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Fabry Disease: Twenty Novel α-Galactosidase A Mutations and Genotype–Phenotype Correlations in Classical and Variant Phenotypes

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Abstract

Background: Fabry disease (OMIM 301500) is an X-linked inborn error of glycosphingolipid metabolism resulting from mutations in the α-galactosidase A (α-Gal A) gene. The disease is phenotypically heterogeneous with classic and variant phenotypes. To assess the molecular heterogeneity, define genotype/phenotype correlations, and for precise carrier identification, the nature of the molecular lesions in the α-Gal A gene was determined in 40 unrelated families with Fabry disease.
Materials and Methods: Genomic DNA was isolated from affected males or obligate carrier females and the entire α-Gal A coding region and flanking sequences were amplified by PCR and analyzed by automated sequencing. Haplotype analyses were performed with polymorphisms within and flanking the α-Gal A gene.
Results: Twenty new mutations were identified (G43R, R49G, M72I, G138E, W236X, L243F, W245X, S247C, D266E, W287C, S297C, N355K, E358G, P409S, g1237del15, g10274insG, g10679insG, g10702delA, g11018insA, g11185-delT), each in a single family. In the remaining 20 Fabry families, 18 previously reported mutations were detected (R49P, D92N, C94Y, R112C [two families], F113S, W162X, G183D, R220X, R227X, R227Q, Q250X, R301X, R301Q, G328R, R342Q, E358K, P409A, g10208delAA [two families]). Haplotype analyses indicated that the families with the R112C or g10208delAA mutations were not related. The proband with the D266E lesion had a severe classic phenotype, having developed renal failure at 15 years. In contrast, the patient with the S247C mutation had a variant phenotype, lacking the classic manifestations and having mild renal involvement at 64 years.
Conclusions: These results further define the heterogeneity of α-Gal A mutations causing Fabry disease, permit precise heterozygote detection and prenatal diagnosis in these families, and provide additional genotype/phenotype correlations in this lysosomal storage disease.

Introduction

Fabry disease (MIM 301500) is an X-linked recessive inborn error of glycosphingolipid metabolism due to the deficient activity of the lysosomal enzyme, α-galactosidase A (α-*D*-galactoside galactohydrolase, EC 3.2.1.22; α-Gal A) (1,2). The enzymatic defect results in the accumulation of neutral glycosphingolipids with terminal α-galactosyl moieties, particularly globotriaosylceramide (GL-3) (3). Although the glycosphingolipid deposition occurs systemically, the major disease manifestations in classically affected males, who have absent or nondetectable levels of α-Gal A activity, primarily result from the progressive accumulation in the microvascular endothelium, leading to ischemia and vascular occlusions. The resultant clinical manifestations include acroparesthesias, which usually begin early in childhood, angiokeratoma, hypohidrosis, and the characteristic corneal and lenticular opacities. With advancing age, progressive glycosphingolipid deposition in the microvasculature leads to renal failure, cardiac involvement, and cerebrovascular disease.

In classically affected families, heterozygous females for this X-linked disease may have a range of manifestations from asymptomatic to the full blown disease, due to random X-chromosomal inactivation. Most carriers are asymptomatic and live a normal lifespan. Although many carriers have the corneal opacity (~80%) that does not affect vision, some have significant cutaneous involvement or report the occurrence of acroparesthesias in childhood or adolescence. Rare carriers with very low or no detectable α-Gal A activity have been reported with manifestations as severe as affected males, including the development of renal failure (3).

In contrast, male variants with residual α-Gal A activity (~1–5% of normal) also have been described (4–7). These males present later in life and lack the classic manifestations of angiokeratoma, acroparesthesias, hypohidrosis, and the characteristic ophthalmologic findings. Among these mildly affected males are the "cardiac variants" who lack the vascular endothelial pathology (4–7) and present in the

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fifth or sixth decade of life with cardiac involvement including left ventricular hypertrophy, cardiomegaly, and conduction abnormalities. They may have proteinuria, but usually do not develop renal failure.

Until recently, the medical management of affected males or symptomatic females with the classic phenotype has consisted of prophylaxis for the acroparesthesias with carbamazepine, diphenylhydantoin, and gabapentin; antihypertensive drugs; and dialysis or renal transplantation for patients experiencing end-stage renal failure. However, enzyme replacement therapy has been shown in recent clinical trials to reverse the disease pathology and markedly improve the well-being and quality of life for classically affected Fabry patients (8–10).

