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Identification of Point Mutations in the α-Galactosidase A Gene in Classical and Atypical Hemizygotes with Fabry Disease

Hitoshi Sakuraba,* Akihiro Oshima,* Yukiko Fukuhara,* Michie Shimmoto,* Yoshiro Nagao,* David F. Bishop,† Robert J. Desnick,† and Yoshiyuki Suzuki*

*Department of Clinical Genetics, Tokyo Metropolitan Institute of Medical Science, Tokyo; and †Division of Medical and Molecular Genetics, Mount Sinai School of Medicine, New York

Summary

Efforts were directed to identify the specific mutations in the α-galactosidase A (α-Gal A) gene which cause Fabry disease in families of Japanese origin. By polymerase-chain-reaction-amplification of DNA from reverse-transcribed mRNA and genomic DNA, different point mutations were found in two unrelated Fabry hemizygotes. A hemizygote with classic disease manifestations and no detectable α-Gal A activity had a G-to-A transition in exon 1 (codon 44) which substituted a termination codon (TAG) for a tryptophan codon (TGG) and created an *Nhe*I restriction site. This point mutation would predict a truncated α-Gal A polypeptide, consistent with the observed absence of enzymatic activity and a classic Fabry phenotype. In an unrelated Japanese hemizygote who had an atypical clinical course characterized by late-onset cardiac involvement and significant residual α-Gal activity, a G-to-A transition in exon 6 (codon 301) resulted in the replacement of a glutamine for an arginine residue. This amino acid substitution apparently altered the properties of the enzyme such that sufficient enzymatic activity was retained to markedly alter the disease course. Identification of these mutations permitted accurate molecular heterozygote diagnosis in these families.

Introduction

Fabry disease is an X-linked recessive disorder caused by the deficient activity of the lysosomal hydrolase, α-galactosidase A (α-Gal A; E.C.3.2.1.22) (Desnick and Bishop 1989). In affected hemizygotes, the accumulation of glycosphingolipids with terminal α-linked galactosyl moieties, predominantly globotriaosylceramide, occurs in various tissues and body fluids (Sweeley and Klionsky 1963). This leads to the major disease manifestations, including acroparethesias, angiokeratoma, hypohidrosis, corneal opacities, and progressive vascular disease of the kidney, heart, and central nervous system. Classically affected males have no detectable α-Gal A activity, while atypical variants, who have milder phenotypes, have residual α-Gal A activity (Clark et al. 1971; Romeo et al. 1975; Bishop et al. 1981; Bach et al. 1982; Kobayashi et al. 1985). Although most heterozygous females are asymptomatic, about 70% have the Fabry keratopathy, and some have mild clinical manifestations (Desnick and Bishop 1989). The enzymatic diagnosis of heterozygotes by intermediate levels of α-Gal A activity is unreliable because of random X-chromosome inactivation (Lyon 1961; Rietra et al. 1976; Desnick et al. 1987).

The recent isolation of both the full-length cDNA and the entire genomic sequence encoding α-Gal A has provided fundamental information concerning the structure of the α-Gal A gene and enzyme protein (Bishop et al. 1986, 1988*a*, 1988*b*; Kornreich et al. 1989). The 12-kb genomic sequence has seven exons with a size range of 92–291 bp and introns of 0.2–3.7 kb. The full-length 1,397-bp α-Gal A cDNA encodes a precursor peptide of 429 amino acids, including a 31-residue signal peptide. The precursor is cotranslationally glycosylated and then modified in the Golgi

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Address for correspondence and reprints: Hitoshi Sakuraba, M.D., Department of Clinical Genetics, Tokyo Metropolitan Institute of Medical Science, 3–18–22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

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apparatus and lysosomes to the 398-residue mature polypeptide which dimerizes to form the 101-kD active lysosomal glycoprotein. Studies of 130 unrelated Fabry families by Southern hybridization analysis identified six gene rearrangements, including five partial deletions and one partial duplication (Bernstein et al. 1989). Each of the rearrangement breakpoints has been sequenced (Kornreich et al. 1990). In addition, amplification of an α-Gal A genomic sequence revealed in codon 356 the first exonic point mutation, which altered the kinetic and physical properties of the enzyme and caused a variant form of the disease (Bernstein et al. 1989).

