



Future clinical and biochemical predictions of Fabry disease in females by methylation studies of the *GLA* gene



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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder caused by a deficiency of α -galactosidase A (α -gal A). The clinical variability of the phenotypes of Fabry disease in females is still poorly understood. The degree of aberrant methylation of non-mutated alleles is thought to have significant effects on X-chromosome inactivation (XCI). We previously reported that one heterozygous Fabry female showing classical phenotypes had complete methylation of the non-mutated allele of the *GLA* gene. In this report, we summarized 36 heterozygous females with a clinical severity score based on the FABry STabilization index (FASTEX). We measured their α -gal A activity and plasma/serum globotriaosylsphingosine (lyso-Gb3) accumulation and performed electron microscopy of skin biopsies. We analyzed the methylation-sensitive restriction enzyme sites throughout the *GLA* gene, including the 5'UTR, and found a single *Sac*II site and multiple *Hha*I and *Hpa*II sites aggregated in exon 1 and the 5'UTR. One *Hpa*II sequence in exon 7 was also detected as a methylation-sensitive site. With methylation-sensitive restriction enzymes, methylated and non-methylated alleles could be separated, and the ratio of the methylation was quantified. We found a clear correlation between the severity of the phenotype and lyso-Gb3 accumulation for heterozygous Fabry disease in females. Methylation of the non-mutated allele was also proportionately correlated to the clinical severity score measured by FASTEX.

1. Introduction

Fabry disease (OMIM #301500) is an X-linked disorder characterized by the deficiency of the lysosomal hydrolase α -galactosidase A (α -gal A, E.C. 3.2.1.22) due to mutations in the *GLA* gene [1,2]. Patients with partial or complete deficiency of α -gal A are unable to degrade glycosphingolipids, such as globotriaosylceramide (Gb3), and related glycosphingolipids, such as globotriaosylsphingosine (Lyso-Gb3), effectively, and these lipids consequently accumulate in body fluids and the lysosomes of a variety of cell types, including the vascular endothelium, kidney, heart, eye, and nerve cells [3,4].

Clinical manifestations in classically affected hemizygotes who have no detectable α -gal A activity include pain and paresthesia in the extremities, angiokeratoma, and hypo- or anhidrosis. Corneal and lenticular opacities are also early findings. With increasing age, proteinuria, hyposthenuria, and lymphedema appear [2]. Severe renal impairment leads to hypertension and uremia. Death usually occurs from renal

failure or cardiac or cerebrovascular disease [1].

The clinical course and prognosis of heterozygous females differ from those of hemizygotes [5]. Approximately 70% of heterozygous Caucasians have whorl-like corneal dystrophy as an early manifestation of the disease [6,7]. However, Japanese female Fabry patients have a heterogeneous presentation. The age at onset of disease varies from 4 to 68 years. > 50% of the heterozygotes present with acroparesthesia and corneal opacities. Approximately 40% of the patients have proteinuria and left ventricular hypertrophy (LVH). The incidence of LVH in women over 68 years of age is approximately 100% [8]. The diagnoses of heterozygous females are also complicated because of residual α -Gal A activity [9]. Even genetic testing of at-risk patients or patients suspicious for Fabry disease has been compromised by the recently recognized occurrence of various benign missense mutations [10–12].

Various Fabry disease severity scoring systems, such as the Mainz Severity Score Index (MSSI) [13], the Fabry Disease Severity Scoring System (DS3) [14] provide an index of disease severity at a single time

Abbreviations: α -gal A, α -galactosidase A; Gb3, globotriaosylceramide; lyso-Gb3, globotriaosylsphingosine; XCI, X-chromosomal inactivation

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Table 1
Summary of patients' clinical, biochemical and genetic information

