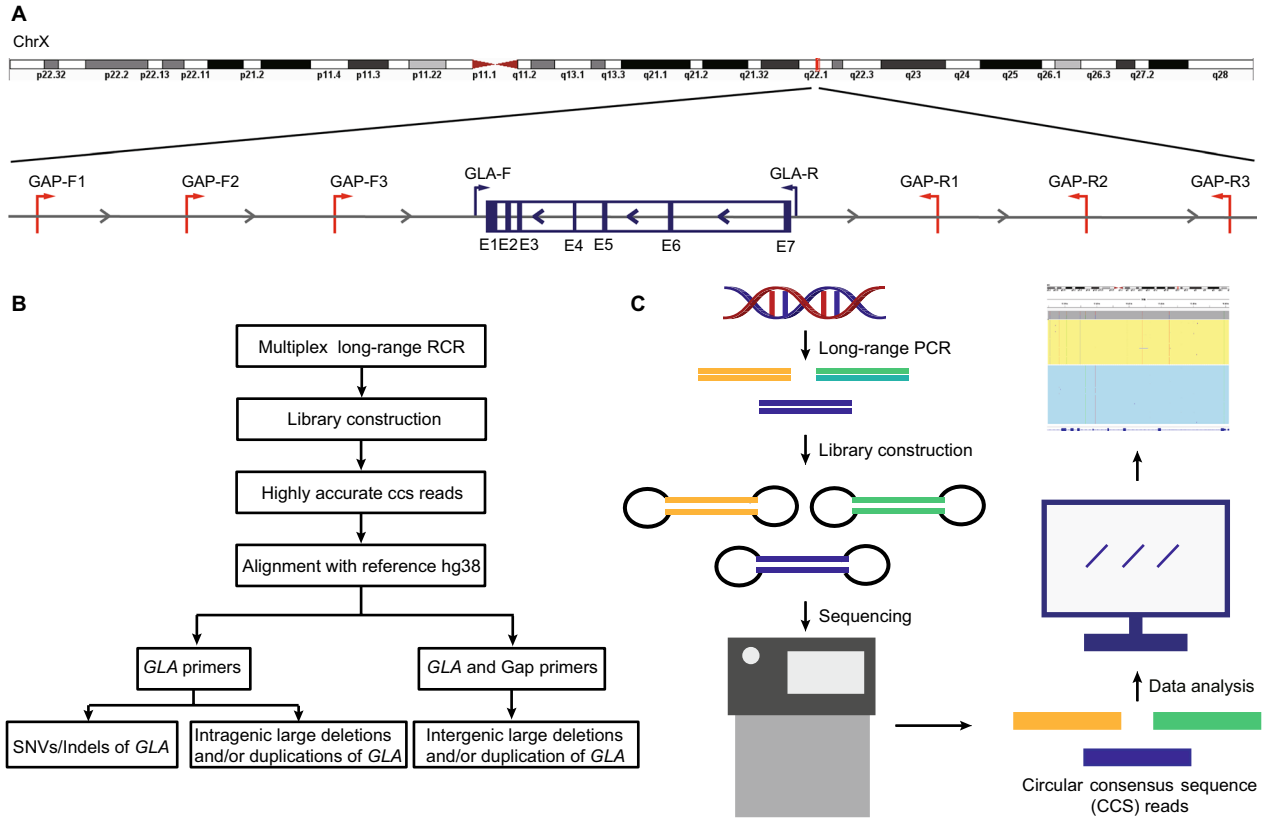

Fig. 1 The design of molecular testing for the *GLA* gene. **A** The location of the *GLA* gene and two primer sets. **B** The procedure of the experimental design. **C** Flowchart of the molecular genetic diagnosis of FD

