MUTATION IN BRIEF

Novel Mutations of the GLA Gene in Japanese Patients with Fabry Disease and Their Functional Characterization by Active Site Specific Chaperone

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Fabry disease is an X-linked recessive inborn metabolic disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A (EC 3.2.1.22). The causative mutations are diverse, include both large rearrangements and single-base substitutions, and are dispersed

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throughout the 7 exons of the α-galactosidase A gene (*GLA*). Mutation hotspots for Fabry disease do not exist. We examined 62 Fabry patients in Japan and found 24 *GLA* mutations, including 11 novel ones. A potential treatment reported for Fabry disease is active site specific chaperone (ASSC) therapy using 1-deoxygalactonojirimycin (DGJ), an inhibitor of α-galactosidase A, at subinhibitory concentrations. We transfected COS-7 cells with the 24 mutant *GLA*s and analyzed the α-galactosidase A activities. We then treated the transfected COS-7 cells with DGJ and analyzed its effect on the mutant enzyme activities. The activity of 11 missense mutants increased significantly with DGJ. Although ASSC therapy is useful only for misfolding mutants and therefore not applicable to all cases, it may be useful for treating many Japanese patients with Fabry disease.

KEY WORDS: α-galactosidase A; GLA; lysosomal storage disease; 1-deoxygalactonojirimycin; chaperone therapy

INTRODUCTION

Fabry disease (FD; MIM# 301500) is a pan-ethnic, X-linked, lysosomal storage disorder caused by a deficiency in the lysosomal enzyme α-galactosidase A (EC 3.2.1.22) (Brady et al., 1967). Human α-galactosidase A is a homodimeric glycoprotein with three N-linked oligosaccharide chains on each subunit. Enzyme deficiency results in a systemic lysosomal accumulation of glycolipids, primarily globotriosylceramide (Gb3), in the vascular endothelium and other tissues. In the classical form of the disease, the patient develops angiokeratoma, hypohidrosis, and episodic pain crises in the extremities during childhood or adolescence. With advancing age, the morbidity of renal failure, cardiac disease, and early onset of stroke increases. The severity of the clinical manifestations depends on the amount of residual α-galactosidase A activity. Hemizygous male patients with no or very low α-galactosidase A activity usually have severe clinical symptoms and die as young adults. Heterozygous female Fabry patients exhibit a wide range of severity, from a virtually symptom-free course (Marguery et al., 1993) to one comparable to that of their male counterparts (Whybra et al., 2001), although they usually have no symptoms or very mild manifestations.

We examined Fabry patients in Japan and sequenced the patients' *α-galactosidase A* gene (*GLA*) (MIM# 300644). To analyze the mutant α-galactosidase A activities, we transfected COS-7 cells with the mutant *GLA*s. In addition, since 1-deoxygalactonojirimycin (DGJ) stabilizes the α-galactosidase A conformation and improves its stability (Asano et al., 2000; Ishii et al., 2000; Yam et al., 2006), we added DGJ to the incubation medium and examined its effect on the mutant α-galactosidase A activities.

MATERIALS AND METHODS

Patients

We examined 62 Fabry patients from 31 unrelated families. Diagnosis was based on reduced or absent α-galactosidase A activity and typical signs and symptoms of the disease. We received approval to use the patients' DNA for this study from The Ethics Committee on Genetics of the Niigata University School of Medicine, and obtained informed consent from the patients.

Mutation analysis

Blood samples obtained from patients with Fabry disease were transferred into blood-collecting tubes containing ethylene diamine tetraacetic acid (EDTA) and stored at 4°C. Unlike heparin, EDTA does not inhibit the activity of reverse transcriptase, which is used for the reverse transcriptase polymerase chain reaction (RT-PCR). The human *GLA* consists of 7 exons. We found it difficult to obtain full-length *GLA* cDNA (RefSeq BC_002689.2) by performing a single RT-PCR. We therefore obtained exon 1 and exon 7 by PCR from genomic DNA, and the region between exons 1 and 7, containing the full-length exons 2-6 by RT-PCR, from total RNA.

White blood cells were collected using Lymphoprep (Axis-Shield PoC, Oslo, Norway). The genomic DNA of the white blood cells was isolated using an automatic isolation system (NA1000; Kurabo, Osaka, Japan) within 1 week after blood sampling and stored at -20°C until use.

