Identification of Cryptic Novel α -Galactosidase A Gene Mutations: Abnormal mRNA Splicing and Large Deletions

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Abstract Anderson-Fabry (FD) disease is an inborn error of metabolism caused by a deficiency of α -galactosidase A (*GLA*), a lysosomal enzyme. Many male FD patients display a classic FD phenotype; however, some female patients have neither reduced leukocyte *GLA* enzyme activity level nor FD symptoms. Thus, *GLA* gene analysis is especially important for diagnosing suspected FD in female subjects. In this study, we revealed 4 novel *GLA* gene mutations in 5 independent families using *GLA* cDNA analysis and multiplex ligation-dependent probe amplification (MLPA) analysis. These distinct mutations included a large deletion mutation from intron 1 to exon 5 (c.195-471_c.691del5.5k,

corresponding to g.8508_g.14069del5.5k), an insertion mutation of splicing enhancer sequence in intron 4 (c.639+329_c.639+330ins113, corresponding to g.12627_g.12628ins113), an insertion mutation of retrotransposon L1 in exon 4 (c.634_c.635, corresponding to g.12293_g.12294), and a non-SNP deep intronic point mutation in intron 3 (c.547+395G>C, corresponding to g.11727G>C). It is difficult to detect these mutations with direct sequencing of only the exonic element. When exonic mutations are not found in the *GLA* gene from suspected FD patients, *GLA* cDNA and MLPA analyses should be performed to detect large deletion/insertion and intronic mutations including transcription abnormalities.

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Introduction

Anderson-Fabry disease (FD [OMIM 301500 (<http://www.omim.org/>)] an X-linked lysosomal storage disorder caused by mutation of the α -galactosidase A (*GLA*) gene (Reference Sequence accession number NM_000619.2 (<http://www.ncbi.nlm.nih.gov/nucore>), MIM 300644) resulting in deficient activity of GLA (Desnick et al. 2001). This causes storage of various glycolipids such as globotriaosylceramide in many tissues, including vascular endothelium, renal glomeruli and tubules, dorsal root ganglia, cardiomyocytes, cornea, and skin. The main clinical symptoms of FD are neuropathic pain, hypohidrosis, and cerebrovascular, renal, and cardiac disease (Desnick et al. 2001). Although FD is inherited in an X-linked recessive manner, heterozygous female patients often develop clinical symptoms (Kobayashi et al. 2008). Recently, enzyme replacement therapy (ERT) using recombinant human GLA was developed and found to improve various clinical, pathologic,

Table 1 Patients' symptoms and GLA enzyme activities

Family		Sex/age	Pain	Hypohidrosis	LVH	Renal disease	Eye disease (corneal opacity)	WBC GLA enzyme activity (nmol/mg/h)	Healthy control's GLA enzyme activity range (nmol/mg/h)
Family A	I-2	F/75	+	+	+	—	+	68.7	56.3–190.6
	II-1	F/48	—	—	—	—	—	61.6	"
	II-2	M/45	—	—	—	—	—	123.4	"
	II-3	M/43	+	+	+	—	+	0.13	"
	II-4	F/40	—	—	—	—	—	124.9	"
	II-5	F/38	—	—	—	—	—	86.4	"
	II-6	F/36	+	—	—	—	+	32.9	"
Family B	I-2	F/71	+	—	+	—	—	231.0	157–259
	II-3	F/40	+	—	+	—	—	160.6	"
	III-1	M/11	+	—	—	—	—	9.6	152–281
Family C	II-2	F/52	+	—	+	—	—	29.4	121.6–152.8
	III-1	F/22	+	+	—	—	—	47.9	"
	III-2	F/19	+	—	—	—	—	21.6	160.7–345.6
Family D	II-2	M/12	+	+	—	—	+	2.04	152–169
Family E	I-2	F/59	+	—	+	—	—	120.3	169.5–212.7
	II-1	M/27	+	—	—	—	—	5.06	"
	II-2	F/26	+	—	—	+	+	101.6	"

LVH left ventricular hypertrophy, +/— positive or negative symptom

and biochemical characteristics of FD (Desnick et al. 2001). Thus, correct diagnosis of FD and early initiation of ERT are very important to improve prognosis. In male patients, enzyme analysis is usually sufficient for diagnosis. However, in female patients, especially those without a family history of the disease, detection of the pathologic *GLA* mutation is often necessary for definitive diagnosis.