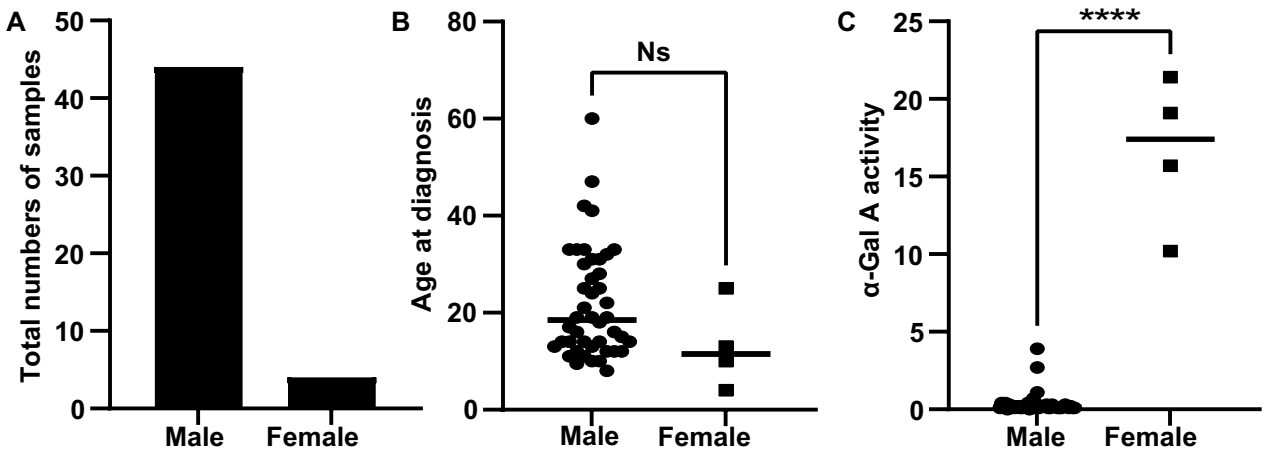

Fig. 2 The demographic information and enzyme activity assay of the enrolled 48 probands. **A** Histogram showing the numbers of males and females in the enrolled samples. **B** A scatter plot visualizing the age at diagnosis in male and female samples. **C** A scatter plot showing the α-Gal A activity in male and female patients. "Ns" indicates no significant difference ($p = 0.1426$). "****" indicates an extremely significant difference ($p < 0.0001$)

significant decrease ($p < 0.0001$). In males, α-Gal A activity ranged from 0.1 to 2.7 nmol/ml, markedly below the reference range (29.0–64.4 nmol/ml). In females, α-Gal A

activity ranged from 10.2 to 21.4 nmol/ml, higher than in male but still below the normal range.

CAFD improved genetic diagnosis of FD compared with sanger sequencing

All individuals enrolled in this study were tested using both CAFD and Sanger sequencing. A comparative analysis of these two diagnostic methods was conducted (Table 1). CAFD accurately identified three variants that were missed by Sanger sequencing in two probands. In family F47, a male proband, P47-1, carried a novel 1715 bp intronic insertion in intron 4 (Fig. 3A–D). In family F48, the female proband, F48-1, had two deep intronic variants on one allele (Fig. 3E and F).

In family F47, CAFD identified a novel pathogenic variant with a 1715 bp insertion in intron 4 of the male proband, which the control method did not detect due to its location in a deep intronic region. Interestingly, the proband's mother didnot have this pathogenic variant (Fig. 3A and B), suggesting it was a de novo variant