Since it would be useful to identify mutations that cause the classic and/or atypical disease phenotypes and since molecular detection of potential heterozygotes would be valuable for accurate genetic counseling, efforts have been directed to determine the specific α-Gal A mutations in unrelated Fabry families. In the present paper, we report two exonic point mutations in Fabry families of Japanese origin. Of particular interest, the absence of α-Gal A activity in a hemizygote with classical disease manifestations was due to a base substitution which introduced a termination codon and resulted in the early truncation of the enzyme's polypeptide chain, whereas the residual α-Gal A activity in an unrelated atypical hemizygote with a markedly milder phenotype was caused by an amino acid substitution in residue 301 of the enzyme subunit. In both families, the identification of the specific point mutation permitted molecular heterozygote detection.

Material and Methods

Patients

Hemizygote 1 (family O), a 32-year-old Japanese male, had a classic disease course with acroparethesias, angiokeratoma, hypohidrosis, and corneal opacities since 10 years of age. As an adult, he experienced progressive renal deterioration and moderate hypertrophy of the left ventricle.

Hemizygote 2 (family M), a 52-year-old Japanese male, was found to have an electrocardiogram abnormality with hypertrophy of both the ventricular septum and the left ventricle when he was evaluated for chest pain at the age of 52 years. Biopsy of the left-ventricular endocardium revealed vacuolar degeneration of myocardial cells and cytoplasmic lamellar inclusion bodies. He had no other signs or symptoms of Fabry disease. Screening of various lysosomal enzymes revealed a specific decrease of α-Gal A activity in cultured lymphoblasts (hemizygote 2, 3.3 nmol/h/mg protein; classic Fabry hemizygotes, 0.4 ± 0.1 nmol/h/mg protein [n = 13]; normal control values, 92.0 ± 21.7 nmol/h/mg protein (n = 10]).

Preparation of DNA, Poly(A)$^+$ RNA, and Synthetic Oligonucleotides

Genomic DNA was isolated from cultured lymphoblasts (Aldridge et al. 1984). Total RNA was isolated from cultured lymphoblasts by using the guanidine isothiocyanate procedure, and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography (Maniatis et al. 1982). Table 1 lists the oligonucleotide primers for cDNA amplification (oligonucleotides 1–6), genomic amplification (oligonucleotides 7–10) and dideoxy sequencing (oligonucleotides 11–16), which were synthesized with an Applied Biosystems DNA synthesizer Model 381 (Foster City, CA).

Southern and Northern Hybridization Analyses

Genomic DNA (5 μg) was digested with *Sac*I, *Taq*I, *Msp*I, *Pvu*II, *Hin*dIII, or *Nhe*I (New England Biolabs, Beverly, MA), and aliquots were electrophoresed in 1% agarose gels, were transferred to a nylon membrane (Hybond-N; Amersham, Buckinghamshire, England) by the method of Southern (1975), and were analyzed with the ^{32}P-labeled α-Gal A cDNA according to a method described by Bernstein et al. (1989). Northern hybridization analysis was performed with total RNA according to a method described by Maniatis et al. (1982) by using the ^{32}P-labeled human α-Gal A cDNA as probe. After autoradiography the filter was washed with 0.5% SDS in water at 95°C for 10 min, and a second hybridization was done with the ^{32}P-labeled human β-galactosidase cDNA (Oshima et al. 1988) as a control. The densities of the autoradiographic signals were determined using a Molecular Dynamics 300A Scanning Imager (Sunnyvale, CA).