To determine the sequence of exons 1 and 7 of *GLA*, we performed PCR on genomic DNA with the following specific primers: Sense Ex1-1(+), 5'-CCAGTTGCCAGAGAAACA-3'; Antisense Ex1-2(-), 5'-GAGACTCTCCAGTTCCC-3'; Sense Ex7-5(+), 5'-ACAAGTGCTTGATAGTTCTGA-3'; Antisense Ex7-6(-), 5'-CAGGAAGTAGTAGTTGGCAA-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 59°C, and 30 sec at 68°C with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The lengths of the expected products were 390 bp for exon 1 and 420 bp for exon 7. The PCR products were analyzed by 2% NuSieve GTG agarose (Takara, Shiga, Japan) gel electrophoresis and then eluted using the Gene Clean Spin Kit (Q-BIOgene, Irvine, CA, U.S.A.).

To determine the sequence of the cDNA region of *GLA* that included exons 2 to 6, we first amplified it by RT-PCR. We generated first-strand cDNA by RT (ReverTra Ace -α-, Toyobo) using random 9-mers. The reaction consisted of 10 min at 30°C, 20 min at 42°C, 5 min at 99°C, and 5 min at 4°C. To amplify the cDNA of *GLA*, we performed the first PCR with specific primers as follows: 5' #1 primer: 5'-TATGCTGTCCGGTCACC-3', #66 (AG66) primer: 5'-TTAAAGTAAGTCTTTTAATGACAT-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 55°C, and 1 min 30 sec at 68°C with KOD-Plus DNA polymerase (Toyobo). The first PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). The length of the expected products was 1.3 kbp. To amplify the *GLA* cDNA, we performed a second (nested) PCR with the following specific primers: cDNA#1, 5'-TTGGCAAGGACGCCTAC-3'; cDNA#2, 5'-TGCGATGGTATAAGAGCG-3'. The PCR protocol consisted of 2 min at 94°C, then 30 cycles of 15 sec at 94°C, 30 sec at 55°C, and 1 min at 68°C with KOD-Plus DNA polymerase (Toyobo). The length of the expected product was 1 kbp. The PCR products were analyzed by 2% NuSieve GTG agarose (Takara) gel electrophoresis and then eluted using the Gene Clean Spin Kit (Q-BIO gene).

We analyzed both strands of the *GLA* sequence in the PCR and RT-PCR products by direct sequencing using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster, CA, U.S.A.).

Construction of pKSCX-*GLA*

We constructed plasmid pKSCX-*GLA* by inserting the normal human *GLA* cDNA in pCXN2Gal (Ishii et al., 1993) into the unique EcoRI site of the pKSCX expression vector, which bears the cytomegalovirus immediate-early enhancer/chicken β-actin hybrid promoter, and the kanamycin-resistance gene for selection (Niwa et al., 1991). We confirmed the pKSCX-GLA construct by sequencing both DNA strands.

Construction of pKSCX-mutant-*GLA*

To create point mutations or to delete or insert single or multiple amino acids, we used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) (Yasuda et al., 2004). Because pKSCX contains many GC-rich sequences, we constructed plasmid pCI-*GLA* by inserting the normal human *GLA* cDNA of pCXN2Gal (Ishii et al., 1993) into the unique *Eco*RI site of the pCI mammalian expression vector (Promega, Madison, WI, U.S.A.). We then used pCI-*GLA* as the mutagenesis template. We designed specific primer pairs according to each mutation. To clone the mutant-*GLA* cDNA derived from patients with Fabry disease, we incorporated the mutation into the pCI-*GLA* cDNA using the site-directed mutagenesis kit with the specific primer pairs. We confirmed the mutant *GLA*s by sequencing both DNA strands. We digested the pCI-mutant-*GLA* with *Eco*RI, eluted the *Eco*RI-*Eco*RI fragment containing the mutant-*GLA* cDNA, and ligated it into the like-digested cloning site of pKSCX, yielding pKSCX-mutant-*GLA*.

Cell culture and transfection

We prepared pKSCX-*GLA* or pKSCX-mutant-*GLA* using the Qiagen EndoFree plasmid Giga kit (Qiagen GmbH) (Maruyama et al., 2000), as described previously, and then subjected it to ethanol precipitation.

