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Short Communication

TWO NOVEL MUTATIONS IN THE α-GALACTOSIDASE GENE IN JAPANESE CLASSICAL HEMIZYGOTES WITH FABRY DISEASE

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Summary Four α-galactosidase gene mutations were identified in Japanese male patients with Fabry disease who had no detectable α-galactosidase activity. Two of them were novel mutations, an 11-bp deletion in exon 2 and a g^{-1} to t substitution at the 3′ end of the splice acceptor site in intron 1. The former caused a frameshift and led to the creation of a new stop codon at codon 118. The latter was predicted to provoke aberrant mRNA splicing followed by accelerated degradation of the mRNA. A nonsense mutation, R301X, and a 2-bp deletion starting at nucleotide position 718, which were reported previously, were also identified in unrelated patients.

Key Words Fabry disease, α-galactosidase, gene mutation, partial deletion, splice site mutation

Introduction

Fabry disease is an X-linked recessive disorder caused by a genetic defect of lysosomal α-galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22; α-Gal). α-Gal catalyses the hydrolysis of the α-linked galactose residues in the non-reducing terminals of glycosphingolipids (*e.g.* predominantly globotriaosylceramide). In Fabry disease, deficient α-Gal activity results in the progressive accumulation

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of natural substrates in various tissues, mainly in lysosomes of the vascular endothelium. The major clinical manifestations in hemizygotes with no detectable α-Gal activity include pain in the distal extremities and acroparesthesia, angiokeratoma, hypohidrosis, corneal opacity in childhood, and progressive vasculopathy of the kidneys, heart, and central nervous system (Desnick *et al.*, 1995). Recently, atypical hemizygotes with residual α-Gal activity and progressive cardiomyopathy of late onset were reported (Sakuraba *et al.*, 1990; von Scheidt *et al.*, 1991; Nagao *et al.*, 1991; Nakao *et al.*, 1995).

The α-Gal gene, which is located at Xq22, is approximately 12-kbp long and consists of 7 exons, ranging from 92-bp to 291-bp in length (Bishop *et al.*, 1988; Kornreich *et al.*, 1990). These exons are suitable for amplification by means of the polymerase chain reaction (PCR) with intronic oligonucleotide primers. So far, more than 80 α-Gal gene mutations have been identified in patients with Fabry disease (Sakuraba *et al.*, 1990, 1992; Fukuhara *et al.*, 1990; Koide *et al.*, 1990; Yokoi *et al.*, 1991; Ishii *et al.*, 1991, 1992; von Scheidt *et al.*, 1991; Eng *et al.*, 1993, 1994; Davies *et al.*, 1993, 1994; Ploos van Amstel *et al.*, 1994; Okumiya *et al.*, 1995a, b) including at least 27 Japanese patients. These mutations were heterogeneous, and about half of them were point mutations causing single amino acid substitutions. In this communication, sequencing analysis was performed with amplified genomic DNA including an exon and small portion of the adjacent intron sequence. Two novel mutations and two reported mutations were identified in Japanese male patients with Fabry disease.

Patients

The patients were unrelated Japanese Fabry hemizygotes, and their clinical manifestations are briefly summarized in Table 1. All patients had the classical form of the disease, α-Gal activity being deficient in leukocytes.

Materials and Methods

Cell culture and isolation of genomic DNA and poly(A)$^+$RNA. Lympho-

Table 1. Clinical summary of the patients examined in this study.

Patient no.	1	2	3	4
Sex	Male	Male	Male	Male
Age (year)	47	25	42	25
Pain/Acroparesthesia	+	+	+	+
Angiokeratoma/Telangiectasis	+	+	+	+
Hypohidrosis	NE	+	+	+
Proteinuria	+	NE	+	+
Cardiac involvement	+	+	+	+
α-Galactosidase activity[a]	<1	<1	<1	<1

[a] Enzyme activity in leukocytes was expressed in munits/mg protein; normal control (mean$\pm$SD), 98.6$\pm$27.3, n=37. NE, not examined.

blast lines were established by transformation with Epstain-Barr virus, and cultured at 37°C under 5% CO_2 in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum and antibiotics. Genomic DNA and poly(A)$^+$RNA were isolated from the cultured cells by the standard techniques (Sambrook *et al.*, 1989).