Patient No.	Age	Major clinical characteristics	α-gal A activity ^a		Plasma Lyso-Gb3 ^b	FASTEX Raw Score	Methylation of normal (non-mutated) allele (%)		GLA genotypes	Reference (GLA mutation)		
			Lym	SFs			Blood DNA	SFs DNA				
			Hthal (exon 1) site/ HpaII (exon 7) site ^c		Hthal (exon 1) site/ HpaII (exon 7) site ^c		SacII site					
1	37y	Acroparesthesia, dysmorphism, intellectual disabilities, LVH	0	0	61	11	97.9	84.6	92.3	83.6	c.36C > A(p.C12X)	[27]
2	44y	Mild cardiomegaly	9.9	2.5	5.6	5	46.7	29.7	35.1	21.6	c.36C > A(p.C12X)	[27]
3	42y	Mild pain	9.1	12.9	8	2	10.8	7.4	1.5	44.3	c.36C > A(p.C12X)	[27]
4	11y	Mild acroparesthesia	2.8	0.8	28.7	1	70.6	52.5	56.8	40	c.36C > A(p.C12X)	[27]
5	51y	Chest pain, acroparesthesia, LVH	8.2	4.7	21.3	11	46.7	69.2	60.1	70.7	c.97G > T(p.D33Y)	This study
6	42y	Chest pain, LVH	4.4	-	10.6	11	52.3	69.2	-	-	c.97G > T(p.D33Y)	This study
7	39y	Chest pain, CCF, acroparesthesia	5.6	1.9	25	12	72.2	89.5	35.5	59.2	c.97G > T(p.D33Y)	This study
8	66y	Cerebral infraction, CCF, renal failure	5.3	-	35.5	18	53	56.9	-	-	c.97G > T(p.D33Y)	This study
9	53y	Chest pain, CCF, acroparesthesia	8.5	-	7.1	12	41.3	81.7	-	-	c.97G > T(p.D33Y)	This study
10	55y	Chest pain, cardiomegaly, proteinuria (+)	7	-	7.7	10	28.3	99.9	-	-	c.668G > C(p.C223S)	[28]
11	22y	Pain in extremities	8.8	-	13.1 (S)	1	31.8	-	-	-	c.668G > C(p.C223S)	[28]
12	45y	Pain, mild cardiomegaly, proteinuria (++)	4.1	3.7	17.1	13	74.1	45.2	4.8	-	c.950 T > C (p.I317T)	[44]
13	53y	Mild pain, mild cardiomegaly, proteinuria (+)	4.5	3.6	5.8	12	49.7	74.1	-	99.9	c.950 T > C (p.I317T)	[44]
14	35y	Pain, cardiomegaly	8.3	0.1	40	9	82.2	38.2	93.8	-	c.901C > T (p.R301X)	[45]
15	28y	Acroparesthesia	3.1	6.8	26.1	5	77.9	68.3	48.7	-	c.901C > T (p.R301X)	[45]
16	45y	Headache, acroparesthesia, chest pain, LVH	4.2	6.0	18.2	14	66.7	-	-	-	c.334C > T (p.R112C)	[46]
17	37y	Mild pain	5.5	5.5	5.2	1	81	45.5	-	-	c.1124G > A(p.G375E)	[47]
18	40y	Mild pain	8.9	1.6	25.2	2	26.7	60.6	-	-	c.749A > C(p.Q250P)	[48]
19	37y	Mild pain	4	-	14.2	2	57.4	-	-	-	c.749A > C(p.Q250P)	[48]
20	50y	Mild proteinuria, mild cardiomegaly	9.5	-	21.5	4	13.9	52.5	-	-	c.1208 T > C(p.L403S)	[49]
21	29y	Mild cardiomegaly	8.8	-	10.5	6	6.9	78.4	-	-	c.658C > T(p.R220X)	[50]
22	30y	Asymptomatic	3.8	-	9.7	1	39.7	-	-	-	c.658C > T(p.R220X)	[50]
23	62y	Cardiomyopathy, pain, proteinuria (+)	8.9	-	15.1	16	52	-	-	-	c.658C > T(p.R220X)	[50]
24	5y	Asymptomatic	4.5	-	6.2 (S)	0	Non-meth	-	-	-	c.761delTTG	This study
25	7y	Asymptomatic	5.5	-	8.9	0	Non-meth	-	-	-	c.761delTTG	This study
26	53y	Acroparesthesia, headache, cardiac hypertrophy	1.8	-	24.5	14	Meth	-	-	-	c.802delTTAG	This study
27	72y	Proteinuria (++++), creatinine clearance (L), chest pain, cardiomegaly	3.4	1.7	14	15	92.1	-	79.2	-	c.902G > A(p.R301Q)	[51]
28	74y	CCF, cardiac arrhythmia, pacemaker in situ, proteinuria (++)	5.1	3.6	48.7	21	82.8	-	-	-	c.679C > T(p.R227X)	[52]
29	45y	Mild pain	6.0	-	25.8	7	94.3	99.9	-	-	c.679C > T(p.R227X)	[52]
30	62y	Mild pain, LVH	8.2	-	25	13	22.5	-	-	-	IVS3 + 1G > A	[53]
31	44y	Asymptomatic	-	-	23.1	0	49.4	-	-	-	c.658C > T(p.R220X)	[50]
32	45y	Mild cardiomegaly	4.5	-	4.1	5	40	-	-	-	c.118C > T(p.P40S)	[54]
33	6y	Asymptomatic	5.4	-	18.5	0	47.7	-	-	-	c.1019G > A(p.W340X)	[55]
34	6y	Asymptomatic	7.5	-	13	0	37.5	-	-	-	c.1019G > A(p.W340X)	[55]
35	6y	Asymptomatic	2.2	-	27	2	83.8	81.4	-	-	c.3G > A(p.M1I)	[56]
36	8y	Asymptomatic	4.5	-	7.4	1	63.9	74.9	-	-	c.3G > A(p.M1I)	[56]

LVH, left ventricular hypertrophy; α-gal A, α-galactosidase A; LYM, lymphocyte; SFs, skin fibroblasts; y, year; -, not measured; Met, methylated; Non-met, non-methylated.

^a α-gal A activity was measured with 4-MU substrate and the normal range of α-gal A activity was 10–12 nmol/h/mg protein in lymphocytes and skin fibroblasts. Blood lymphocytes were collected to measure the enzyme activity before two weekly enzyme infusions for patients who were in ERT.

^b Lyso-Gb3 was measured in the plasma of all the patients except in cases 11 and 24. Their Lyso-Gb3 was measured in blood serum. The normal range of Lyso-Gb3 in plasma/serum was considered < 2.1 ± 2 nmol/L.

^c Methylation of the Hthal site was measured in exon 1 for the patients who have exon 1 mutations including cases 1–9, and 35–36, and methylation of the HpaII site was measured in exon 7 for the patients who have exon 5–7 mutations including cases 10, 12–15, 17–18, 20–21, and 29.

point. In our daily practice in Japan, the FABry STabilization indEX (FASTEX) [15] is commonly used as a mathematical model in order to objectively verify the clinical severity and stability of patients with Fabry disease.

The pathophysiology for the severity of phenotypes in heterozygous Fabry females is still poorly understood. XCI occurs at random within females resulting in an average inactivation ratio of 50:50 with normal distribution [16]. Skewed XCI is described for some other diseases [17]. The existence of skewed XCI in females with Fabry disease was found in a recent study by analyzing the human androgen receptor (*HUMARA*) gene [18] which demonstrated the existence of skewed inactivation (29%) of heterozygotes females with Fabry disease correlating to Fabry disease severity scores (MSSI and DS3).

In higher order eukaryotes, DNA is methylated only at 5'-CpG-3' dinucleotides (CpGs), and this DNA modification has important regulatory effects on gene expression, especially in CpG-rich areas (known as CpG islands) located in the promoter regions of many genes [19–21]. As extensive methylation of CpG islands is associated with transcriptional inactivation of several genes on one of the two X-chromosomes in females [22,23], such X chromosomes result in an inactive condition. XCI is accompanied by the changes in DNA methylation at CpG islands, and the methylation of *HpaII* and *HhaI* sites correlates with XCI [24,25]. The 5'UTR of the *GLA* gene contains a CpG island composed of 474 base pairs (chrX:101407840–101,408,313) [26]. Our previous study showed that the methylation of *HpaII* and *HhaI* sites in exon 1 of *GLA* correlates with XCI and the severity of the disease [27].

In this study, we summarized 36 heterozygous Fabry females with their clinical severity score and detected their lyso-Gb3 accumulation in plasma and sphingolipid accumulation with skin biopsies. We obtained the methylation-sensitive restriction enzyme sites (*RE*) in exon 1, 5'UTR and exon 7 of the *GLA* gene. We found a clear correlation between the severity of the phenotype, lyso-Gb3 accumulation and methylation of the normal allele detected by nondigestion with methylation-sensitive *RE SacII*, *HhaI* and *HpaII*.

2. Materials and methods

2.1. Patients' clinical summary

The information of the 36 heterozygous Japanese Fabry females was summarized based on their previous family history, careful physical examinations, and biochemical and molecular findings. The findings were then categorized to fit FASTEX scoring including the nervous, cardiac and renal systems.