Although the diagnosis of affected males with the classic or variant phenotype can be made reliably by demonstrating the markedly deficient α-Gal A activity in plasma, leukocytes, or cultured cells, the enzymatic identification of heterozygous females is less reliable because of random X-chromosome inactivation (11,12). In fact, the finding of normal α-Gal A activity in an at-risk female does not exclude heterozygosity, and only the presence of an α-Gal A mutation provides precise carrier identification. Thus, molecular testing is required for accurate carrier detection, appropriate genetic counseling, and prenatal diagnosis in affected families.

The isolation and sequencing of the full-length cDNA and entire ~12 kb genomic sequence encoding α-Gal A (13,14) (Genbank X14448) has facilitated characterization of the mutations causing Fabry disease. Of the mutations described to date (Human Gene Mutation Database http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html), most have been unique to each family or "private," with the exception of a few mutations found in several unrelated individuals that occurred at CpG dinucleotides, known hotspots for mutation (15,16).

To investigate the molecular heterogeneity of mutations causing Fabry disease, and to delineate possible phenotype–genotype correlations, mutation analysis of the α-Gal A gene was performed in 40 unrelated Fabry families. In 20 families novel mutations were identified; 18 previously reported mutations were detected in the other families. Patients with early-onset classical disease or a mild variant phenotype were identified, thereby providing additional genotype–phenotype information.

Materials and Methods

Patient Specimens

Thirty-eight affected males and two obligate heterozygotes (probands 4 and 38) from 40 unrelated families with Fabry disease were evaluated. EDTA-anticoagulated whole-blood samples were obtained with informed consent. For each affected male, the enzymatic diagnosis was established by demonstration of deficient α-Gal A activity in plasma and/or peripheral leukocytes.

Mutation Analysis

High-molecular-weight genomic DNA was isolated from leukocytes according to standard procedures. The α-Gal A gene, which consists of seven exons distributed over ~12,500 bp, was PCR- amplified in four amplicons (17) using the oligonucleotide primers listed in Table 1. The amplicons were purified using exonuclease I and shrimp alkaline phosphatase on a RoboAmp 4200 (MWG Biotech, Courtabeuf, France), and each was sequenced with an ABI Prism 3700 Capillary Array Sequencer using the ABI Prism BigDye Terminator Ready Reaction Mix (Perkin-Elmer-Cetus, Norwalk, CT, USA). DNA sequences were analyzed using Navigator 2.0 software (PE Biosystems, Norwalk, CT, USA).

Computer Analysis

Each of the missense lesions was analyzed to determine the relative conservation of the substituted amino acid by comparison with 42 α-Gal A orthologs (27 eukaryotic and 12 prokaryotic) and five α-Gal B orthologs in the GenBank database (http://www.ncbi.nih.gov/Entrez/nucleotide.html).

Table 1. Oligonucleotide primers used for PCR amplification and sequencing of the α-Gal A gene

Amplicon	Exon(s) Amplified	Primer Sequence (5′ → 3′)	Orientation	Genomic Sequence*
1	Promoter and exon 1	AAGCACGCATTTGCCTAGAT	Forward	330–349
		TTTAGGGCTAGTCCTGATTC	Reverse	1526–1545
2	Exon 2	GCATTTTAGGTTGTTCAGTTG	Forward	4715–4735
		ACAGAAGTGCTTACAGTCCTC	Reverse	5301–5321
3	Exons 3 and 4	CCTAGTTTGAACTAGCTCAG	Forward	7059–7078
		GACAGGACATGAATGGTGAA	Reverse	8623–8642
4	Exons 5–7	CTTTAAGGGATTTCAAGTTA	Forward	9930–9949
		TTGAGATGGAATCTTTGTTG	Reverse	11588–11607

*α-Gal A genomic residues numbered according to Kornreich et al. (14).

These searches were performed using the MacVector program (Oxford Molecular Group). Highly conserved residues were defined as those that were present in all three mammalian orthologs, in at least 20 of 27 eukaryotic orthologs, and in 4 of 5 α-Gal B orthologs, except for the lesions that occurred in α-Gal A exon 7, which has little (16%), if any, amino acid identity with the corresponding region of α-Gal B (18).