More than 750 variants and mutations (missense/non-sense (68.8%), splicing (4.7%), regulatory (0.4%), small deletions (14.2%), small insertions (5.0%), small indels (1.4%), large deletions (4.2%), large insertions (0.5%), and complex (0.8%); no repeats detected) have been identified in the *GLA* gene from FD patients in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/>). In the present study, using multiplex ligation-dependent probe amplification (MLPA) methods, mRNA analysis, and intronic sequencing, we examined *GLA* genes from FD patients who did not carry mutations in exons or exon/intron boundaries. We found 4 novel cryptic mutations related to FD: a 5.5 kb deletion mutation, an insertion mutation of splicing enhancer (SE) sequence, an insertion mutation of exon-skipping element by long interspersed nuclear element-1 (L1) retrotransposon element, and a point mutation in a non-protein-coding region.

Materials and Methods

Patients

A total of 17 individuals from 5 families (Family A–Family E) in which at least one member had FD were analyzed for *GLA* variants (Table 1). Of note, all male patients had classic, not late-onset, FD. A total of three individuals without FD were also examined as controls.

Genomic DNA Analysis and Sequence

Genomic DNA analysis was performed as previously described (Kobayashi et al. 2012). Briefly, genomic DNA was extracted from white blood cells (WBCs) in the blood using a DNA Midi Kit (Qiagen). Exon and flanking intron elements of the *GLA* gene were amplified by polymerase chain reaction (PCR), using AmpliTaq Gold 360 Master Mix (Life Technologies), and sequenced using the BigDye Terminator Kit (ver. 3.1) (Life Technologies) and ABI PRISM 3100 Genetic Analyzer (Life Technologies). PCR temperature cycles of 94°C>55°C>72°C were used. PCR products were separated with 10% polyacrylamide gel containing Tris/borate/EDTA electrophoresis buffer. The PCR primers used for *GLA* genomic DNA analysis and the cDNA sequencing primers are shown in Table S1.

MLPA Assay

In order to find long deletions, the MLPA assay was carried out following the manufacturer's instructions using the SALSA MLPA P159-A2 *GLA* kit probe mix (FALCO bio systems). The PCR products were run on the ABI PRISM 3100 Genetic Analyzer with GeneMapper Software ver. 4.0 using GeneScan LIZ 500 size standards (Life Technologies). Data obtained were analyzed using Peak Scanner Software v1.0 (Life Technologies). MLPA raw data were normalized and analyzed with Coffalyser.NET software.

Total RNA Extraction, cDNA Synthesis, and RT-PCR of *GLA*

Whole blood was collected into EDTA-2Na tubes (TERUMO) or Tempus Blood RNA Tubes (Life Technologies). Total RNA was extracted from whole blood using TRIzol Reagent (Life Technologies), followed by phenol-chloroform. After ethanol precipitation, RNA was dissolved in water. For RT-PCR, first-strand cDNA was synthesized with PrimeScript II 1st strand cDNA Synthesis Kit (TAKARA BIO) according to the manufacturer's protocol. *GLA* cDNA was amplified with KOD FX Neo (TOYOBO). RT-PCR primers and the nested PCR primers used for *GLA* cDNA are shown in Table S1. These primer sequences were designed based on previously reported sequences (Shimotori et al. 2008).

cDNA Cloning and Transformation

All nested PCR products were re-amplified using Platinum Taq DNA polymerase (Life Technologies) to add a 3' A overhang to PCR products for TA cloning. cDNA and genomic DNA cloning were performed with a TOPO TA cloning kit with pCR2.1 TOPO vector (Life Technologies), which was transformed into competent DH5 α (TAKARA BIO) following the manufacturers' protocols.

GLA Enzyme Activity

WBCs were extracted from heparinized whole blood from the patient and from a healthy volunteer, and GLA enzyme activity in the WBCs was analyzed using a fluorogenic substrate, 4-methylumbelliferyl- α -D-galactopyranoside, as described previously (Kobayashi et al. 2012).