2.2. Calculation of the clinical severity score by FASTEX

The FASTEX score was calculated as described before [15] with some modification. In short, nervous events included pain and other neural events; cardiac events included LVH, ECG/arrhythmia and cardiac functioning categorized by the New York Heart Association (NYHA), and renal events included the albumin-creatinine ratio (ACR)/protein-creatinine ratio (PCR) and eGFR. One of seven events ranged from 0–4. The total raw score was then summed.

2.3. Measurement of α -gal A activity using 4MUGal substrate

The level of α -gal A activity was measured as previously reported [28]. In short, lymphocytes and skin fibroblasts (SFs) were sonicated, and the whole cell lysates were incubated with a mixture containing 700 mM 4-methylumbelliferyl- α -D-galactopyranoside (4 MUGal; Sigma), 0.5 M N-acetyl-D-galactosamine (GalNAc; Sigma), and 50 mM citrate-phosphate (pH 4.5) buffer in a water bath at 37 °C for 3 h. The reactions were stopped with 150 mM ethylenediaminetetraacetic acid (pH 11.5) buffer. The fluorescence (excitation at 355 nm/emission at 460 nm) was measured with a microplate reader. The enzyme activity

was calculated as $\text{nmol}\cdot\text{h}^{-1}\cdot\text{mg protein}^{-1}$.

2.4. Quantification of lyso-Gb3 by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Quantification of lyso-Gb3 was performed as described before [28]. In short, 60 μL fresh plasma was separated from whole blood and placed into a 1.5 mL tube containing 540 μL 2-propanol (HPLC-grade, Kanto, Japan) and 20 μL internal standard. After mixing for 2 min with a microtube mixer (Tommy, Japan), the extraction was centrifuged for 20 min at 12,000g. The supernatant from the extraction was dried under an air stream and reconstituted with 20 μL methanol. The final solution was transferred to autosampler micro vials for measurement by the LCMS-8040 system (Shimadzu, Japan).

The calibration set consisted of LysoGb3 (Matreya LLC, USA) standards with the same amount of N-glycinated LysoGb3 (internal standard, Matreya LLC, USA) in the methanol solvent. The calibrated amount of LysoGb3 was 0.0–0.8 ng ($R^2 = 0.996$), and a calibration set was performed for every 50 samples.

Five microliters of the solution were measured by high-performance liquid chromatography (HPLC) connected to a tandem mass spectrometer (LCMS-8040, Shimadzu, Japan). The mixture was eluted with mobile phase A (10 mM NH_4COOH , Wako, Japan) and phase B (10% 10 mM $\text{NH}_4\text{COOH}/\text{MeOH}$) through a C8 column (Inertsil C8–3, 3 μm , 2.1 \times 50 mm, GL Sciences Inc., Japan) connected to an in-line filter unit (ACQUITY, Waters, USA). The multiple reaction monitoring (MRM) transitions monitored were 786.5 > 264.3 m/z for LysoGb3 and 843.5 > 264.3 m/z for N-glycinated LysoGb3. The quantification of LysoGb3 was performed by comparing the peak molecular target area with the internal standard area.

2.5. Electron microscopy of skin biopsies

Electron microscopy for skin biopsies for cases 1–5, 7, 12–16, 18, 27–28 was done as described before [29]. In short, a 2 mm skin punch was taken from the left elbow, and small blocks of tissue were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2). Some blocks were directly embedded in Epon or glycol methacrylate. Ultrathin sections were examined either unstained or after various treatments: uranyl acetate and lead citrate, periodic acid-silver methenamine, periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP), and hydrogen peroxide-phosphotungstic acid (H_2O_2 -PTA).

2.6. Detection of methylation sensitivity for *SacII*, *HhaI* and *HpaII* sites in the *GLA* gene

Methylation-sensitive sites were detected as described before [27]. In short, DNA from peripheral blood and SFs was collected using the Qiagen blood and tissue kit (Cat. No. 69504) and an amount of 400 to 1200 ng of DNA was used for bisulfite modification following the MethylEasy Xceed (Human Genetic Signatures Pty Ltd) protocol. Bisulfite-modified DNA, 40 to 50 ng, was amplified for each exon and the 5'UTR of the *GLA* gene with respective primer sets. The amplified DNA was then purified and sequenced.

2.7. Quantification of methylation of each allele in the *GLA* gene

One microgram of DNA from peripheral blood and SFs containing *SacII* was incubated for 20 h at 37 °C. The digested DNA was then purified by a high pure PCR product purification kit (ref. 11,732,676,001). Purified DNA fragments were used for long PCR amplification with PrimeSTAR GXL (Takara cat# R050A). A pair of primers (forward 5'-AGCGAGACGGTAGACGACGACCAGAACTAC TTC-3', reverse 5'-GGGGTGGGTATCTGGATGAGTAAATATGGGTT-3') was used to amplify the whole *GLA* gene 11 kb including the 5'UTR for all the cases. For *HhaI* and *HpaII* sites, 200 ng of DNA were used for the

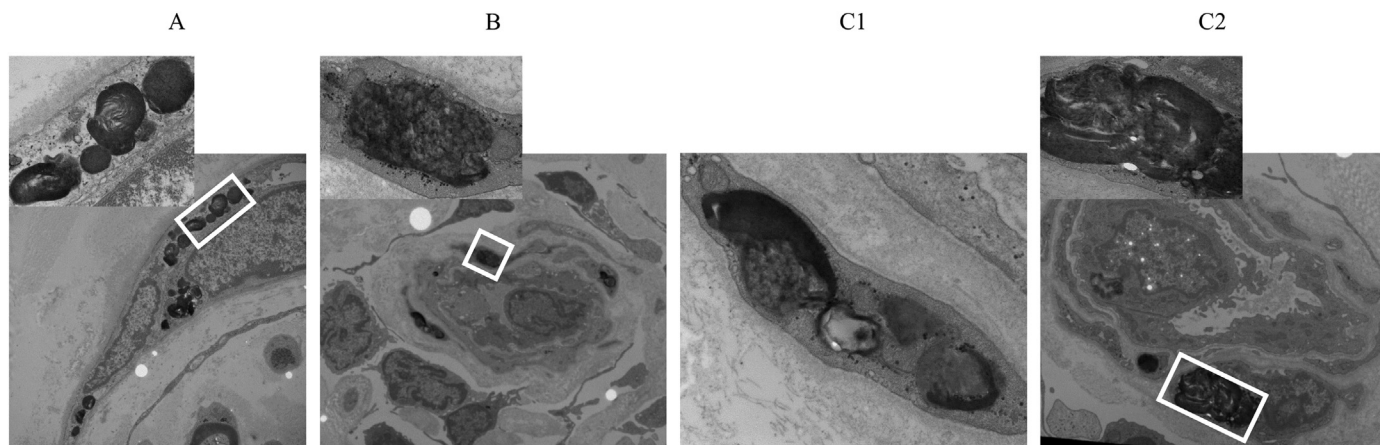


Fig. 1. Electron micrograph of a section of skin tissue from case 1 showing massive lysosomal accumulation in peripheral neuronal Schwann cells (A), vascular endothelium (B) and fibroblasts (C).