Results

To identify the α-Gal A mutations in 40 unrelated families with Fabry disease, four amplicons containing the seven α-Gal A exons and their adjacent flanking and intronic regions were PCR-amplified and sequenced. No size abnormalities were detected when the amplicons were analyzed by agarose gel electrophoresis. In contrast, direct automated sequencing of each amplicon detected a single mutation in each Fabry proband, including 20 novel and 18 previously reported mutations (Table 2). The 20 novel mutations included 12 missense and 2 nonsense mutations, 3 small deletions, and 3 small insertions as described below. The previously reported lesions included 12 missense mutations—R49P, D92N, C94Y, R112C (two families), F113S, G183D, R227Q, R301Q, G328R, R342Q, E358K, and P409A—5 nonsense mutations—W162X, R220X, R227X, Q250X, and R301X—and 1 small deletion g10208delAA (two families) (Fig. 1).

As indicated in Table 2, the novel missense mutations included:

1. a G-to-C transversion of genomic nucleotide (g) 1306 in codon 43 of exon 1 (GGC→CGC), replacing a neutral, polar highly conserved (Table 3) glycine with a basic arginine (G43R);
2. a C-to-G transversion of g1324 in codon 49 of exon 1 (CGC→GGC), replacing a basic arginine with a neutral, polar glycine (R49G);
3. a G-to-A transition of g5115 in codon 72 of exon 2 (ATG→ATA), replacing a neutral, polar methionine with an isoleucine (M72I);
4. a G-to-A transition of g7312 in codon 138 of exon 3 (GGA→GAA), substituting the neutral, polar, highly conserved glycine with a glutamic acid (G138E);
5. a G-to-C transversion of g10220 in codon 243 of exon 5 (TTG→TTC), changing a leucine to a phenylalanine (L243F);
6. a C-to-G transversion of g10231 in codon 247 of exon 5 (TCT→TGT), resulting in the replacement of a serine by a cysteine (S247C);
7. a T-to-A transversion of g10289 in codon 266 of exon 5 (GAT→GAA), resulting in the substitution of glutamic acid for a highly conserved aspartic acid (D266E);
8. a G-to-T transversion of g10569 in codon 287 of exon 6 (TGG→TGT), substituting a cysteine for tryptophan (W287C);
9. a C-to-G transversion of g10598 in codon 297 of exon 6 (TCT→TGT), substituting a serine by a cysteine (S297C);
10. a C-to-A transversion of g11043 in codon 355 of exon 7 (AAC→AAA), resulting in the replacement of a neutral, nonpolar asparagine with a neutral, polar lysine (N355K);
11. an A-to-G transition of g11051 in codon 358 of exon 7 (GAG→GGG), replacing a glutamic acid with a glycine (E358G). This mutation differs from the already described E358K (GAG→AAG) allele, which occurs in the same codon (19); and
12. a C-to-T transition of g11203 in codon 409 of exon 7 (CCC→TCC), replacing a neutral, polar

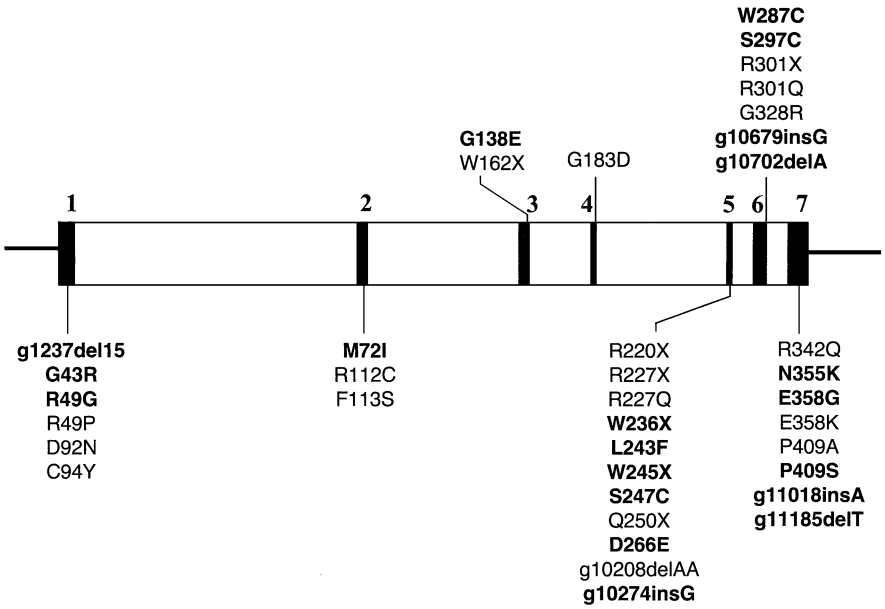

Fig. 1. Distribution of the 20 novel and 18 previously reported mutations in the α-Gal A gene identified in this study. Exons are indicated as solid rectangles and the novel mutations are indicated in bold.