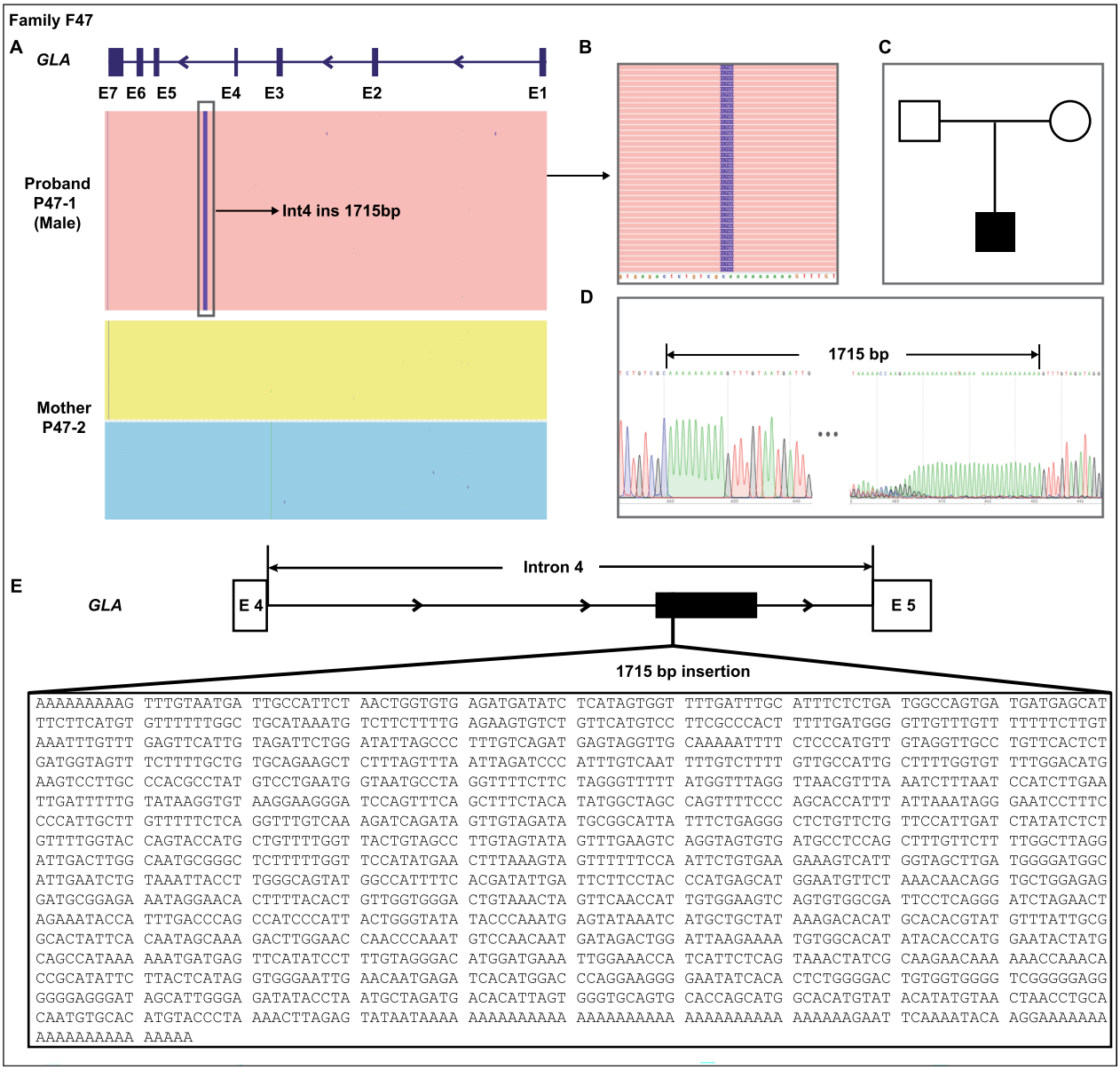

Fig. 3 The variants identified in family F47 by CAFD. **A** The Integrative Genomics Viewer (IGV) plots of genotype detected by CAFD in family F47. **B** The IGV plots of the insertion region for proband 47–1 in family F47. **C** Pedigree of family F47. **D** The insertion variant detected by CAFD in family F47 was confirmed by Sanger sequencing. **E** The location of AluSx-mediated intron4 insertion

(Fig. 3C). The insertion sequence was later confirmed by Sanger sequencing (Fig. 3D). Further analysis indicated that this large insertion is located in the region of AluSx element, suggesting that it is caused by AluSx-mediated gene rearrangement (Fig. 3E).

In family F48, CAFD identified two deep intronic variants in cis configuration in the female proband, which were not detected by the control method (Fig. 4A). Both two variants were located in a deep intronic region and were inherited from her mother (Fig. 4B). Sanger sequencing confirmed these variants (Fig. 4C). These variants were classified as variants of unknown significance (VUS). The clinical characteristics were documented for further analysis.

The characteristics of variants detected by LRS

Overall, 47 variants were identified by CAFD in 48 probands (Table 2). Except for the proband in family F48, who had two variants in cis-configuration (Fig. 3E), all other probands carried a single variant. The characteristics of these 47 variants are visualized in Fig. 5. Variants c.658C > T and c.644A > G had a 4% frequency, higher than that of other variants (2%). The pathogenicity of variants was predicted based on the guidelines of the American College of Medical Genetics and Genomics (ACMG) [31]. Detailed ACMG classification and evidence levels for each of the 47 variants were listed in Table S1. Of the 47 variants, 42 (89.36%) were pathogenic, while 5 (10.64%) were classified as VUS. Sixteen (34.04%) variants were novel and previously unreported, including 15 novel SNV/Indels and one novel large intronic insertion (Fig. 5A).