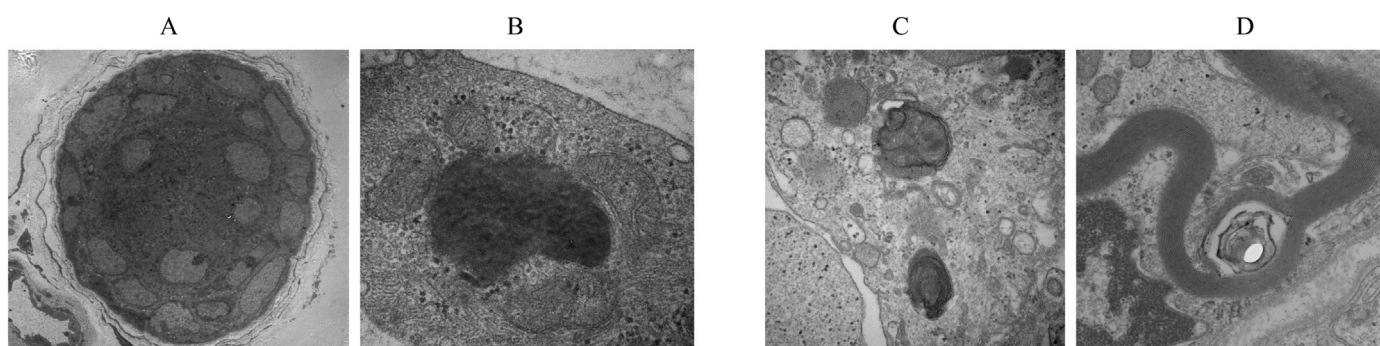


Fig. 2. In case 7, deposition was detected in the exocrine gland and fibroblastic cells (A, B). Massive and myelin-like depositions were observed in case 27 (C, D).

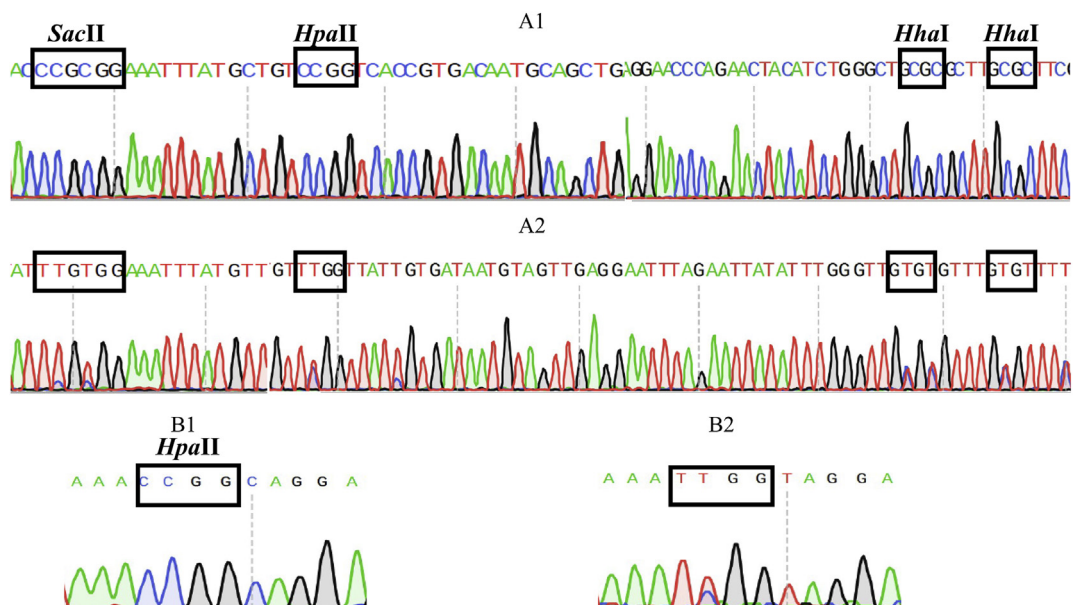


Fig. 3. Genomic DNA was treated with methylation-sensitive endonuclease and PCR-based amplification was done. Methylation-sensitive *SacII*, *HpaII* and *HhaI* were detected in exon 1, the 5'UTR (before and after treatment, A1 and A2, respectively) and exon 7 (before and after treatment, B1 and B2, respectively).

digestion reaction. Purified DNA fragments were used for amplification of exon 1 of the *GLA* gene for cases 1–9, 32 and 35–36 who had exon 1 mutations by the primers described before [27]. For other cases that had exon 5–7 mutations, exons 5 to 7 were amplified. All amplified PCR fragments were purified by Wizard SV gel and PCR (Promega), and direct sequencing was performed.

Quantification of methylation was performed based on digestion of mutated or non-mutated alleles by methylation-sensitive restriction enzymes. The quantification of methylation was measured by the Mutation Surveyor Softgenetics software (version 5.1.0). In short, the calculation of methylation of the non-mutated allele was performed in digested fragments as the length of non-mutated allele/ (length of

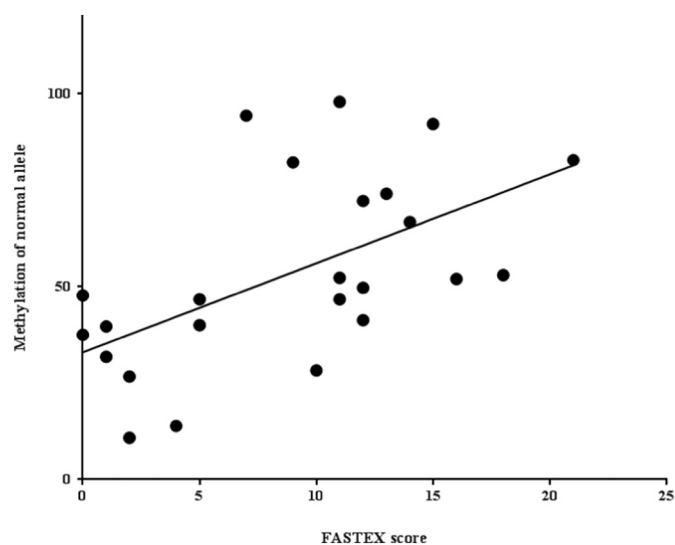


Fig. 4. Correlation between the patient severity (FASTEX) score and methylation of the normal allele measured by the digestion ratio of mutated and non-mutated alleles by methylation-sensitive *RE* *SacII*. The DNA was extracted from blood lymphocytes. Statistical significance was valued as Spearman (ρ) = 0.6333, p = .0004.