Amplification and sequencing of genomic DNA. The seven exons that encode the entire α-Gal mRNA and their flanking sequences were amplified by PCR using seven sets of intronic oligonucleotide primers under appropriate PCR conditions for each exon (Takenaka *et al.*, 1996). The PCR products were subcloned with a pGEM-T Vector System (Promega, Madison, WI, USA). Nucleotide sequences were determined with a DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). At least two clones were sequenced for each patient, and both forward and reverse strand sequencing reactions were carried out for each clone.

First-strand cDNA synthesis and α-Gal cDNA amplification. The first-strand cDNA was synthesized using First-strand cDNA Synthesis Kit (Amersham,

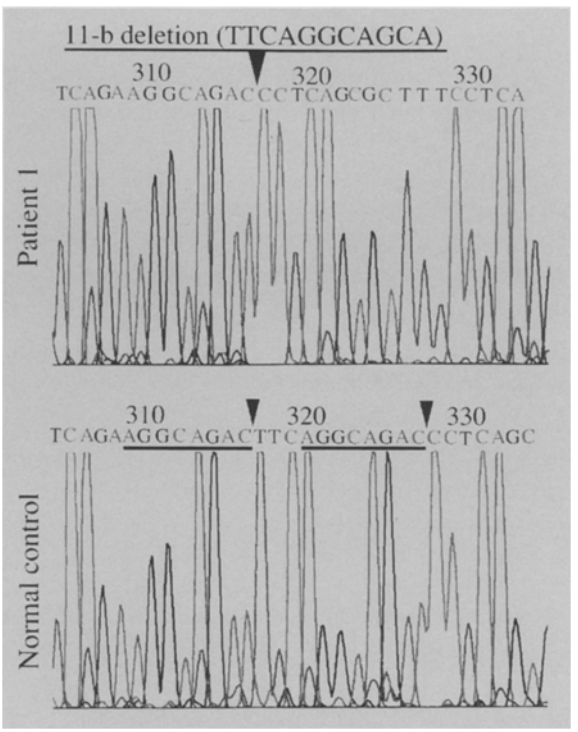

Fig. 1. Nucleotide sequence showing an 11-base deletion in the α-Gal gene (Patient 1). The 11-bp deletion is indicated by the arrowheads. The 8-base direct repeat sequences are underlined. The numbers indicate nucleotide position according to Bishop *et al.* (1988).

Buckinghamshire, UK) according to the manufacturer's instructions. For reverse transcription, 1.0 μg of poly(A)$^+$RNA was mixed with 8 pmol of oligo(dT) primer and 15 units of avian myeloblastosis virus reverse transcriptase in a total volume of 20 μl. Then the mixture was incubated at 42°C for 40 min. The single-stranded cDNA was converted to double-stranded cDNA, and α-Gal cDNA fragment comprising exon 1–4 and a part of exon 5 was amplified by PCR using GeneAmp DNA Amplification Reagent Kit (Perkin Elmer-Cetus, Norwalk, CT, USA) with a set of oligonucleotide primers; 5′-TGAGGATCCTTTATGCTGTCCGGTC-AC-3′ for sense primer, 5′-TGAGAATTCTGGTCCAGCAACATCAACAA-3′ for antisense primer. PCR was performed with 5 μl of the reverse transcriptase reaction mixture as a template DNA according to the manufacturer's instructions. The reaction was carried out in 100 μl of reaction mixture containing 0.5 μg of each PCR primer, and 2.5 units of *Taq* DNA polymerase (AmpliTaq; Perkin Elmer-Cetus). The conditions for thermal cycling with a DNA Thermal Cycler (Perkin Elmer-Cetus) were; 35 cycles of denaturing for 1 min at 94°C, annealing for 2 min at 56°C and extension for 3 min at 72°C. The PCR product was subjected to