The variants identified in this study were further categorized as frameshift, missense, nonsense, intronic variants, and in-frame insertions (Table 2 and Fig. 5). The distribution of variant types was shown in Fig. 4B. Missense variants were the most common, accounting for 54.17%. Eight frameshift variants (17.02%) and seven nonsense variants (14.89%) were also detected. Five intronic variants were identified, three of which (60%)

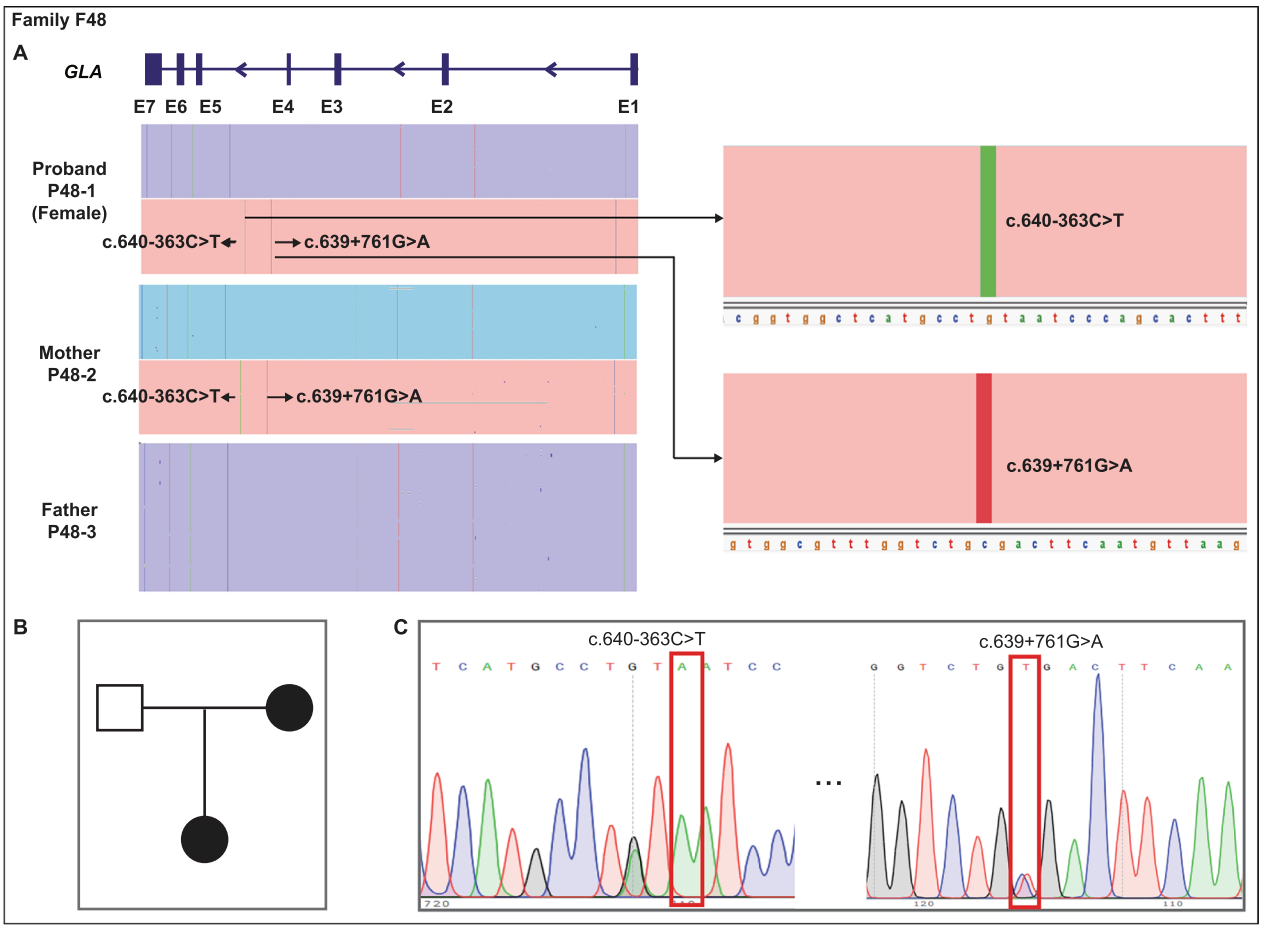

Fig. 4 The variants identified in family F48 by CAFD. **A** The Integrative Genomics Viewer (IGV) plots of genotype detected by CAFD in family F48. **B** Pedigree of family F48. **C** The SNVs detected by CAFD in F48 were confirmed by Sanger sequencing