COS-7 cells (Originator, Gluzman Y; Riken Cell Bank, RCB 0539) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD, U.S.A.) containing 10% fetal bovine serum (FBS; Gibco) (Yasuda et al., 2003). The cells were harvested by trypsin treatment, washed in 10 ml of DMEM containing 10% FBS, and resuspended at 5×10^6 cells/ml in DMEM containing 10% FBS. The cells were

transfected by the LipofectAMINE 2000 method (Invitrogen, Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. The cells were cultured in DMEM containing 10% FBS at 37°C and 5% CO_2. The COS-7 cells were transfected with pKSCX plasmid *in vitro* using the LipofectAMINE2000 cationic lipid reagent. Briefly, 8 μg of DNA (4 μg pKSCX-mutant-*GLA* and 4 μg pKSCX-luciferase) were mixed with 20 μL of LipofectAMINE2000 reagent and added to the medium in 60-mm dishes. After the transfection, the transformants were cultured in 5 ml of DMEM containing 10% FBS with or without 10 μM DGJ (Sigma Chemical, St. Louis, MO, U.S.A.) at 37°C for 3 days before the enzyme assay. The cells were harvested by trypsin treatment, rinsed with phosphate-buffered saline, and homogenized with 0.2 ml of water with a Physcotron (NS-310E, Niti-on, Chiba, Japan). The sample was then subjected to centrifugation at 10000×g for 5 min. The water-soluble extract was used as the enzyme source.

α-Galactosidase A activity assay

The α-Galactosidase A activity of the water-soluble extract was determined using an artificial substrate, 4-methyl-umbelliferyl α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan), as described previously (Fan et al., 1999). The protein content of the water-soluble extract was measured by the method of Lowry (DC protein assay; Bio-Rad, Hercules, CA, U.S.A.).

Luciferase assay

Briefly, 20 μl of the water-soluble extract was added to 100 μl of firefly luciferin solution (PicaGene, Toyo Ink, Tokyo, Japan). The luciferase activity was measured for 10 sec using a luminometer (GENE LIGHT, Microtec Nition, Chiba, Japan), according to the manufacturer's instructions.

Statistical analysis

The data are presented as the mean values ± the standard deviation of the mean. We analyzed all data using the Statcel2 Microsoft Office Excel Add-in Software (Hisae Yanai, Department of Mathematics, Faculty of Science Saitama University). Statistical significance was evaluated using the unpaired *t*-test. We considered *P* values of < 0.05 to be statistically significant.

RESULTS AND DISCUSSION

We detected 24 mutations in 62 Fabry patients from 31 unrelated families in Japan (Table 1). The patients' clinical characteristics are shown in Table 2; the numbers correspond to those of the mutant *GLA*s in Table 1. The mutations consisted of 17 missense substitutions (p.M42V, p.E66Q, p.M76T, p.D93V, p.R112H, p.S148N, p.P205T, p.D231V, p.S235F, p.G258V, p.G260A, p.T282A, p.K308N, p.Q312R, p.G328R, p.L403S, p.T410P), 3 nonsense substitutions (p.S102X, p.R227X, p.W399X), 2 small deletions (c.718_719delAA, c.1020delG), 1 insertion (c.323_324insCAGA), and 1 double missense mutation (p.E66Q, p.R112C). Importantly, 11 of the mutations (p.M76T, p.D231V, p.S235F, p.G258V, p.T282A, p.K308N, p.Q312R, p.L403S, p.T410P, p.S102X, c.323_324insCAGA) were novel. The mutant *GLA* p. E66Q, which is a G-to-C transversion at nucleotide 196 (cDNA) of exon 2, was observed in seven putatively unrelated families, and the mutant *GLA* c.1020Gdel-deletion, was observed in two putatively unrelated families.

To date, more than 20 mutant *GLA*s have been reported in Japan (Takata et al., 1997; Okumiya et al., 1995; Miyamura et al., 1996). Of the 24 mutant *GLA*s we examined, 4 (p. E66Q, p. G260A, p. G328R, c. 718_719delAA) are the same as the known mutant *GLA*s in Japan, and 20 are different. Therefore, in the present study, we examined over half the mutant *GLA*s in Japan.

To examine the activities of the α-galactosidase A produced by these mutant genes, COS-7 cells were transfected with the mutant *GLA*s and cultured with or without 10 μM DGJ for 3 days. All the mutant-α-galactosidase A activities were low, with 0.1% to 50% of the mean normal activity. We examined the relationship between the enzyme activity and clinical symptoms. As expected from previous studies, a one-seventh value of normal α-galactosidase A activity was sufficient to suppress clinical symptoms, and 8 of the 24 mutations reached an activity at least one-seventh that of normal α-galactosidase A activity with DGJ treatment (Table 2) (Nakao et al., 1995; Nakao et al., 2003; Yoshitama et al., 2001). The patients with a one-seventh value of normal α-galactosidase A activity or more did not exhibit the classic symptoms of the disease (angiokeratoma, hypohidrosis,

and episodic pain crises), nor did they develop renal failure and/or cardiac disease later in life (Nakao et al., 1995; Nakao et al., 2003; Yoshitama et al., 2001).