Single-Nucleotide Polymorphism Genotyping Assay

In samples from Family E, we additionally performed a single-nucleotide polymorphism (SNP) genotyping assay to detect whether the variant was SNP or non-SNP. This assay

was a high-throughput SNP genotyping system that combined the Invader assay with multiplex PCRs (Ohnishi et al. 2001). We screened II-1 in Family E, and the genotype and/or allele frequencies were compared with those of a cohort of Japanese subjects ($n = 1,492$) selected from the general population.

Results

Patient Profiles and Results of DNA Analysis

Family A

The proband (II-3) had typical classic FD symptoms and his WBC GLA activity was profoundly decreased (Table 1 and Fig. 1a). Thus, the diagnosis of FD was confirmed. PCR showed that exon 2–5 elements were not amplified; therefore, a large deletion was suspected. II-2 had normal GLA activity; thus, FD was excluded. For the male family members (I-2, II-1, II-4, II-5, and II-6), enzyme activities were in the normal range or marginally decreased. I-2 and II-6 had clinical symptoms consistent with FD, whereas II-1, II-4, and II-5 did not have any clinical symptoms of FD. The variant in this family was considered to be a large deletion variant; therefore, we carried out MPLA analysis.

Signals of exon 2–5 elements could not be detected in II-3 (lane 5, Fig. 1b). In I-2, II-5, and II-6, signal peaks of exon 2–5 elements were almost half those of the female control (lanes 3, 7, and 8, Fig. 1b). In contrast, signal peaks of II-1 and II-4 showed the same peak signal level as the female control for all exons (lanes 4 and 6, Fig. 1b). The relative peak areas determined in each *GLA* exon region in female patients (I-2, II-1, II-4, II-5, and II-6) were consistent with this observation (Fig. S1a). To identify the exact deletion area, we performed direct sequence genomic DNA analysis from II-3. We identified a deletion from c.195-471 (g.8508) within intron 1 to c.691 (g.14069) within exon 5 (Fig. 1c and Fig. S1b). Using cDNA sequence analysis, we showed that exons 2–5 were deleted. As a result, this leads to a frameshift and premature insertion of a stop codon at codon 66.

Family B

The clinical symptoms and WBC GLA activities are summarized in Table 1, and the family pedigree is shown in Fig. 2a. III-1 was considered at risk for FD because of his family history of FD and he therefore visited our clinic. He has experienced pain from childhood and his GLA activity was very low (Table 1). Thus, diagnosis of FD was confirmed. For further confirmation of FD in this patient and for diagnosis of female family members at risk, DNA

Fig. 1 Locate in *GLA* gene mutation in Family A. **(a)** Pedigree of Family A, *arrow*, proband; *filled symbol*, clinically and genetically affected patient; *small black dot*, asymptomatic mutation (*GLA*) carrier. **(b)** Detection of all *GLA* exon elements by MLPA analysis. Probe mix (P159-A2) contains 25 probes with 8 probes recognizing

the *GLA* exons located in 7 different *GLA* exons. *Hash symbol*, not detected; *asterisk*, heterozygous peak; *x-axis*, nucleotide fragment size (nt.); *y-axis*, fluorescence intensity. **(c)** *GLA* genomic DNA structure (*upper panel*) and cDNA structure (*lower panel*) in II-3

analysis was carried out for III-1. However, all of the exons were successfully amplified, and there was no sequence change among exons and exon/intron boundaries in the *GLA* gene. Female family members I-2 and II-3 had left ventricular hypertrophy and low WBC *GLA* activities and were therefore considered to have FD. For further genetic

analysis of this family, *GLA* cDNA was synthesized from III-1 and cloned into a cloning vector for sequencing.