Table 2 The characteristic of 47 variants detected by CAFD

Variants	Changed amino acid	Variants type	Location	Variant numbers	Frequency of variants	References
SNVs/Indels						
c.644A > G	p.N215S	Missense	Exon 5	2	4.26%	[32]
c.658C > T	p.R220*	Nonsense	Exon 5	2	4.26%	[33]
c.796G > A	p.D266N	Missense	Exon 5	1	2.13%	[34]
c.1213_1217del AGTCA	p.S405Hfs*	Frameshift	Exon 7	1	2.13%	This study
c.769G > C	p.A257P	Missense	Exon 5	1	2.13%	Published[a]
c.886A > G	p.M296V	Missense	Exon 6	1	2.13%	[35]
c.305C > A	p.S102*	Nonsense	Exon 2	1	2.13%	This study
c.1095dup	p.T366Yfs*9	Frameshift	Exon 7	1	2.13%	This study
c.1067G > A	p.R356Q	Missense	Exon 7	1	2.13%	[10]
c.130 T > G (VUS)	p.W44G	Missense	Exon 1	1	2.13%	This study
c.779G > C	p.G260A	Missense	Exon 5	1	2.13%	[36]
c.77dup	p.G28Wfs*3	Frameshift	Exon 1	1	2.13%	[37]
c.1094dup	p.Y365*	Nonsense	Exon 7	1	2.13%	[38]
c.408 T > G	p.D136E	Missense	Exon 3	1	2.13%	This study
c.548-1G > A	NA	Intronic variant	Intron 3	1	2.13%	[39]
c.425G > T	p.C142F	Missense	Exon 3	1	2.13%	This study
c.782G > A	p.G261D	Missense	Exon 5	1	2.13%	[40]
c.1021G > A	p.E341K	Missense	Exon 7	1	2.13%	[41]
c.266 T > C	p.L89P	Missense	Exon 2	1	2.13%	[42]
c.892A > G	p.N298D	Missense	Exon 6	1	2.13%	This study
c.613C > A	p.P205T	Missense	Exon 4	1	2.13%	[43]
c.424 T > C	p.C142R	Missense	Exon 3	1	2.13%	[44]
c.195-1G > A	NA	Intronic variant	Intron 1	1	2.13%	[45]
c.931C > T	p.L311F	Missense	Exon 6	1	2.13%	[46]
c.1188_1208dup (VUS)	p.F396_R402dup	Inframe insertion	Exon 7	1	2.13%	This study
c.388_390dup (VUS)	p.K130dup	Inframe insertion	Exon 3	1	2.13%	This study
c.896A > G	p.D299G	Missense	Exon 6	1	2.13%	Published[b]
c.1235_1236del	p.T412Sfs*	Frameshift	Exon 7	1	2.13%	[45]
c.778G > C	p.G260R	Missense	Exon 5	1	2.13%	This study
c.837G > T	p.Q279H	Missense	Exon 6	1	2.13%	[47]
c.334C > T	p.R112C	Missense	Exon 2	1	2.13%	[48]
c.1220 T > A	p.I407K	Missense	Exon 7	1	2.13%	[49]
c.718_719del	p.K240Efs*9	Frameshift	Exon 5	1	2.13%	[50, 51]
c.719dup	p.S241Efs*9	Frameshift	Exon 5	1	2.13%	This study
c.678G > A	p.W226*	Nonsense	Exon 5	1	2.13%	[51, 52]
c.647A > G	p.Y216C	Missense	Exon 5	1	2.13%	[53]
c.927del	p.L310Sfs*7	Frameshift	Exon 6	1	2.13%	This study
c.1196G > A	p.W399*	Nonsense	Exon 7	1	2.13%	[42]
c.738_775del	p.S247Rfs*5	Frameshift	Exon 5	1	2.13%	This study
c.485G > A	p.W162*	Nonsense	Exon 3	1	2.13%	[32]
c.773G > T	p.G258V	Missense	Exon 5	1	2.13%	[50]
c.901C > T	p.R301*	Nonsense	Exon 6	1	2.13%	[54]
c.1022A > G	p.E341G	Missense	Exon 7	1	2.13%	[53]
c.119C > A	p.P40H	Missense	Exon 1	1	2.13%	[45]
c.640-363C > T (VUS)	NA	Intronic variant	Intron 4	1	2.13%	This study
c.639 + 761G > A (VUS)	NA	Intronic variant	Intron 4	1	2.13%	This study
Large insertion						
Int4 ins 1715 bp (chrX:101399559ins1715 bp)	NA	Intronic variant (Large insertion)	Intron 4	1	2.13%	This study

Table 2 (continued)

[a] The variant is listed in LOVD (https://www.lovd.nl/). The reference is cited as Cooper (2000), but the full paper was not found

[b] The variant is listed in LOVD (https://www.lovd.nl/). The reference is cited as Kwan (2007), but the full paper was not found

were undetected by conventional Sanger sequencing due to their location in deep intronic regions. Only two variants were in-frame insertions. Most variants were located in exon 5 (27.66%), followed by exon 7 (21.28%) and exon 6 (14.89%).