First-Strand cDNA Synthesis and cDNA Amplification

Three overlapping regions of the α-Gal A transcript were synthesized using an Amersham cDNA Synthesis System Plus kit according to the manufacturer's instructions. For each synthesis, poly(A)$^+$ RNA (2 μg) was mixed at 4°C first with an antisense oligonucleotide primer (oligonucleotide 1, 2, or 3; 0.5 μg each) in a total volume of 18 μl and then with avian myeloblastosis virus reverse transcriptase (2 μl, 30 units/μl; Stratagene, La Jolla, CA. The mixture was incubated at 42°C for 90 min. The single-stranded α-Gal A cDNA fragments were converted to double-stranded DNA and then were amplified in vitro by the polymerase chain

Table 1

Oligonucleotides Used for α-Gal A cDNA Synthesis, cDNA and Genomic Amplification, and Sequencing

Oligonucleotide Number	Sequence[a]	Orientation[b]	cDNA (or genomic) Coordinates[c]
1	5′-TGAG*AATTCCAAAACTCCCAGGGAA-3′	−	433 to 450
2	5′-TGAG*AATTCCAGCTGAGGCCAAAG-3′	−	816 to 834
3	5′-TGAG*AATTCTTAAAGTAAGTCTTTTAATG-3′	−	1271 to 1290
4	5′-CCGG*TCGACAATTTATGCTGTCCGGT-3′	+	−11 to −27
5	5′-CCGG*TCGACTGAAGCTAGGGATTT-3′	+	383 to 400
6	5′-CCGG*TCGACCCAGATATGTTAGTG-3′	+	790 to 807
7	5′-CCGG*TCGACGGTTAATCTTAAAAGCCC-3′	+	−42 to −59
8	5′-TGAG*AATTCCTGATGCAGGAATCTGGC-3′	−	177 to 194
9	5′-CCGG*TCGACTGGGTCATCTAGGTAACT-3′	+	(1441) to (1458)
10	5′-TGAG*AATTCTGATAGTAACATCAAGA-3′	−	(10792) to (10809)
11	5′-TTCTGAGACCATGAGCTC-3′	−	220 to 237
12	5′-GAGCTCATGGTCTCAGAA-3′	+	220 to 237
13	5′-TATAAAGAGGCCACTCAC-3′	−	605 to 622
14	5′-GTGAGTGGCCTCTTTATA-3′	+	605 to 622
15	5′-TACAGCCCAGGCTAAGCC-3′	−	1036 to 1053
16	5′-GGCTTAGCCTGGGCTGTA-3′	+	1036 to 1053

[a] Asterisks denote *SalI* or *Eco*RI restriction-cleavage sites.
[b] Indicated as sense (+) or antisense (−).
[c] As numbered for sequences of full-length α-Gal A cDNA (Bishop et al. (1988*b*) and 12-kb gene (Kornreich et al. 1989).

reaction (PCR) by using the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer–Cetus, Norwalk, CT) and appropriate oligonucleotide primers, which were constructed so that *SalI* and *Eco*RI sequences would be present at the 5′ and 3′ ends of the PCR products, respectively (table 1). These primers were designed to amplify the entire α-Gal A coding region in three overlapping fragments: the 5′ region (oligonucleotides 1 and 4 for nucleotides −27 to 450), the center region (oligonucleotides 2 and 5 for nucleotides 383 to 834), and the 3′ region (oligonucleotides 3 and 6 for nucleotides 790 to 1290). PCR was performed according to the method of Saiki et al. (1988); each 100-μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, template DNA (2 μl reverse transcriptase reaction mixture), 1 μg each of the appropriate sense and antisense PCR primers, 0.1 mM of each dNTP, and 5 units *Taq* DNA polymerase (Perkin Elmer–Cetus). The reaction was amplified for 30 cycles by denaturing at 94°C for 1.5 min, annealing at 55°C for 2 min, and extending at 72°C for 4 min. The aqueous layer was removed, the PCR product was extracted with phenol/chloroform and was precipitated with ethanol, and the pellet was resuspended in 20 μl of TE buffer (10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA). The amplified product was separated by agarose gel electrophoresis and was electroeluted to an NA45 DEAE-cellulose membrane (Schleicher and Schuell, Dassel, West Germany).