Some mutant-α-galactosidase A variants form aggregates in the endoplasmic reticulum (ER), due to their failure to fold properly during synthesis, and they may lose their enzyme activity. Such mutant-α-galactosidase A variants may be degraded by the ER quality control system (Yam et al., 2006). Moreover, misfolded α-galactosidase A variants appear to stay within the ER regardless of their catalytic competence, leading to their possible degradation or aggregate formation. As a result, mutant α-galactosidase A variants with defective folding tend to be retained in the ER and not reach the lysosomes, where the enzyme functions. DGJ functions as an active-site-specific chaperone (ASSC) to stabilize the conformation of mutant-α-galactosidase A variants and preserve at least some of their enzyme activity. In some cases, DGJ allows mutant-α-galactosidase A variants to pass through the ER quality control system and be sorted into lysosomes, resulting in an increase in the mutant's α-galactosidase A activity. This suggests that the stability of mutant α-galactosidase A is a critical factor that determines its further transportation and function as residual enzyme activity. However, DGJ does not have a measurable effect on all mutant α-galactosidase A activities, particularly those with frame-shift or early termination mutations.

In this study, the activities of 11 of the 24 α-galactosidase A mutants significantly increased after incubation with 10 μM DGJ for 3 days (Table 1). The activity of 11 missense mutants significantly increased with DGJ treatment, while that of the other missense, and the nonsense, insertion, and deletion mutants did not increase. Because the No. 7 mutation causes the incorrect synthesis of most of the protein, including the active site, the apparent enzyme activity of No. 7, 4% of the normal enzyme activity, may be experimental noise. Therefore, some of the other mutations with less than 4% enzyme activity need to be interpreted carefully. Nevertheless, these findings indicate that although ASSC therapy is not applicable to all patients with Fabry disease, many Japanese patients might respond to such treatment. Importantly, a preliminary study of DGJ use in transgenic mice showed no general signs of toxicity (Fan et al., 1999), supporting this compound's promise as a treatment for human Fabry disease.

Our findings support the potential usage of ASSC as an alternative treatment for Fabry disease. It is likely that by correcting the trafficking defect with DGJ, the misfolded α-galactosidase A variants are directed to lysosomes, thereby increasing their residual enzymatic activities. Our results showed that DGJ specifically affects misfolded α-galactosidase A, since it reproducibly increased the activity of different *GLA* variants. We also showed that DGJ causes normal α-galactosidase A activity to increase, although the reason is not known. Therefore, DGJ may be a useful treatment for every heterozygous patient.

Recently, enzyme replacement therapy (ERT) was approved for patients with Fabry disease: two recombinant glycoprotein products, Replagal® (Transkaryotic Therapies, Cambridge, MA, U.S.A.) and Fabrazyme® (Genzyme, Cambridge, MA, U.S.A.), are now available (Goi et al., 2005; Hoffmann et al., 2006). Because kidney and heart involvements are important causes of the morbidity and mortality in patients with Fabry disease, they should be the main targeted organs in therapies designed to treat it. However, many investigations have reported that in Fabry disease the kidney and heart are highly resistant to ERT with either enzyme treatment (Brady et al., 2001; Ioannou et al., 2001). Furthermore, most of the injected enzyme is delivered to the liver and has difficulty reaching other organs, such as the kidney and heart (Lee et al., 2003; Sakuraba et al., 2006). In addition, the Gb3 in the Fabry mouse kidney is highly resistant to ERT by systemic intravenous injection. In ERT of the Fabry mouse, most (65~70%) of the injected enzyme is recovered in the liver, and less than roughly 1% of the injected dose is detected in the kidney and heart (Lee et al., 2003). An examination of ERT in the Fabry mouse also showed that only a very small quantity of the recombinant enzyme remains in tissues hours after the infusion.

DGJ treatment, in contrast, increased the enzyme activity of normal α-galactosidase A and of several mutant-α-galactosidase A variants (Table1, No.1, 4, 8, 10, 13, 15, 16, 17,18, 19, 23) enough to extend the persistence of enzyme activity *in vitro*. Using DGJ as an ASSC may resolve the problems caused by mutant α-galactosidase A effectively. Increasing the activity of both normal and mutant α-galactosidase A with DGJ treatment may prove clinically useful.