Among the 48 families, 21 included samples from both probands and their parents. Family analysis indicated that four probands (19.05%, 4/21) carried de novo variants (Table 1), including c.769G > C (p.A257P), c.548-1G > A, c.424 T > C (p.C142R) and a novel 1715 bp intronic insertion.

Outcomes of diagnostic testing in probands

Diagnostic testing based on the diagnostic criteria was performed on 48 probands with a clinical suspicion of FD. Of these, 97.92% (47/48) were diagnosed with FD, while 2.08% (1/48) had an uncertain diagnosis. Therefore, the diagnostic yield of LRS and conventional Sanger sequencing was 97.92% (47/48) and 95.83% (46/48), respectively. Clinical symptoms of the four probands with VUS identified in this study were detailed in Table S2. Three probands with VUS were confirmed to have FD. Among them, two male probands, P8-1 (c.130 T > G, p.W44G) and P25-1 (c.388_390dup, p.K130dup), were diagnosed with FD based on extremely low or absent α-Gal A activity and specific symptoms of FD. Additionally, a female proband, P24-1 (c.1188_1208dup, p.F396_R402dup), with deficient α-Gal A activity, specific symptoms, and a positive family history, was also diagnosed with FD. However, a diagnosis of FD could not be confirmed in the female proband P48-1 (c.640-363C > T, c.639 + 761G > A), and an uncertain diagnosis was determined. This proband exhibited deficient α-Gal A activity and a history of non-specific symptoms, including persistent headache and hypohidrosis.

Discussion

In this study, the utility of CAFD was evaluated in a cohort of 48 probands who were clinically suspected of FD. Molecular genetic diagnosis was performed using both CAFD and conventional Sanger sequencing, and diagnosis of FD was determined based on the diagnostic criteria. The diagnostic yield of CAFD and conventional Sanger sequencing was 97.92% and 95.83%, respectively, with a 2.09% increase with CAFD. In family F47, the male proband F47-1, with a novel intronic insertion variant caused by AluSx-mediated gene rearrangement, was newly diagnosed by CAFD. This suggested that CAFD can identify missing variants and provide a more precise genetic diagnosis for FD. Although a female proband, F48-1, from family F48, with deep intronic variants, was identified by CAFD, the diagnosis of FD cannot be confirmed without further supportive data.

The diagnosis of FD is challenging due to variable presentation and progressive cardiac, renal, and cerebral involvement. Diagnosis relies on clinical characteristics, which are often variable and non-specific. Initially, α-Gal A activity was used in the diagnosis of FD [7]. Male patients with classic form exhibited extremely low or undetectable α-Gal A activity.However, the α-Gal A activity in patients with late-onset form can overlap with the reference range, especially in females, due to random X chromosome inactivation [55].Plasma lyso-Gb3 has high sensitivity and specificity for FD in both males and females and provides supportive diagnostic information, especially in cases where α-Gal A activity or gene sequencing results are inconclusive [24]. It should be noted, however, that normal levels of plasma lyso-Gb3 have been detected in infant FD patients with a likely pathogenic *GLA* variant, highlighting the limitation of this biomarker. Therefore, precise genetic diagnosis plays a prominent role in FD diagnosis and is considered the gold standard. Enzymatic and biomedical diagnosis must be confirmed through genetic testing [2, 18, 23, 56]. A accurate diagnostic program for individuals with clinical suspicion of FD could include enzyme activity, plasma lyso-Gb3, clinical features and *GLA* gene sequencing [2, 23].