Cloning and Sequencing of Amplified cDNA

The PCR products from the three overlapping α-Gal A coding regions from each hemizygote were digested with *Eco*RI and *SalI* (New England Biolabs) and then were subcloned into M13mp18 and M13mp19 vectors. To eliminate possible PCR errors, six clones for each of the three amplified α-Gal A regions were sequenced individually by the dideoxy chain-termination method (Sanger et al. 1977) by using an M13 sequencing system (Takara Shuzo Co., Kyoto, Japan) with universal and unique α-Gal A primers (oligonucleotides 11–16).

Amplification and Sequencing of Genomic DNA

To confirm the α-Gal A point mutations identified in the PCR-amplified reverse-transcribed mRNAs from the Fabry hemizygotes, genomic DNA (2 μg) was PCR amplified using sense and antisense primers for exon 1 (oligonucleotides 7 and 8) and for exon 6 (oligonucleotides 9 and 10), respectively. The conditions for PCR amplification and sequencing of the PCR products were as described above.

Results

Southern and Northern Hybridization Analyses

In 20 unrelated Fabry hemizygotes of Japanese origin, Southern hybridization analysis with six restriction endonucleases did not reveal any gross abnormalities in gene structure. In contrast, northern hybridization analysis of the α-Gal A transcript revealed that 11 (55%) had mRNAs of normal size (~1.4 kb) and amount. These findings suggested that the mutations in these unrelated hemizygotes were due to either point mutations or small deletions or insertions. Among these 11 unrelated affected hemizygotes were hemizygotes 1 and 2, who had classic and atypical disease phenotypes, respectively. To investigate the nature of the mutations responsible for the classic and atypical phenotypes, efforts were undertaken to reverse transcribe, PCR amplify, and sequence their respective α-Gal A transcripts.

cDNA Sequence Analysis

The PCR products of the three overlapping regions of the reverse-transcribed α-Gal A from the classic and atypical hemizygotes were of the expected size as assessed by agarose gel electrophoresis. Sequencing of six clones from each of the three overlapping α-Gal A cDNA regions revealed a single unique base substitution in each hemizygote. The classic hemizygote had in codon 44 a G-to-A transition which changed a tryptophan codon (TGG) to a termination codon (TAG). In hemizygote 2 with the atypical phenotype, a G-to-A transition in codon 301 resulted in an arginine-to-glutamine substitution.