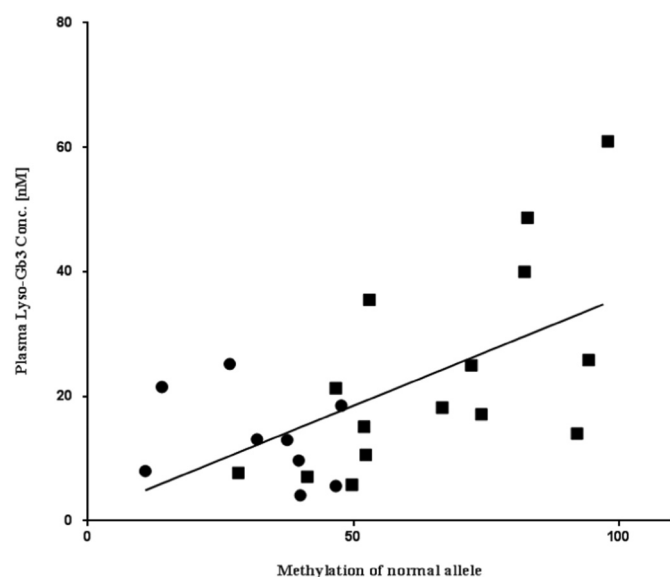


Fig. 5. Correlation between the patient lyso-Gb3 accumulation and methylation of the normal allele measured by the digestion ratio of mutated and non-mutated alleles by methylation-sensitive *RE* *SacII*. The DNA was extracted from blood lymphocytes. The circle represents mild cases and the square represents severe cases (FASTEX raw score > 10). Statistical significance was valued as Spearman (ρ) = 0.5271, p = .004.

mutated allele + length of non-mutated allele).

3. Results

3.1. Clinical characteristics of heterozygous Fabry females

The clinical presentations were widely varied. Some cases were found to have no phenotype, e.g., cases 22, 24–25, 31, 33–36, and some cases were showing a severe phenotype as Hemizygous Fabry males, e.g., cases 1, 4–10, 12–13, 16, 23, 26–28, 30.

3.2. Calculation of the FASTEX score

Based on the clinical characteristics, the FASTEX raw score was summed. Most of the heterozygous female scores (21/36) ranged from 5 to 21. Some of them scored < 5, e.g., case 3, 4, 11, 17–20, 22, 24–25, 31, 33–36.

3.3. α -gal A activity

Whole cell lysates from blood lymphocytes and SFs were used to detect α -gal A activity. The enzyme activity was widely varied and irrelevant to clinical severity, and it is listed in Table 1.

3.4. Quantification of lyso-Gb3

Fresh plasma/serum were collected from all subjects, and lyso-Gb3 was extracted in methanol. Three consecutive measurements were performed and averaged. The list is summarized in Table 1.

3.5. Electron microscopy

Skin biopsies were taken from the left elbow and prepared for visualization by electron microscopy. Sphingolipid accumulation also varied case by case. Diffuse accumulation was detected in the lysosomes of almost all kind of cells including neuronal Schwann cells, vascular endothelium and fibroblasts (Fig. 1) in case 1. Mainly vascular endothelial and fibroblastic accumulation were found in cases 5, 7 (Fig. 2). Sphingolipid accumulation was detected predominantly in exocrine and fibroblastic cells in case 7 and myelin-like deposition was detected primarily in fibroblasts in case 27 (Fig. 2). Other cases were found to have heterogeneous and irregular or no accumulation (results not shown).

3.6. Detection of methylation sensitivity for restriction enzyme sites in the *GLA* gene

To assess differential methylation, genomic DNA was first treated with a methylation-sensitive endonuclease and then PCR-amplified. After PCR of bisulfite-reacted DNA, almost all the unmethylated cytosines (C) were converted to thymine (T), while methylcytosines were not. When we searched a single methylation-sensitive restriction enzyme site, one *SacII* site was detected in the 5'UTR, and a cluster of *HhaI* and *HpaII* sites were detected in exon 1 and 5'UTR of the *GLA* gene. Other exons also contained *HhaI* and *HpaII* sites; however, only one methylation-sensitive *HpaI* *RE* site was detected in exon 7 (Fig. 3).

3.7. Quantification of methylation of individual alleles

The quantification of methylation was performed based on digestion of mutated or non-mutated alleles by methylation-sensitive *RE*. The digestion of an allele indicated non-methylation. The quantification of methylation of the normal (non-mutated) allele for heterozygous Fabry females was calculated and plotted as a percentage. The quantification is listed in Table 1.

4. Discussion

After the approval of enzyme replacement therapy (ERT), it became the most popular treatment option for Fabry disease. Orally administered pharmacological chaperone migalastat hydrochloride also provide a beneficial effect on *GLA* amenable mutations [30]. Recent study showed that > 28% Japanese families had amenable mutations to migalastat [31]. However, timing the initiation of therapy is most crucial to get maximum beneficial effects [30,32,33]. The international guidelines for ERT are also being set up for presymptomatic patients [34]. The heterozygous female patients manifest wide variety of clinical

features, some have mild and some manifest severe phenotypes in early childhood [7,8,27]. Our current study also showed a heterogeneous clinical presentation; one was asymptomatic, and others showed classical phenotypes as Hemizygous males (Table 1).