A 304 bp insertion between exons 4 and 5 was identified by cDNA analysis. This insertion was from intron 4, c.639 +240_c.639+430 191bp (g.12538_g.12728) with a purine-rich additional 113 bp unknown sequence between c.639

Fig. 2 Locate in *GLA* gene mutation in Family B. **(a)** Pedigree of Family B, *arrow*, proband; *filled symbol*, clinically and genetically affected patient; *small black dot*, asymptomatic mutation (*GLA*)

carrier. **(b)** *GLA* cDNA structure between exon 4 and 5 (*upper panel*) and intron 4 genomic DNA structure (*lower panel*) in III-1. *Thick line*: alternative exon region involved in *GLA* cDNA in III-1

+329 (g.12627) and c.639+330 (g.12628) (Fig. 2b and Fig. S2). As a result, the *GLA* mRNA leads to frameshift and premature insertion of a stop codon at codon 220. We also analyzed genomic DNA and found a purine-rich 113 bp sequence between c.639+329 and c.639+330 (Fig. 2b and Fig. S2). Female members of this family I-2 and II-3 had the same variant heterozygously in *GLA* and were confirmed to have FD.

Family C

III-1 and III-2 were considered at risk of FD because their grandfather (I-1) was diagnosed with FD at another

hospital (Fig. 3a). II-2 was an obligate heterozygote. Three female family members, II-2, III-1, and III-2, had experienced pain from childhood, and their WBC *GLA* activities were low (Table 1). No exonic mutations were found in their *GLA* gene. *GLA* cDNA from III-2 was sequenced, and a 304 bp insertion was found between exon 4 and 5 (data not shown). This insertion sequence was the same as for Family B. We amplified intron 4 in genomic DNA from II-2, III-1, and III-2. Two types of bands were seen in all family members (Fig. 3b). The smaller band was exactly the same size as for normal controls. By contrast, the larger band was the same size as that for III-1 in Family B. This fragment contained a

Fig. 3 Detection of *GLA* gene mutation in Family C. **(a)** Pedigree of Family C, arrow, proband; filled symbol, clinically and genetically affected patient. **(b)** Detection of inserted region with PCR analysis in Family C. Gel electrophoresis data show PCR-amplified product of

intron 4 element. Arrowhead, normal sequence (660 bp); arrow, abnormal sequence (773 bp); lane 1, DNA size marker (ϕ X174 DNA-HaeIII digest); lane 2, male control; lane 3, III-1 (Family B); lane 4, female control; lane 5, II-2; lane 6, III-1; lane 7, III-2

304 bp insertion. Thus, II-2, III-1, and III-2 had heterozygous FD.

Family D

The proband (II-2) had typical clinical signs of FD and his *GLA* activity was very low (Table 1 and Fig. 4a). We successfully amplified all exons and exon/intron boundaries (except exon 4) by PCR. The patient had no exonic mutations in any of the amplified DNA. Exon 4 element was completely skipped in *GLA* cDNA, as determined by cDNA sequence analysis (Fig. 4b and Fig. S3). His *GLA* mRNA leads to frameshift and premature insertion of a stop codon at codon 200. After TA cloning of genomic DNA of exon 4, each clone was sequenced. We found that poly (T) oligonucleotides and 139 base inverted long interspersed nuclear element-1 (LINE 1, L1) retrotransposon sequence were inserted after c.634 (g.12293). After the 139 bp insertion, a normal exon 4 beginning from c.620 (g.12279) followed. Thus, from c.620 to c.634 was duplicated.

Family E

The male family member, II-1, visited our clinic because he was at risk for FD (Fig. 5a). He had

typical classic FD symptoms, and his WBC *GLA* enzyme activity was very low, indicating that he had FD (Table 1). No sequence change was found in the exons and exon/intron boundaries. Female family members, I-2 and II-2, had FD symptoms (Table 1). A 115 bp insertion sequence between exon 3 and 4 element was identified by cDNA sequence analysis. This insertion sequence was from intron 3, c.547+285_c.547+399 (g.11617_g.11731). This *GLA* mRNA leads to a frameshift and premature insertion of a stop codon at codon 223. We analyzed intron 3 and found one variant at the corresponding position c.547+395G>C (g.11727G>C) (Fig. 5b and Fig. S4). This variant was not found in the analysis of members from the general Japanese population by Multiplex-PCR Invasion Assay, indicating that this variant is pathogenic. I-2 and II-2 also had the same heterozygous sequence variation in *GLA*.