Table 2. α-Gal A mutations causing Fabry disease in 40 unrelated families

Proband	Mutation	Location	Genomic Nucleotide Change	cDNA Codon Change	Age (Yr)	Origin	Phenotype
Novel mutations							
Missense mutations							
1	G43R	Exon 1	g1306 G > C	127 GGC > CGC	22	British	Classic
2	R49G	Exon 1	g1324 C > G	145 CGC > GGC	57	French	Classic
3	M72I	Exon 2	g5115 G > A	216 ATG > ATA	61	French	Classic
4	G138E	Exon 3	g7312 G > A	413 GGA > GAA	56	German	Classic
5	L243F	Exon 5	g10220 G > C	729 TTG > TTC	37	French	Classic
6	S247C	Exon 5	g10231 C > G	740 TCT > TGT	64	French	Variant
7	D266E	Exon 5	g10289 T > A	798 GAT > GAA	31	French	Severe classic
8	W287C	Exon 6	g10569 G > T	861 TGG > TGT	29	British	Classic
9	S297C	Exon 6	g10598 C > G	890 TCT > TGT	20	Belgian	Classic
10	N355K	Exon 7	g11043 C > A	1065 AAC > AAA	16	French	Classic
11	E358G	Exon 7	g11051 A > G	1073 GAG > GGG	41	West Indian	Classic
12	P409S	Exon 7	g11203 C > T	1225 CCC > TCC	38	British	Classic
Nonsense mutations							
13	W236X	Exon 5	g10198 G > A	707 TGG > TAG	32	British	Classic
14	W245X	Exon 5	g10225 G > A	734 TGG > TAG	50	French	Classic
Small deletions							
15	58del15	Exon 1	g1237delGCCCTCG TTTCCTGG	58delGCCCTCG TTTCCTGG	40	Kurdish	Classic
16	CTT AGA CAG*	Exon 6	g10702delA	994delA	19	Iraqi	Classic
17	AGG TTA AGA	Exon 7	g11185delT	1207delT	28	French	Classic
Small insertions							
18	CCA GGG GGG	Exon 5	g10274insG	778insG	29	French	Classic
19	TTG GGG CAA	Exon 6	g10679insG	972insG	21	French	Classic
20	TTA AGC CTG	Exon 7	g11018insA	1041insA	50	British	Classic
Previously reported mutations							
Missense mutations							
21	R49P	Exon 1	g1325 G > C	146 CGC > CCC	24	British	Classic
22	D92N	Exon 1	g5176 G > A	274 GAT > AAT	30	British	Classic
23	C94Y	Exon 1	g5180 G > C	281 TGT > TCT	13	Hispanic	Classic
24	R112C	Exon 2	g5233 C > T	334 CGC > TGC	33	French	Classic
25	R112C	Exon 2	g5233 C > T	334 CGC > TGC	47	French	Classic
26	F113S	Exon 2	g5237 T > C	338 TTT > TCT	47	British	Classic
27	G183D	Exon 4	g8321 G > A	548 GGT > GAT	31	French	Classic
28	R227Q	Exon 5	g10171 G > A	680 CGA > CAA	39	British	Classic
29	R301Q	Exon 6	g10610 G > A	902 CGA > CAA	60	French	Intermediate
30	G328R	Exon 6	g10690 G > A	982 GGG > AGG	26	Arab	Classic
31	R342Q	Exon 7	g11003 G > A	1025 CGA > CAA	33	French	Classic
32	E358K	Exon 7	g11050 G > A	1072 GAG > AAG	39	French	Classic
33	P409A	Exon 7	g11203 C > G	1225 CCC > GCC	29	British	Classic
Nonsense mutations							
34	W162X	Exon 3	g7384 G > A	485 TGG > TAG	39	British	Classic
35	R220X	Exon 5	g10149 C > T	658 CGA > TGA	36	British	Classic
36	R227X	Exon 5	g10170 C > T	679 CGA > TGA	59	French	Classic
37	Q250X	Exon 5	g10239 C > T	748 CAG > TAG	53	French	Classic
38	R301X	Exon 6	g10609 C > T	901 CGA > TGA	48	French Canadian	Classic
Small deletions							
39	AGT ATA AAG AGT	Exon 5	g10208delAA	717delAA	50	French	Classic
40	AGT ATA AAG AGT	Exon 5	g10208delAA	717delAA	20	French	Classic