By measuring α -gal A enzyme activity, the clinical phenotype of heterozygous females cannot be predicted, and lyso-Gb3 was thought to be a useful biomarker for the diagnosis of Fabry disease heterozygotes [35]. In this study, we also found similar pictures showed in some severe cases of high enzyme activity (Table 1). Because we used whole cell lysates, which contained both the precursor and mature forms, we could not separate their activities [36,37]. However, the clinical severity of heterozygous females correlated to the accumulation of lyso-Gb3 (Table 1) and massive sphingolipid accumulation detected by electron microscopy in different cells of skin biopsies (Figs. 1 and 2).

XCI and skewed XCI favoring the expression of the mutant allele has been proposed as a mechanism to explain the development of severe clinical symptoms in heterozygous Fabry disease and some other X-linked diseases [18,38,39]. The skewed XCI patterns were determined by studying the methylation status of the polymorphic (CAG)_n repeat region located within exon 1 of the *HUMARA* gene [18] for 29% cases. Our study demonstrated direct evidence of methylation in the *GLA* gene. In most of the cases 27/36 (75%), the methylation of the normal allele (non-mutated allele) directly correlated to the FASTEX severity score (Fig. 4), probably due to DNA methylation in the *GLA* gene suppressing transcription [40]. One allele was mutated, and another allele was methylated in affected females, which caused complete suppression of *GLA* gene transcription and mature α -gal A synthesis. We recently reported that the aberrant DNA methylation of the *GLA* gene is directly associated with autophagy dysfunction [41], which caused massive accumulation of sphingolipids and their derivatives. In this study, we found a clear correlation between methylation of the normal allele and accumulation of lyso-Gb3 (Figs. 1, 2 and 5). Except in cases 4, 24, 25, 28, 29, all the cases were receiving ERT for 3–10 years, and have a significant correlation between the clinical severity FASTEX score, methylation of the normal allele and deposition of lyso-Gb3.

Methylation of the *SacII* and *HhaI* sites for heterozygous females is highly correlated to the FASTEX severity score and lyso-Gb3 accumulation (statistically significant) [Figs. 4 and 5], probably because these sites were close to the promoter region, which contains 474 base pairs of CpG island [26]. In some cases (cases 10, 13, 15, 17, 21, and 29) the methylation of the normal allele measured in the *HpaII* site in exon 7 was correlated to the FASTEX severity score although the statistical significance could not be detected due to a small number of cases.

Several limitations existed in our study. We could not elucidate the phenotype-specific methylation effects, and the severity scores were not recorded when the cases were first diagnosed. We could also not confirm the progression of cardiomyopathy, renal failure or related effects in relation to the methylation ratio of the heterozygous females. However, our current study, which correlated the clinical severity to methylation of the normal allele and accumulation of lyso-Gb3, would be an effective tool for early prediction of phenotypes and early initiation of therapy in females heterozygous for Fabry disease. In addition, several targeted genetic editing techniques were reported in recent years [42,43]. By detecting the methylation sites in the *GLA* gene, the methylation site can be edited and might allow gene therapy applications for Fabry disease patients.

5. Conclusion

We summarized 36 heterozygous Fabry females with their clinical severity score, their lyso-Gb3 accumulation in plasma and sphingolipid accumulation with skin biopsy. We had detected methylation sensitive *RE* site in exon 7 along with exon 1 and 5' UTR and of the *GLA* gene. We found a clear correlation between the severity of the phenotype, lyso-Gb3 accumulation and methylation of normal allele detected by non-digestion with methylation sensitive *RE*.

Declaration of Competing Interest

Nothing to be declared for MAH, CW, HY, TM and KA. YE has been awarded grants, research support and honoraria from Actelion, BioMarin, Sanofi, Shire and Dainippon Sumitomo Pharmaceuticals Limited.

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References

- [1] R.O. Brady, A.E. Gal, R.M. Bradley, E. Martensson, A.L. Warshaw, L. Laster, Enzymatic defect in Fabry's disease: ceramide trihexosidase deficiency, *New Eng. J. Med.* 276 (1967) 1163–1167.
- [2] R.J. Desnick, Y.A. Ioannou, C.M. Eng, A-Galactosidase a deficiency: Fabry disease, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease*, eighth ed., McGraw Hill, New York, 2001, pp. 3733–3774.
- [3] C.S. Nance, C.J. Klein, M. Banikazemi, S.H. Dikman, R.G. Phelps, J.C. McArthur, M. Rodriguez, R.J. Desnick, Later-onset Fabry disease: an adult variant presenting with the cramp-fasciculation syndrome, *Arch. Neurol.* 63 (2006) 453–457.
- [4] B.E. Smid, L. van der Tol, M. Biegstraaten, G.E. Linthorst, C.E. Hollak, B.J. Poorthuis, Plasma globotriaosylsphingosine in relation to phenotypes of Fabry disease, *J. Med. Genet.* 52 (2015) 262–268.
- [5] R.J. Desnick, D.F. Bishop, Fabry disease α -galactosidase a deficiency and Schindler disease. α -N-acetylgalactosaminidase deficiency, in: J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, J.L. Goldstein, M.S. Brown (Eds.), *The Metabolic Basis of Inherited Disease*, 6th ed., McGraw-Hill, New York, 1989, p. 1751.
- [6] A.T. Franceschetti, Fabry disease ocular manifestations, *Birth Defects Orig Artic Ser.* 12 (1976) 195–208.
- [7] N. Guffon, Clinical presentation in female patients with Fabry disease, *J. Med. Genet.* 40 (2003) e38.
- [8] M. Kobayashi, T. Ohashi, M. Sakuma, H. Ida, Y. Eto, Clinical manifestations and natural history of Japanese heterozygous females with Fabry disease, *J. Inher. Met. Dis.* 31 (Suppl. 3) (2008) S483–S487.
- [9] G.E. Linthorst, B.J. Poorthuis, C.E. Hollak, Enzyme activity for determination of presence of Fabry disease in women results in 40% false-negative results, *J. Am. Coll. Cardiol.* 51 (2008) 2082–2083.
- [10] M. Yasuda, J. Shabbeer, S.D. Benson, I. Maire, R.M. Burnett, R.J. Desnick, Fabry disease: characterization of alpha-galactosidase a double mutations and the D313Y plasma enzyme pseudodeficiency allele, *Hum. Mutat.* 22 (2003) 486–492.
- [11] B.E. Smid, C.E. Hollak, B.J. Poorthuis, M.A. van den Bergh Weerman, S. Florquin, W.E. Kok, R.H. Lekanne Deprez, J. Timmermans, G.E. Linthorst, Diagnostic dilemmas in Fabry disease: a case series study on *GLA* mutations of unknown clinical significance, *Clin. Genet.* 88 (2015) 161–166.
- [12] D. Oder, N. Uceyler, D. Liu, K. Hu, B. Petritsch, C. Sommer, G. Ertl, C. Wanner, P. Nordbeck, Organ manifestations and long-term outcome of Fabry disease in patients with the *GLA* haplotype D313Y, *BMJ Open* 6 (2016) e010422.
- [13] E.H. Giannini, A.B. Mehta, M.J. Hilz, et al., A validated disease severity scoring system for Fabry disease, *Mol. Genet. Metab.* 99 (2010) 283–290.
- [14] C. Whybra, C. Kampmann, F. Krummenauer, et al., The mainz severity score index: a new instrument for quantifying the Anderson–Fabry disease phenotype, and the response of patients to enzyme replacement therapy, *Clin. Genet.* 65 (2004) 299–307.
- [15] R. Mignani, F. Pieruzzi, F. Berri, A. Burlina, B. Chinea, M. Gallieni, M. Pieroni, A. Salvati, M. Spada, FABry STabilization inbDEX (FASTEX): an innovative tool for the assessment of clinical stabilization in Fabry disease, *Clin. Kidney J.* 9 (2016) 739–747.
- [16] J.M. Amos-Landgraf, A. Cottle, R.M. Plenge, M. Friez, C.E. Schwartz, J. Longshore, H.F. Willard, X chromosome-inactivation patterns of 1,005 phenotypically unaffected females, *Am. J. Hum. Genet.* 79 (2006) 493–499.
- [17] J. Minks, W.P. Robinson, C.J. Brown, A skewed view of X chromosome inactivation, *J. Clin. Invest.* 118 (2008) 20–23.
- [18] L. Echevarria, K. Benistan, A. Toussaint, O. Dubourg, A.A. Hagege, D. Eladari, F. Jabbour, C. Beldjord, P. De Mazancourt, D.P. Germain, X-chromosome inactivation in female patients with Fabry disease, *Clin. Genet.* 89 (2016) 44–54.
- [19] A. Razin, A.D. Riggs, DNA methylation and gene function, *Science* 210 (1980) 604–610.
- [20] A. Bird, CpG-rich islands and the function of DNA methylation, *Nature* 321 (1986) 209–213.
- [21] A. Bird, The essentials of DNA methylation, *Cell* 70 (1992) 5–8.
- [22] G.P. Pfeifer, S.D. Steigerwald, P.R. Mueller, B. Wold, A.D. Riggs, Genomic sequencing and methylation analysis by ligation mediated PCR, *Science* 246 (1989) 810–813.
- [23] A.D. Riggs, G.P. Pfeifer, X-chromosome inactivation and cell memory, *Trends*