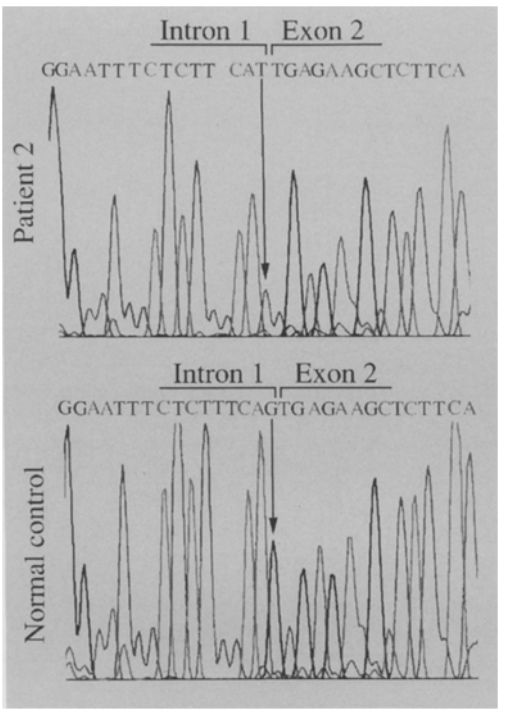

Fig. 2. Nucleotide sequence showing a single base substitution at the boundary between intron 1 and exon 2 in the α-Gal gene (Patient 2). A g^{-1} to t substitution is indicated by the arrows.

agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light.

Northern hybridization analysis of α-Gal mRNA. Northern hybridization analysis was performed with poly(A)$^+$RNA as a sample according to the standard method (Sambrook *et al.*, 1989). Five micrograms of poly(A)$^+$RNA was electrophoretically separated in a formaldehyde-agarose gel, transferred to a nylon membrane (Hybond-N; Amersham), and then hybridized with an entire α-Gal cDNA probe labeled with [α-^{32}P]dCTP. After washing of the filter with 10 mM Tris-HCl (pH 7.4) at 95°C, second hybridization was performed with an [α-^{32}P]-dCTP-labeled β-actin cDNA probe as a sampling control.

Assaying of α-Gal activity and protein determination. α-Gal activity was assayed with an artificial substrate, 4-methylumbelliferyl α-D-galactopyranoside (Nacalai Tesque, Kyoto, Japan) as described previously (Mayes *et al.*, 1981). One unit of enzyme activity was defined as 1.0 μmol 4-methylumbelliferone released per hour at 37°C. Protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Results and Discussion

In this communication, we performed gene analysis on four Japanese male patients with Fabry disease, who had been newly diagnosed on the basis of clinical manifestations and leukocyte α-Gal activity, and identified two novel mutations and two reported ones. In Patient 1, an 11-bp deletion starting at nucleotide position 317 of cDNA, Del 11b (#317–327), was found (Fig. 1). It resulted in replacement of 106-LQADPQRFPHGIR-118 in the normal sequence by 106-

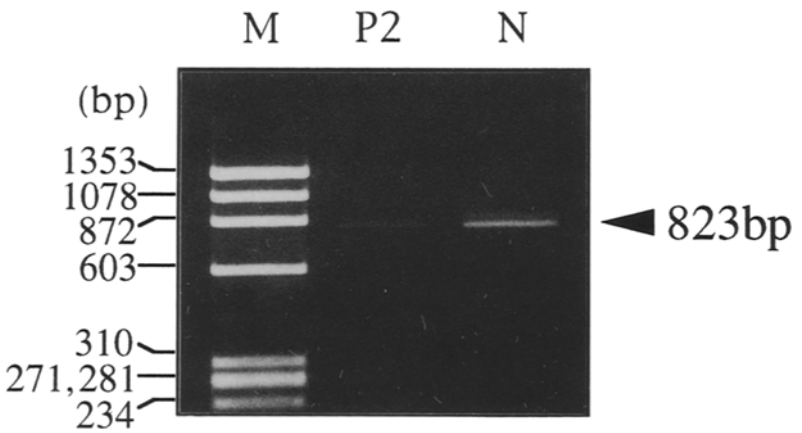

Fig. 3. Agarose gel electrophoresis of α-Gal cDNA fragments amplified by PCR. α-Gal cDNA fragment comprising exon 1–4 and a part of exon 5 was prepared from a normal control (N) and Patient 2 (P2). Lane M, molecular weight marker. The fragments were stained with ethidium bromide and visualized under ultraviolet light after electrophoresis in 1.5% agarose gel.