*Inserted or deleted bases underlined.

proline with a neutral, nonpolar serine (P409S).

The relative conservation during evolution of the amino acids substituted by the missense mutations is indicated in Table 3.

The two novel nonsense mutations included:

1. a G-to-A transition of g10198 in codon 236 of exon 5 (TGG to TAG), predicting a termination signal instead of a tryptophan (W236X), and deletion of 194 residues; and

Table 3. Conservation of human α-Gal A missense mutations in α-Gal A and α-Gal B orthologues

			α-Gal A Orthologs			α-Gal B Orthologs	
Proband	Exon	Mutation	Prokaryotic (12)	Eukaryotic (27)	Mammalian (3)	Eukaryotic (5)	Conservation*
1	1	G43R	5	22	3	5	+++
2	1	R49G	1	1	3	4	+
3	2	M72I	0	0	3	4	+
4	3	G138E	3	24	3	5	+++
5	5	L243F	0	4	3	3	—
6	5	S247C	1	3	1	0	—
7	5	D266E	5	22	3	5	+++
8	6	W287C	7	25	3	5	+++
9	6	S297C	2	2	3	5	+
10	7	N355K	2	23	3	2	+++
11	7	E358G	3	1	3	1	—
12	7	P409S	2	6	3	5	+

*For the degree of evolutionary conservation:—, minimal conservation; +, conserved among all three α-Gal A mammalian orthologs and four of five α-Gal B eukaryotic orthologues, with the exception of the α-Gal A exon 7 mutations (18); ++, additionally or alternately conserved among 14–23 of α-Gal A eukaryotic orthologs; +++, conserved among all three α-Gal A mammalian orthologs and five α-Gal B eukaryotic orthologs, and at least 22 α-Gal A eukaryotic orthologues.

2. a G-to-A transition of g10225 in codon 245 of exon 5 (TGG to TAG), substituting a tryptophan codon by a termination signal (W245X), and deletion of 184 residues.

Six novel small gene rearrangements, including two single nucleotide insertions and three deletions were identified in six unrelated classically affected probands (Table 2). The three small insertions were

1. a single nucleotide insertion after g10273 (g10273insG) that caused a frameshift after codon 259, altered residues 262 and 263, and then introduced a termination signal at codon 264;
2. a single nucleotide insertion after g10682 (g10682insG) that caused a frameshift after codon 224, altered residues 326 to 331, and prematurely terminated the polypeptide after residue 332; and
3. a single nucleotide insertion after g11018 (g11018insA) that caused a frameshift after codon 347, altered residues 348–373, and then introduced a termination signal at codon 374.

The three novel gene deletions included:

1. an in-frame 15-bp deletion in exon 1 (58del15) that resulted in the deletion of amino acids 20 to 24 (ALVSW) from the 31 residue leader sequence;
2. a single nucleotide deletion (g11185delT) at codon 403, predicting a frameshift and premature termination in that codon; and
3. a single base deletion (994delA) of g10702 that introduced a frameshift after codon 331, altered residues 332–346, and then introduced a termination signal at codon 347.

Discussion

Sequencing of the α-Gal A gene in 40 unrelated families with Fabry disease revealed 20 novel and 18 previously reported mutations. Each of the 20 novel mutations was identified in a single family, demonstrating the extensive molecular heterogeneity in this disease. The novel mutations were dispersed along the gene, and included 12 missense and 2 nonsense point mutations, 3 small insertions, and 3 small deletions, all in the coding region. No novel mutation occurred at a CpG dinucleotide, whereas 7 (R112C, R220X, R227X, R227Q, R301X, R301Q, R342Q) of the 18 previously reported mutations occurred at CpG dinucleotides, known hotspots for mutation. Of the 12 novel missense mutations, only 5 (G43R, G138E, D266E, W287C, and N355K) occurred at highly conserved residues (Table 3). Notably, among these mutations was D266E, which substituted a highly conserved aspartate with an isofunctional glutamate. The aspartate must be essential for enzyme activity (or stability) because substitution of the same charged, but slightly longer, glutamate residue caused Fabry disease.