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Table 1. Mutations in the *GLA* genes of Fabry patients

No.	Number of Patients (hemizygote/ heterozygote)	Exon	Nucleotide Change (RefSeq BC_002689.2)	Amino acid change (RefSeq AAH02689.1)	GLA activity		GLA DGJ (-) /normal GLA	GLA DGJ (+) /normal GLA	GLA DGJ (+) /GLA DGJ (-)
					DGJ (-)	DGJ (+)			
					nmol/h/ml/10^7RLU		%	%	ratio
normal GLA					3770 ± 1410	*6840 ± 1980		181.0	1.8
1	1 / 0	1	c.124A<G	p.M42V	160 ± 52	*848 ± 245	4.2	22.5	5.3
2	8 / 8	2	c.196G<C	p.E66Q	1883 ± 102	2280 ± 841	49.8	60.5	1.2
3	1 / 0	2 2	c.196G<C c.334C<T	p.E66Q p.R112C	121 ± 53	129 ± 30	3.2	3.4	1.1
4	0 / 1	2	c.227T<C	p.M76T	108 ± 49	*222 ± 31	2.9	5.9	2.1
5	1 / 0	2	c.278A<T	p.D93V	45 ± 7	56 ± 7	1.2	1.5	1.2
6	0 / 1	2	c.305C<G	p.S102X	71 ± 27	74 ± 24	1.9	2.0	1.1
7	1 / 0	2	c.323_324insCAGA		89 ± 60	150 ± 119	2.4	4.0	1.7
8	1 / 0	2	c.335G<A	p.R112H	24 ± 3	*2470 ± 716	0.6	65.5	102.0
9	2 / 1	3	c.443G<A	p.S148N	5 ± 6	49 ± 7	0.1	1.3	9.1
10	0 / 1	4	c.613C<A	p.P205T	321 ± 108	*5640 ± 776	8.5	150.0	17.6
11	1 / 0	5	c.679C<T	p.R227X	81 ± 33	104 ± 29	2.1	2.8	1.3
12	1 / 0	5	c.692A<T	p.D231V	128 ± 32	160 ± 33	3.4	4.2	1.3
13	1 / 0	5	c.704C<T	p.S235F	120 ± 23	*305 ± 63	3.2	8.1	2.5
14	3 / 0	5	c.718_719delAA		19 ± 11	20 ± 13	0.5	0.5	1.0
15	1 / 1	5	c.773G<T	p.G258V	73 ± 12	***385 ± 24	1.9	10.2	5.3
16	1 / 0	5	c.779G<C	p.G260A	406 ± 65	***2230 ± 257	10.8	59.2	5.5
17	1 / 1	6	c.844A<G	p.T282A	9 ± 2	*223 ± 62	0.2	5.9	25.3
18	1 / 0	6	c.924A<T	p.K308N	175 ± 38	***1770 ± 125	4.6	46.9	10.1
19	2 / 0	6	c.935A<G	p.Q312R	381 ± 57	***1640 ± 197	10.1	43.5	4.3
20	1 / 0	6	c.982G<A	p.G328R	8 ± 12	9 ± 6	0.2	0.2	1.1
21	1 / 2	7	c.1021delG		27 ± 10	78 ± 11	0.7	2.1	2.9
22	1 / 3	7	c.1196G<A	p.W399X	76 ± 31	86 ± 28	2.0	2.3	1.1
23	2 / 0	7	c.1208T<C	p.L403S	857 ± 262	*1440 ± 176	22.7	38.2	1.7
24	6 / 5	7	c.1228A<C	p.T410P	6 ± 3	8 ± 3	0.2	0.2	1.3

*, $p < 0.05$ **, $p < 0.005$ ***, $p < 0.0005$.

The α-galactosidase A activity of COS-7 origin is subtracted from the results of the α-galactosidase A activity assay.