Conventional Sanger sequencing and NGS are usually applied for the genetic diagnosis of FD. However, deep intronic variants cannot be detected by conventional Sanger sequencing and NGS based WES, as evidenced by the fact that 60% of intronic variants were undetected by conventional Sanger sequencing in this study. While the variants in both exons and introns can be identified using nested PCR or long-range PCR based Sanger sequencing, this approach is labor-intensive and unsuitable for large-scale applications. Furthermore, Sanger sequencing struggles to resolve the cis/trans configuration of multiple variants. With respect to large deletions or insertions caused by gene rearrangements, conventional Sanger sequencing may yield negative results in heterozygous females, as the normal allele can mask the pathogenic allele. WGS, WES and targeted NGS, have been successfully applied to detect various types of variants including SNVs/indels and large deletions/duplications

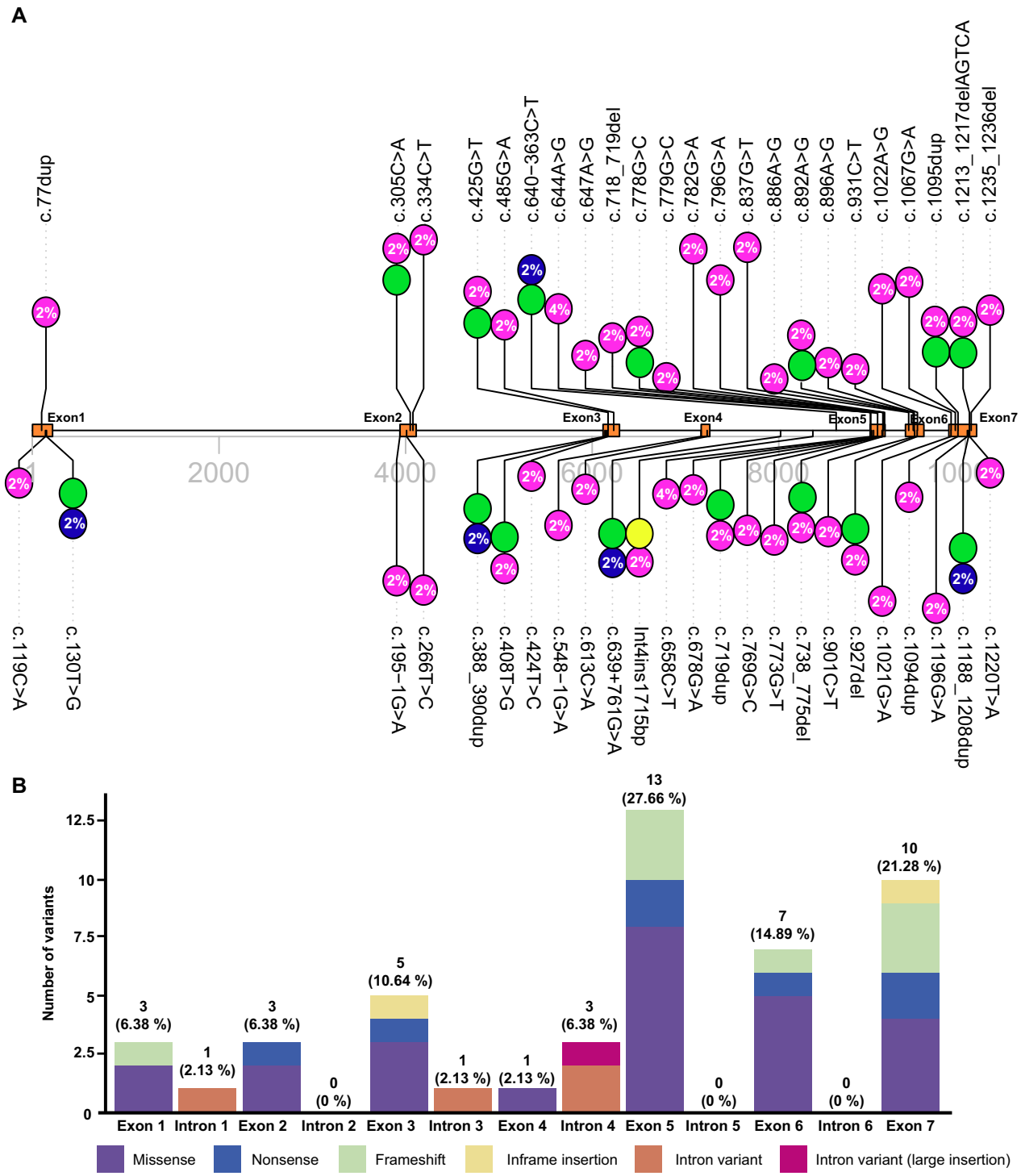

Fig. 5 The characteristics of variants in 48 probands. **A** The distribution of variants in the *GLA* gene. **B** Stacked chart showing the distribution of variant types. The percentages in the circles exhibit the frequency of variants. The circles filled with purple and blue represent pathogenic variants and VUS, respectively. The variants with yellow or green circles indicate novel variants. Yellow circles indicated a novel 1715 bp intronic insertion. Green circles indicate novel SNV/Indels

resulting from gene rearrangements. However, these strategies, which cover numerous genes, may not be the best choice for clinicians who ask for a fast and cost-effective method. Compared to other methods, CAFD exhibits significant advantages in identifying deep intronic variants, large deletion/insertion variants and the cis/trans configuration of multiple variants. Furthermore, the targeted sequencing strategy of CAFD at high-depth (ranges from 30 to 11,960) allows the accurate discovery of variants, sequencing data was faster and easier to analyze. Above all, CAFD may actually be a promising alternative to sanger sequencing for first-line genetic testing.