Of the six novel gene rearrangements, exons 6 and 7 each had two. The identification of these small deletions and insertions supports the previous suggestion that exons 6 and 7 are regions prone to small gene rearrangements (20,21). Two of the three small insertions involved the addition of an extra G to a

small series of Gs (5 in 778insG and 3 in 972insG), presumably due to DNA replication errors. The 58del15 mutation, which eliminated five amino acids in the leader sequence, resulted in an in-frame deletion of 15 bp, presumably due to slipped mispairing during replication between 7-bp direct repeats (TTCTGG) such that one direct repeat and the intervening sequence were deleted. The five deleted residues were in the central hydrophobic core of the leader. When evaluated by the von Heijne algorithm (22), loss of these residues altered the most likely leader sequence cleavage site from the normal site after residue 31 to several sites in exon 7. Thus, the polypeptide may not be targeted to the endoplasmic reticulum, or if it is, it would be miscleaved.

These studies provided additional genotype–phenotype information for Fabry disease. Most of the affected males described had the classic phenotype. Although the age of onset and/or severity of the manifestations varied, these patients presented with angiokeratoma, hypohidrosis, and acroparesthesias in childhood, and developed renal insufficiency, cardiac, or cerebrovascular disease in the fourth or fifth decades of life. Notably, several variants were identified. Proband number 7 with the D266E mutation progressed to renal failure early; he began dialysis at the age of 15 years, and at 16 years received a kidney allograft, which has remained functional for 15 years. Only a few other affected males whose genotypes were not reported have experienced early renal failure (23,24). Although the aspartic acid to glutamic acid substitution at residue 266 is isofunctional, the aspartate is highly conserved in evolution, being present among all the higher eukaryotes, the majority (19 of 24) of the lower eukaryotes, and all of the α-Gal B orthologs (Table 3). In contrast, the affected male with the S247C mutation had a milder variant phenotype. He did not have the classic disease manifestations (acroparesthesia, angiokeratoma, hypohidrosis, or corneal dystrophy). Instead, he presented at age 64 with mild proteinuria and moderate renal insufficiency (serum creatinine, 1.7 mg/dl; glomerular filtration rate by EDTA-^{51}Cr, 41.2 ml/min/1.73 m^2; normal, 63–119 ml/min/1.73 m^2). He had low, but detectable, plasma and leukocyte α-Gal A levels, which were consistent with his mild phenotype. These findings suggested that he has an intermediate phenotype, perhaps similar to the recently described renal variant (Nakao et al., in review). Of note, the serine at residue 247 is not evolutionarily conserved, only being present in the spider monkey, three lower eukaryotes, and the bacterium *Thermotoga maritima* (Table 3).

Among the previously reported mutations detected in this study, it is interesting to note that the R301Q mutation was initially identified in a patient with the cardiac variant (25). However, the patient reported here developed renal insufficiency and received a kidney transplant at age 59 (6). This mutation apparently produces a small amount of residual activity, and presumably its synthesis and/or stability are subject to variation. A similar clinical course was reported for an unrelated classically affected male (26) and a mildly affected male with an "intermediate" phenotype (8) who had the R301Q mutation and developed renal failure.

Among mutation detection methods, single-strand conformational polymorphism (27) and chemical cleavage of mismatches (28) have been used effectively to detect α-Gal A mutations. However, DNA sequencing remains the "gold standard" for mutation detection. With the improvement in both sequencing chemistries and attendant softwares for mutation detection, direct automated DNA sequencing has now become even more practical for routine mutation detection involving genes with a moderate number of exons, like α-Gal A (20,26,29).

In summary, the identification of 20 novel α-Gal A mutations increased our understanding of the molecular heterogeneity and the genotype/phenotype correlations in Fabry disease. In addition, these studies permit precise carrier detection, genetic counseling, and prenatal diagnosis in these 40 families with this X-linked lysosomal disease.

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