PSALSSWDSPASX-118 in the patient. Since an 8-bp direct repeat sequence (5′-AGGCAGAC-3′) was observed at the sequence flanking the breakpoint junction, it seems to have been caused by slipped mispairing (Krawczak and Cooper, 1991) during DNA replication. In Patient 2, a g^{-1} to t substitution at the 3′ end of the splice acceptor site in intron 1, Int 1-SA (#195-1g→t), was found (Fig. 2). On PCR-amplification for α-Gal cDNA, trace level of normal size fragment (823 bp) was detected, but other fragment (*e.g.* truncated fragment resulting from exon skipping) was not observed for the patient under the condition described before (Fig. 3). Furthermore, Northern hybridization analysis revealed that the amount of α-Gal mRNA had markedly decreased in the patient as compared with that in normal control (Fig. 4). These findings suggest that Int 1-SA mutation provokes aberrant pre-mRNA splicing, and abnormally processed RNA is subjected to accelerated degradation in cells due to a conformational change of RNA molecule, resulting in marked decrease in the amount of mRNA. Some mutations at the splice acceptor site, which abolished proper RNA splicing, were reported in inherited diseases; β-thalassemia in which the processed mRNA contained an extended exon (Antonarakis *et al.*, 1984), Tay-Sachs disease in which an exon skipping occurred (Tanaka *et al.*, 1993). Three mutations at the splice acceptor site in the α-Gal gene

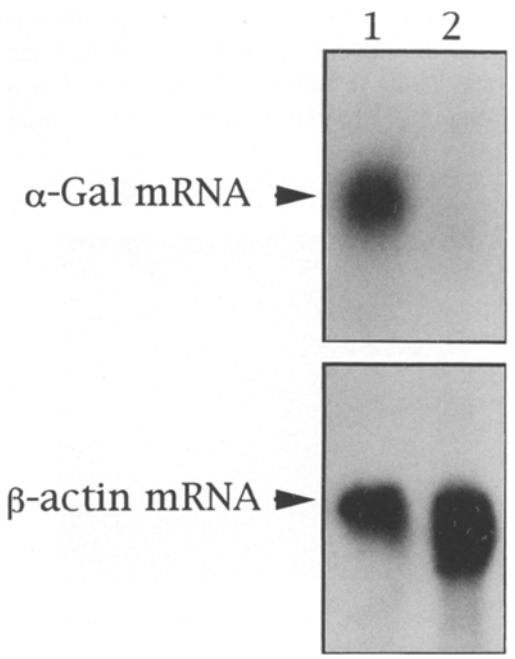

Fig. 4. Northern hybridization analyses of α-Gal mRNA (upper panel) and β-actin mRNA (lower panel) in lymphoblasts. Lane 1, normal control; lane 2, Patient 2. The amount of α-Gal mRNA was markedly decreased in the patient cells.

Table 2. Gene analysis in the present patients with Fabry disease.

Patient	Location	Allele designation (Base change[a])	Notes
1	Exon 2	Del 11b (#317-327)	106-PSALSSWDSPASX-118[b]
2	Intron 1	Int 1-SA[c] (#195-1g→t)	marked decrease in α-Gal mRNA
3	Exon 6	R301X (#901C→T)	Arg→Stop at codon 301
4	Exon 5	Del 2b (#718-719)	240-EYLGLDIFX-248[b]

[a] The base number of α-Gal cDNA according to Bishop *et al.* (1988).
[b] Created amino acid sequence at the indicated codon number.
[c] Splice acceptor site.

were also reported in Fabry disease; one mutation which was occurred in intron 3 resulted in a complete deletion of exon 4 (Yokoi *et al.*, 1991), others were not investigated for RNA processing (Eng *et al.*, 1993; Davies *et al.*, 1994). In our case, since the amount of α-Gal mRNA markedly decreased in Patient 2, it seems that α-Gal enzyme protein is hardly synthesized in the cells.

Two reported mutations were identified in Patients 3 and 4 (Table 2). Patient 3 had a nonsense mutation, R301X, which was also found in an English patient (Eng *et al.*, 1994). Patient 4 had a 2-bp deletion starting at nucleotide position 718 in exon 5, Del 2b (#718-719) (Davies *et al.*, 1994).

These findings suggest molecular genetic heterogeneity underlying Fabry disease among different ethnic groups. The accumulation of such data will provide us with significant information for understanding the molecular basis and pathogenesis of Fabry disease.

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