Table 2. Clinical characteristics of the study patients

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
1	hemizygote	unknown	10	-	-	+	+	protein2+,occult blood1+	left ventricular hypertrophy
2	hemizygote	3.4	26	-	-	-	-	-	normal
	hemizygote	2.3	45	-	-	-	-	-	left ventricular hypertrophy
	hemizygote	1	-	-	-	-	-	-	normal
	hemizygote	3.5	-	-	-	-	-	-	normal
	hemizygote	unknown	-	-	-	-	-	-	normal
	hemizygote	unknown	50	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	unknown	-	-	-	-	-	-	normal
	heterozygote	unknown	-	-	-	-	-	-	normal
	heterozygote	unknown	-	-	-	-	-	-	unknown
	heterozygote	unknown	50	-	-	-	-	chronic renal failure on HD	-
	heterozygote	2.5	44	-	-	-	-	-	unknown
	heterozygote	4.3	47	-	-	+	-	unknown	unknown
	heterozygote	4.5	21	-	-	-	-	unknown	unknown
	heterozygote	4	24	-	-	-	-	unknown	unknown
	heterozygote	unknown	40	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
	heterozygote	4	40	-	-	-	-	chronic renal failure	left ventricular hypertrophy
3	hemizygote	unknown	8	+	+	-	-	protein2+	left ventricular hypertrophy
4	hemizygote	unknown	20	-	-	-	-	protein2+	normal
5	heterozygote	11.1	unknown	-	-	-	-	unknown	normal
6	heterozygote	8.4	11	+	-	-	+	-	normal
7	hemizygote	0.2	9	+	-	+	+	protein2+	left ventricular hypertrophy
8	hemizygote	unknown	50	-	-	-	-	chronic renal failure on HD	left ventricular hypertrophy
9	hemizygote	0.8	childhood	+	-	+	+	-	normal
	hemizygote	1.6	childhood	+	-	+	unknown	protein2+	normal
	heterozygote	11.4		+	-	+		-	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
10	heterozygote	unknown	40	unknown	-	unknown	unknown	-	left ventricular hypertrophy
11	hemizygote	unknown	11	+	+	-	-	protein3+,occult blood+-	left ventricular hypertrophy
12	hemizygote	3.7	3	+	+	+	+	protein+-	aortic regurgitation 3
13	hemizygote	unknown	14	+	+	+	-	unknown	normal
14	hemizygote	2	9	+	unknown	-	-	-	normal
	hemizygote	2.2	10	+	unknown	-	+	-	normal
	hemizygote	2.5	13	+	unknown	-	-	-	normal
15	heterozygote	4.3	16	-	-	-	+	-	normal
	hemizygote	3.4		+	+	+	-	protein2+	left ventricular hypertrophy
16	hemizygote	1.2	7	+	+	-	+	protein+,occult blood+	left ventricular hypertrophy
17	heterozygote	5.25	45	-	-	-	-	-	mitral regurgitation 3
	hemizygote	3	12	+	+	-	-	-	left ventricular hypertrophy
18	hemizygote	unknown	childhood	+	-	unknown	unknown	protein2+	left ventricular hypertrophy
19	hemizygote	0.4	-	-	-	-	-	-	-
	hemizygote	1.8	-	-	-	-	-	-	-
20	hemizygote	5	unknown	-	-	-	-	-	normal
21	hemizygote	0.4	25	-	-	-	-	protein2+	left ventricular hypertrophy
	heterozygote	6.3	10	+	-	-	+	protein3+	left ventricular hypertrophy
	heterozygote	11.7	10	+	-	-	+	protein3+	left ventricular hypertrophy
22	hemizygote	0.3	8	+	+	-	-	-	-
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
	heterozygote	unknown	unknown	-	-	-	-	unknown	unknown
23	hemizygote	3.1	unknown	-	-	-	-	protein+	left ventricular hypertrophy
	hemizygote	unknown	unknown	-	-	-	-	-	left ventricular hypertrophy
24	hemizygote	2.4	10	+	+	+	-	protein3+	sick sinus syndrome
	heterozygote	unknown	8	+	+	+	+	chronic renal failure on HD	left ventricular hypertrophy

No*		serum GLA activity (nmol/h/mg)	age at onset	neuropathic pain	hypohidrosis	Angiokeratoma	corneal opacity	urine abnormality	ECG
	heterozygote	5	20	+	-	+	+	-	-
	heterozygote	3.1	10	+	+	+	+	-	-
	hemizygote	2.9	childhood	+	+	+	+	chronic renal failure on HD	chronic heart failure
	hemizygote	2	13	+	+	+	-	-	-
	heterozygote	3.8	8	+	+	-	-	-	-
	heterozygote	3	12	+	-	-	-	-	-
	hemizygote	3	7	+	+	-	-	-	-
	heterozygote	4.2	12	+	-	-	-	-	-
	hemizygote	unknown	12	+	+	+	+	chronic renal failure on HD	-

*The mutation numbers in Table 2 correspond to those in Table 1.
Case numbers in bold italic denote α-galactosidase A activity greater than one-seventh of normal, following DGJ treatment.

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