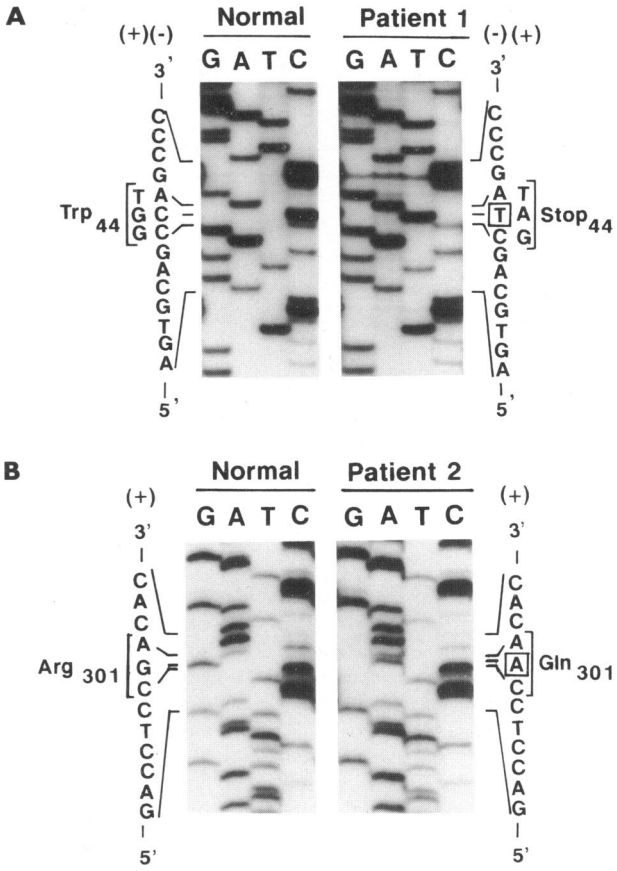

Figure 1 Partial sequence of the amplified α-Gal A genomic DNA, confirming the point mutations (*A*) in exon 1 in hemizygote 1 and (*B*) in exon 6 in hemizygote 2. The sequence from a normal individual is shown in the left gels, and those from hemizygotes 1 and 2 are shown in the right gels.

Genomic DNA Sequence Analysis

To confirm the presence of these mutations in genomic DNA, the exonic regions which contain the predicted mutations were amplified, and the exon 1 PCR product (271 bp) and the exon 6 PCR product (385 bp) were subcloned and six clones of each were sequenced. The results confirmed that the predicted base changes were present in the α-Gal A genes of the respective hemizygotes (figs. 1*A* and 1*B*).

Since the G-to-A base substitution in codon 44 created a new *Nhe*I restriction-endonuclease site in exon 1, hemizygote and heterozygote detection in this family can be accomplished by Southern hybridization analyses using the α-Gal A cDNA as probe. As shown in figure 2, the α-Gal A gene in normal males contains two 9.7-kb *Nhe*I fragments (Kornreich et al. 1989), whereas the Southern blot of the affected hemizygote had only one 9.7-kb fragment and the 5.8- and 3.9-kb fragments resulting from the new *Nhe*I site. The sister of hemizygote 1 had a low normal α-Gal A activity in cultured lymphoblasts and other sources (data not shown), but Southern analysis clearly demonstrated that she did not have the *Nhe*I restriction site generated by the point mutation.

In contrast, the G-to-A transition in α-Gal A codon 301 of atypical hemizygote 2 neither created nor obliterated a known restriction site. Therefore, heterozygote detection could be accomplished either by sequencing the PCR-amplified product from the genomic region containing this mutation or by dot-blot analyses of the amplified genomic region by using normal and mutation-specific oligonucleotides. In the daughter of the atypical hemizygote, both normal and mutant sequences

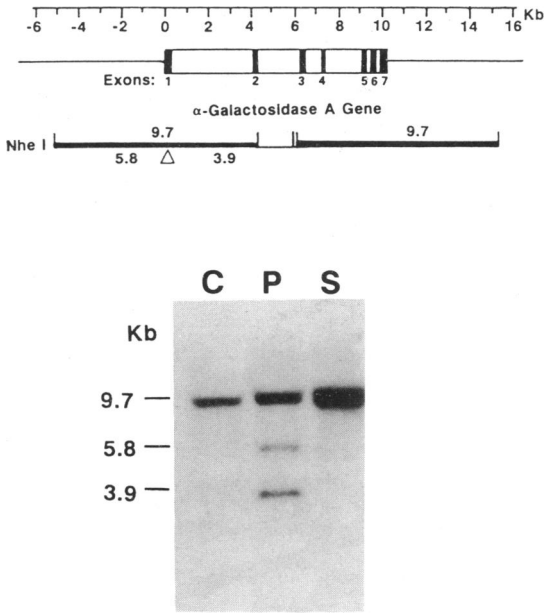

Figure 2 Schematic diagram of the α-Gal A gene, showing the positions of the seven exons and the *Nhe*I restriction sites with exon-containing fragments shown in boldface. In hemizygote 1, the G-to-A creates an *Nhe*I site in exon 1. Southern analysis of DNA from hemizygote 1 (P), the sister (S), and a normal male (C). Note that the 5.8- and 3.9-kb *Nhe*I fragments were derived from the 5′ 9.7-kb *Nhe*I fragment in genomic DNA from hemizygote 1.