Discussion

Family A

It was difficult to diagnose FD due to large deletion of *GLA* in female subjects II-1, II-4, and II-5, as they did not show

Fig. 4 Locate in *GLA* gene mutation in Family D. **(a)** Pedigree of Family D, arrow, proband; filled symbol, clinically and genetically affected patient; small black dot, asymptomatic mutation (*GLA*) carrier. **(b)** *GLA* cDNA structure between exon 3 and 5 (upper panel)

and exon 4 structure (lower panel) in II-2. Dashed line, deletion exon 4 element; thick line, L1 gene; bold highlight, L1 recognition sequence; arrowhead, L1 cutting site; underlined and shaded region, target site duplication (TSD) sequence

symptoms. MLPA analysis is a very effective tool to detect such large gene deletions.

Some previous studies have shown the effectiveness of MLPA analysis to detect large deletions of the *GLA* gene in female FD patients (Marziliano et al. 2012; Yoshimitsu et al. 2011; Schirinzì et al. 2008). We found deletion mutations with multiple exons from exon 2–5 elements using MLPA analysis in this family. Signals of exon 2–5 elements could not be detected in II-3 by MLPA analysis. As the exon 5 signal could not detect by MLPA analysis, we suspected that this subject had no exon 5 elements at all.

However, he had more than half of *GLA* exon 5 element. This result might indicate a limitation of the probe's target in sequence-specific MLPA analysis. Some peaks in I-2 and II-6, and more in II-5, who had normal *GLA* activity and no FD symptoms, showed a pattern indicating heterozygous *GLA* mutation; conversely, II-1 and II-4 were genetically normal.

According to the genomic DNA sequence, II-3 had a 5.5 kb deletion mutation. There are a few reports about rare large deletions in *GLA* (Bernstein et al. 1989; Shabbeer et al. 2006; Rodríguez-Marí et al. 2003). In II-3, *GLA* had

Fig. 5 Locate in *GLA* gene mutation in Family E. **(a)** Pedigree of Family E, arrow, proband; filled symbol, clinically and genetically affected patient. **(b)** *GLA* cDNA structure between exon 3 and 4 (upper panel) and intron 3 genomic DNA structure (lower panel) in II-1. Arrow, c.547+395G>C; thick line, alternative exon 3 element involved in *GLA* cDNA in II-1

8 nucleotide (-AGCAAAAT-) short inverted repeat sequences (IRs) at the 5' and 3' end of the deletion region. The IRs element is usually described as an important sequence for genomic instability and recognized as a hotspot that induces deletion and recombination in mammalian genomic DNA (Voineagu et al. 2008). In this family, 8 nucleotide IRs might involve a 5.5 kb deletion mutation of *GLA*.

II-3 *GLA* cDNA was constructed in exon 1, 6, and 7 elements. However, his *GLA* gene kept a fragment of exon 5. Therefore, we believe that the incomplete exon 5 is spliced out during mRNA mature processing.

In the present study, we revealed novel *GLA* mutations which have a large 5.5 kb deletion mutation. The MLPA method was very effective for use in women suspected of having FD due to a heterozygous large deletion mutation in *GLA*.

Families B and C

We found that an interesting novel long purine-rich insertion mutation involved SE within *GLA* intron 4 in two independent families (B and C). Most of the reported insertion mutations in FD are less than 20 bp and are located within the *GLA* exon element (Rosenberg et al

2000; Glass et al 2004). We identified a relatively long (113 bases) insertion within *GLA* intron 4. The 113 bases had a repeated sequence pattern (-gGAAT-). Such repeated 113 bp sequences are found throughout the human genome, such as in human satellite III-related DNA, SDS-stable vimentin-bound DNA fragments, and sequences of unknown function within chromosomes 16, 7, and 2. This 113 bp insertion element might come from satellite DNA. In addition, a purine-rich element could induce the enhancing pre-mRNA splicing system (Cooper and Mattox 1997; Hastings et al. 2001; Tanaka et al. 1994). We suspect that this 113 bp element within intron 4 plays a role in SE. Thus, 191 bp intronic sequences near the 113 bp element were recognized as new alternative exons in *GLA* cDNA.