This is one of the largest studies on the diagnosis of FD. A novel, de novo pathogenic variant with a 1715 bp insertion located in the AluSx element region of intron 4 in the *GLA* gene was identified using CAFD. This is the first report of AluSx-mediated large insertion in intron 4 [15, 57]. Male patients carrying this variant were hemizygous, exhibiting extremely low enzyme activity (0.7 nmol/ml) at 12 years old. In total, over 1,000 variants have been identified in *GLA*, with approximately 5% causing aberrant splicing [58]. Most splice site variants are found within highly conserved sequences at exon–intron boundaries [59], while only a few variants occur in deep intronic region [25, 60]. The variant IVS4 + 1326C > T, for example, exhibits high residual α-GalA activity (25%) [25]. More severe enzymatic phenotypes were identified for a Chinese hotspot late-onset variant IVS4 + 919G > A [61] and a classic variant IVS4 + 861C > T[62].

CAFD identified 16 novel variants, including 5 VUS and 11 pathogenic or likely pathogenic variants, none of which were registered in the genetic variant databases such as the Human Gene Mutation Database (HGMD), ClinVar Database, and Leiden Open Variation Database (LOVD). The functional role of VUS needs more supportive data in future studies. Notably, CAFD detected 47 variants in 48 probands, highlighting the genetic diversity of *GLA*. Additionally, family pedigree analysis identified four rare de novo pathogenic variants. Hence, FD not only exhibits variable clinical presentations but also has a wide spectrum of variants.

With the development of diagnostic techniques and increased recognition of late-onset disease, FD is now diagnosed more frequently, especially in high-risk populations [8, 13]. High-risk populations include individuals with HCM, end-stage renal disease, and stroke. As previously reported, FD accounts for 1.14% of patients with HCM, 0.3% of patients with end-stage renal disease and 1.6% of patients with stroke [3, 8]. Due to advances in knowledge of FD and the development of disease-specific therapies, timely and accurate genetic diagnosis is extremely important. Although disease management has improved, FD patients spend a lot of time from initial clinical manifestations to diagnosis and appropriate treatment. Timely and accurate diagnosis can accelerate this progress to prevent tissue damage and organ failure. CAFD exactly provides such an accurate and efficient diagnostic strategy to improve the management of FD and ensure prompt treatment for patients.

Overall, CAFD has proven to be an effective genetic diagnostic strategy for FD. A missing, de novo pathogenic variant with large insertion in intron 4 of the *GLA* gene was identified by CAFD. Additionally, a missing female heterozygote with two VUS in cis was also identified by CAFD. There were 47 variants identified by CAFD in 48 probands. Most variants were located in exon 5, followed by exon 7 and exon 6. Four de novo disease-causing variants were confirmed by pedigree analysis. These results suggest that FD has a wide spectrum of variants. According to a comprehensive testing algorithm for the diagnosis of FD including enzyme activity, clinical features and genetic testing, the diagnostic yield of CAFD is 97.92% (47/48), a higher diagnostic rate compared to conventional Sanger sequencing, which was 95.83% (46/48). Genetic testing and screening using CAFD can provide precise diagnosis and timely treatment for FD, greatly improving disease management.

Supplementary Information

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Additional file 1.

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Author contributions
F.Y., N.H. and D.L. contributed equally to this article. They coordinated the project and wrote the manuscript. W.Z and J.W. contributed to patient enrollment. A.M. contributed to the conception and design of the genetic testing. W.M. conducted the experiments and data analysis. Z.Q. contributed to variants interpretation. J.L. conceived the project and revised the manuscript. All authors have read, revised, and approved the final manuscript.

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Availability of data and materials
The original data presented in the study are included in this article.

Declarations

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Peking Union Medical College Hospital. The participants provided their written informed consent to participate in this study.

Competing interests
The authors declare no competing interests.

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