- Genet. 8 (1992) 169–174.
- [24] A.J. Sharp, E. Stathaki, E. Migliavacca, M. Brahmachary, S.B. Montgomery, Y. Dupre, S.E. Antonarakis, DNA methylation profiles of human active and inactive X chromosomes, *Genome Res.* 21 (2011) 1592–1600.
- [25] R.C. Allen, H.Y. Zoghbi, A.B. Moseley, H.M. Rosenblatt, J.W. Belmont, Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation, *Am. J. Hum. Genet.* 51 (1992) 1229–1239.
- [26] M. Gardiner-Garden, M. Frommer, CpG islands in vertebrate genomes, *Mol. Biol.* 196 (1987) 261–282.
- [27] M.A. Hossain, H. Yanagisawa, T. Miyajima, C. Wu, A. Takamura, K. Akiyama, R. Itagaki, K. Eto, T. Iwamoto, T. Yokoi, K. Kurosawa, H. Numabe, Y. Eto, The severe clinical phenotype for a heterozygous Fabry female patient correlates to the methylation of non-mutated allele associated with chromosome 10q26 deletion syndrome, *Mol. Genet. Metab.* 120 (2017) 173–179.
- [28] J. Okada, M.A. Hossain, C. Wu, T. Miyajima, H. Yanagisawa, K. Akiyama, Y. Eto, Ten-year-long enzyme replacement therapy shows a poor effect in alleviating giant leg ulcers in a male with Fabry disease, *Mol. Genet. Metab. Rep.* 14 (2017) 68–72.
- [29] T. Faraggiana, J. Churg, E. Grishman, L. Strauss, A. Prado, D.F. Bishop, E. Schuchman, R.J. Desnick, Light- and electron-microscopic histochemistry of Fabry's disease, *Am. J. Pathol.* 103 (1981) 247–262.
- [30] D.A. Hughes, K. Nicholls, G. Sunder-Plassmann, et al., Safety of switching to migalastat from enzyme replacement therapy in Fabry disease: experience from the phase 3 ATTRACT study, *Am. J. Med. Genet. A* 179 (2019) 1069–1073.
- [31] M. Kobayashi, T. Ohashi, E. Kaneshiro, T. Higuchi, H. Ida, Mutation spectrum of α -galactosidase gene in Japanese patients with Fabry disease, *J. Hum. Genet.* 64 (2019) 695–699.
- [32] M. Biegstraaten, R. Arngriímsson, F. Barbey, et al., Recommendations for initiation and cessation of enzyme replacement therapy in patients with Fabry disease: the European Fabry working group consensus document, *Orphanet. J. Rare Dis.* 10 (2015) 36.
- [33] R.J. Hopkin, J.L. Jefferies, D.A. Laney, et al., The management and treatment of children with Fabry disease: a United States-based perspective, *Mol. Genet. Metab.* 117 (2016) 104–113.
- [34] A. Ortiz, D.P. Germain, R.J. Desnick, J. Politei, M. Mauer, A. Burlina, C. Eng, R.J. Hopkin, D. Laney, A. Linhart, S. Waldeck, E. Wallace, F. Weidemann, W.R. Wilcox, Fabry disease revisited: management and treatment recommendations for adult patients, *Mol. Genet. Metab.* 123 (2018) 416–427.
- [35] A. Nowak, T.P. Mechtler, R.J. Desnick, D.C. Kasper, Plasma LysoGb3: a useful biomarker for the diagnosis and treatment of Fabry disease heterozygotes, *Mol. Genet. Metab.* 120 (2017) 57–61.
- [36] J.Y. Park, G.H. Kim, S.S. Kim, J.M. Ko, J.J. Lee, H.W. Yoo, Effects of chemical chaperone on genetic mutations in alpha-galactosidase in Korean patients with Fabry disease, *Exp. Mol. Med.* 41 (2009) 1–7.
- [37] M.A. Hossain, K. Higaki, S. Saito, K. Ohno, H. Sakuraba, E. Nanba, Y. Suzuki, K. Ozono, N. Sakai, Chaperone therapy for Krabbe disease: potential for late-onset GALC mutations, *J. Hum. Genet.* 60 (2015) 539–545.
- [38] E. Beutler, M. Yeh, V.F. Fairbanks, The normal human female as a mosaic of X-chromosome activity: studies using the gene for C-6-PD-deficiency as a marker, *Proc. Natl. Acad. Sci. U. S. A.* 48 (1962) 9–16.
- [39] W.B. Dobyns, A. Filauero, B.N. Tomson, A.S. Chan, A.W. Ho, N.T. Ting, J.C. Oosterwijk, C. Ober, Inheritance of most X-linked traits is not dominant or recessive, just X-linked, *Am. J. Med. Genet. A* 129A (2004) 136–143.
- [40] P.H. Tate, A.P. Bird, Effects of DNA methylation on DNA-binding proteins and gene expression, *Curr. Opin. Genet. Dev.* 3 (1993) 226–231.
- [41] H. Yanagisawa, M.A. Hossain, T. Miyajima, K. Nagao, T. Miyashita, Y. Eto, Dysregulated DNA methylation of GLA gene was associated with dysfunction of autophagy, *Mol. Genet. Metab.* 126 (2019) 460–465.