Family D

We found an L1 retrotransposon sequence in the exon 4 of *GLA*. L1 is an abundant transposable element within the human genome. The L1 gene is a retrotransposon capable of copying transposon genome into other parts of the genome, while leaving the transposable elements of the original genome (Kazazian 1998). L1 retrotransposon has a poly (A) tail at the 3' end. This retrotransposon generally recognizes -TTTTTAA- consensus sequence. In this case, L1 recognized -TTTCAA- sequence located near the 3' end of exon 4. L1 was sandwiched between 15 duplicate nucleotides sequence. This overlapped 15 nucleotide element is known as a target site duplication (TSD) sequence. An exonic/intronic L1 insertion mutation has also been found in some other genetic diseases (Awano et al. 2010; Kagawa et al. 2014), immunity disorders (Brouha et al. 2002), cancers (Wimmer et al. 2011), and neurologic diseases (Martínez-Garay et al. 2003). The L1 retrotransposon leads to splicing out of the exon 4 element in pre-mRNA maturation. Therefore, the *GLA* gene of this subject could not be transcribed into normal *GLA* mRNA.

Family E

We identified cDNA with a 115 bp sequence from intron 3 between exon 3 and 4. This 115 bp sequence was already known as cassette-type alternative exon 3 element (ENSE 00001812130 (<http://www.ensembl.org/index.html>)). The proband had a c.547+395G>C point mutation in *GLA* intron 3. This point mutation was not found in the general Japanese population, indicating that it is likely pathologic. In most intronic *GLA* gene mutations in subjects with FD, mutations are located in only the GT/AG exon/intron boundary or near the boundary; however, this mutation is located deep in the intron. There have been several other reports of deep intronic mutation in the *GLA* gene that might affect the mRNA splicing process (Ishii et al. 2002;

Filoni et al. 2008; Pisani et al. 2012). Especially those reports show that deep intronic point mutation could have a function of alternative splicing enhancer switch, because it recruits an alternative exon 4 and downregulates the normal *GLA* mRNA expression level (Ishii et al. 2002; Filoni et al. 2008). Thus, this c.547+395G>C mutation might be considered as an alternative splicing enhancer switch.

Conclusions

We revealed four novel *GLA* mutations in this study. One mutation was a large deletion and the others affected the mRNA splicing mechanism. *GLA* cDNA sequence analysis is an effective tool for finding not only exonic gene mutations but also functional intronic mutations related to the pre-mRNA splicing mechanism. MLPA analysis is a simple and powerful tool for analyzing large deletion mutations in genetic genealogy analysis.

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One-Sentence Take-Home Message

We found intronic novel *GLA* gene mutations related to FD with MLPA and cDNA analysis which are powerful molecular methods, although it is a classic procedure: a 5.5 kb deletion mutation, an insertion mutation of splicing enhancer sequence, an insertion mutation of L1 exon-skipping element, and a point mutation in a non-protein-coding region.

Details of the Contributions of Individual Authors

Conception and design of this study: TH, MK, and TO.

Acquisition of data: genetic analysis data; TH, JO, and EK, *GLA* enzyme activity analysis data; EK, SNPs analysis data; SM.

Interpretation of basic research data: TH, YS, HK, HI, and TO.

Care physician of patient: YE and TO (Family A), AO (Family B), TO (Family C, D, and E).

Drafting of the article: TH, MK, and TO.

Critical revision of the article for important intellectual content: MK, HI, and TO.

Final approval of the article: TH&TO.

Guarantor for the Article

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Conflict of Interest

Higuchi T, Ogata J, Kaneshiro E, Shimada Y, and Maeda S declare that they have no conflict of interest.

Kobayashi M has received a speaker honorarium from Sumitomo Dainippon Pharma Co., Ltd., and Genzyme Japan Co., Ltd.

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Our research activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of The Jikei University.

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Details of Ethics Approval

This study was approved by the ethical committee of The Jikei University School of Medicine (Jikei Ethics Approval No. 19-040-4971).

Informed Consent

Informed consent was obtained from all patients for being included in the study.

Animal Rights

This article does not contain any studies with animal subjects performed by any of the authors.

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