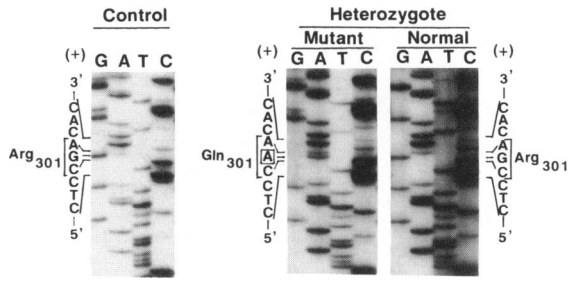

Figure 3 Partial sequence of PCR-amplified genomic DNA from an obligate family M heterozygote. The daughter of hemizygote 2 had both normal and mutant sequences in individual subclones.

were found in individual subclones of amplified genomic DNA, consistent with the fact that she is an obligate heterozygote for Fabry disease (fig. 3).

Discussion

Analysis of the molecular lesions in the α-Gal A gene of the classical and mildly affected Fabry hemizygotes revealed nonsense and missense mutations, respectively. In hemizygote 1 with the classic phenotype, a G-to-A substitution was detected in PCR-amplified cDNA and was confirmed by sequencing the appropriate genomic region. This base change converted a TGG codon for tryptophan to a TAG termination codon. This nonsense mutation, which was the only base change in the entire cDNA, predicted the early truncation of the α-Gal A polypeptide, consistent with the finding of undetectable α-Gal A activity in various cellular sources from this hemizygote. In addition, the creation of an *Nhe*I restriction site by this point mutation was predicted and confirmed in genomic DNA from the affected hemizygote. Southern hybridization analysis for the presence or absence of the *Nhe*I site in an at-risk sister demonstrated that the molecular diagnosis of heterozygotes could be made in this family. Such analyses also can be accomplished conveniently either by dot-blot analyses of amplified genomic DNA by using allele-specific oligonucleotides or, even more easily, by electrophoresis of *Nhe*I-restricted genomic PCR products.

In the atypical variant who had residual α-Gal A activity, a G-to-A transition in codon 301 resulted in an amino acid substitution of a glutamine for an arginine. This base change was the only abnormality detected in the entire coding sequence and was confirmed by sequencing the PCR-amplified genomic region from hemizygote 2. Further characterization of the residual α-Gal A activity resulting from this mutation will require expression, in eukaryotic expression systems (Bishop et al. 1988*b*), of sufficient enzyme for purification and characterization of the altered enzyme's physical and kinetic properties. In this family, sequencing of the amplified genomic DNA region successfully detected an obligate heterozygote. Heterozygotes for this mutation could be detected by dot-blot analyses using allele-specific oligonucleotides.

The identification of these two point mutations in the α-Gal A gene causing Fabry disease in Japanese hemizygotes can be added to the two previously known mutations: in codon 356 of exon 7, a C-to-T transition which caused an arginine-to-tryptophan substitution (Bernstein et al. 1989) and, in codon 40 of exon 1, a C-to-T transition which results in a serine replacing a proline residue (Koide et al. 1990). Of these four α-Gal A base substitutions, it is notable that two (in codons 301 and 356) occur at CpG dinucleotide sites, which are known hot spots for point mutations (Bernstein et al. 1989). Further identification of point mutations in the α-Gal A gene which cause Fabry disease and careful documentation of the disease course and severity in affected hemizygotes will provide genotype-phenotype correlations for this clinically variable lysosomal

storage disease, as well as insights into the structure-function relationships in this lysosomal housekeeping gene. The identification of these heterogeneous mutations will permit molecular heterozygote diagnosis and appropriate genetic counseling in these families.

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