- [42] S. Morita, T. Horii, I. Hatada, Editing of DNA methylation using dCas9- peptide repeat and scFv-TET1 catalytic domain fusions, *Methods Mol. Biol.* 1767 (2018) 419–428.
- [43] X.S. Liu, H. Wu, M. Krzisch, X. Wu, J. Graef, J. Muffat, D. Hnisz, C.H. Li, B. Yuan, C. Xu, Y. Li, D. Vershkov, A. Cacace, R.A. Young, R. Jaenisch, Rescue of fragile X Syndrome neurons by DNA methylation editing of the FMR1 gene, *Cell* 172 (2018) 979–992 (e976).
- [44] J. Shabbeer, M. Yasuda, E. Luca, R.J. Desnick, Fabry disease: 45 novel mutations in the alpha-galactosidase a gene causing the classical phenotype, *Mol. Genet. Metab.* 76 (2002) 23–30.
- [45] C.M. Eng, D.J. Niehaus, A.L. Enriquez, T.S. Burgert, M.D. Ludman, R.J. Desnick, Fabry disease: twenty-three mutations including sense and antisense CpG alterations and identification of a deletional hot-spot in the alpha-galactosidase a gene, *Hum. Mol. Genet.* 3 (1994) 1795–1799.
- [46] S. Ishii, H. Sakuraba, Y. Suzuki, Point mutations in the upstream region of the alpha-galactosidase a gene exon 6 in an atypical variant of Fabry disease, *Hum. Genet.* 89 (1992) 29–32.
- [47] Y. Iwafuchi, H. Maruyama, T. Morioka, S. Noda, H. Nagata, Y. Oyama, I. Narita, Enzyme replacement therapy in a patient of heterozygous Fabry disease: clinical and pathological evaluations by repeat kidney biopsy and a successful pregnancy, *CEN Case Rep.* 6 (2017) 210–214.
- [48] A. Nagasaki, W. Nishie, K. Sato, I. Oba, E. Noguchi, H. Akitsu, D. Sawamura, H. Shimizu, Clinical and genetic analysis of Fabry disease: report of six cases including three heterozygous females, *J. Dermatol. Sci.* 52 (2008) 61–64.
- [49] M. Shimotori, H. Maruyama, G. Nakamura, T. Suyama, F. Sakamoto, M. Itoh, S. Miyabayashi, T. Ohnishi, N. Sakai, M. Wataya-Kaneda, M. Kubota, T. Takahashi, T. Mori, K. Tamura, S. Kageyama, N. Shio, T. Maeba, H. Yahagi, M. Tanaka, M. Oka, H. Sugiyama, T. Sugawara, N. Mori, H. Tsukamoto, K. Tamagaki, S. Tanda, Y. Suzuki, C. Shinonaga, J. Miyazaki, S. Ishii, F. Gejyo, Novel mutations of the GLA gene in Japanese patients with Fabry disease and their functional characterization by active site specific chaperone, *Hum. Mutat.* 29 (2008) 331.
- [50] C. Meaney, L.C. Blanch, C.P. Morris, A nonsense mutation (R220X) in the alpha-galactosidase a gene detected in a female carrier of Fabry disease, *Hum. Mol. Genet.* 3 (1994) 1019–1020.
- [51] H. Sakuraba, A. Oshima, Y. Fukuhara, M. Shimamoto, Y. Nagao, D.F. Bishop, R.J. Desnick, Y. Suzuki, Identification of point mutations in the alpha-galactosidase a gene in classical and atypical hemizygotes with Fabry disease, *Am. J. Hum. Genet.* 47 (1990) 784–789.
- [52] J.P. Davies, B.G. Winchester, S. Malcolm, Mutation analysis in patients with the typical form of Anderson-Fabry disease, *Hum. Mol. Genet.* 2 (1993) 1051–1053.
- [53] P. Ashton-Prolla, B. Tong, J. Shabbeer, K.H. Astrin, C.M. Eng, R.J. Desnick, Fabry disease: twenty-two novel mutations in the alpha-galactosidase a gene and genotype/phenotype correlations in severely and mildly affected hemizygotes and heterozygotes, *J. Investig. Med.* 48 (2000) 227–235.
- [54] T. Koide, M. Ishiura, K. Iwai, M. Inoue, Y. Kaneda, Y. Okada, T. Uchida, A case of Fabry's disease in a patient with no alpha-galactosidase a activity caused by a single amino acid substitution of Pro-40 by Ser, *FEBS Lett.* 259 (1990) 353–356.
- [55] C.M. Eng, L.A. Resnick-Silverman, D.J. Niehaus, K.H. Astrin, R.J. Desnick, Nature and frequency of mutations in the alpha-galactosidase a gene that cause Fabry disease, *Am. J. Hum. Genet.* 53 (1993) 1186–1197.
- [56] L.C. Blanch, C. Meaney, C.P. Morris, A sensitive mutation screening strategy for Fabry disease: detection of nine mutations in the alpha-galactosidase a gene, *Hum. Mutat.* 8 (1996) 38–43.