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Identification of Point Mutations in α-Galactosidase A Gene in Patients with Fabry Disease

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Summary. **Analysis was made of the α-Galactosidase A gene from two patients with Fabry disease using PCR-SSCP (Single Strand Conformation Polymorphism) analysis followed by direct nucleotide sequence analysis. Through PCR-SSCP analysis, we found that the patients' PCR products derived from the exon 7 gave aberrantly moving patterns. Direct nucleotide sequence analysis disclosed two new, previously undescribed point mutations in exon 7. One hemizygote had an A-to-C transition at codon 410 in exon 7 which substituted proline (CCA) for threonine codon (ACA). Another unrelated hemizygote had another different point mutation in exon 7. It showed T-to-C and C-to-T transitions at codon 388 substituting proline (CCT) for leucine codon (CTC). The former mutation was also found in another male member of the patient's family, and his daughter was heterozygous for this mutation. The present findings also indicate heterogeneities of molecular lesions in that there are few common mutations in Fabry disease.**

Key words—Fabry disease, α-Galactosidase A, mutation, single strand conformation polymorphism (SSCP).

INTRODUCTION

Fabry disease is an X-linked hereditary disease caused by a defect of lysosomal hydrolase α-galactosidase A (α-Gal A; EC 3.2.1.22). In affected male hemizygotes, the intracellular accumulation of globotriosylceramide and related glycolipids containing terminal α-galactosyl residues leads to clinical manifestations such as pain and paresthesia in the extremities, angiokeratoma and hypohidrosis of the skin, and progressive vascular disease of the kidney, heart, and central nervous system.[1] The gene encoding α-Gal A has been assigned Xq22 by the mapping of YAC clones containing human α-galactosidase A gene.[2,3] Complementary DNA clones, including full-length cDNAs for human α-galactosidase A, have already been cloned and characterized, and their genomic structural organization has been elucidated.[4-6,7] The α-Gal A gene, which spans 12-kb, contains seven exons and encodes a precursor peptide of 429 amino acids including a 31-residue signal peptide.

Since the description of full-length cDNAs for human α-galactosidase A,[5,6] intensive mutational analyses have been performed and various kinds of mutations including deletions, duplications and missense mutations have been described.[1] In addition to the classical phenotype described above, an atypical phenotype has been described with late-onset cardiac or cardiopulmonary disease. While classic hemizygotes show no detectable α-Gal A activity or enzyme protein, these atypical variants show some residual α-Gal A activity. It has been demonstrated that classical hemizygotes are associated with large and small rearrangements, and nonsense or missense mutations.[1] In contrast, the atypical hemizygotes have been shown to have missense mutations.[8-13] Although these mutations are located only in exons 5 and 6, other mutations causing the classical phenotype have also been identified in exons 5 and 6. Furthermore, attempts to predict clinical phenotypes based on molecular diagnosis have been hampered since most Fabry patients have private mutations.[1] Against this background, there is a need more detailed information not only on molecular lesions for every Fabry patient but also on the correlation of genotypes with phenotypes of Fabry disease. Furthermore, from the viewpoint of genetic counseling, it is not enough to undertake enzymatic analysis of α-Gal A activities, because

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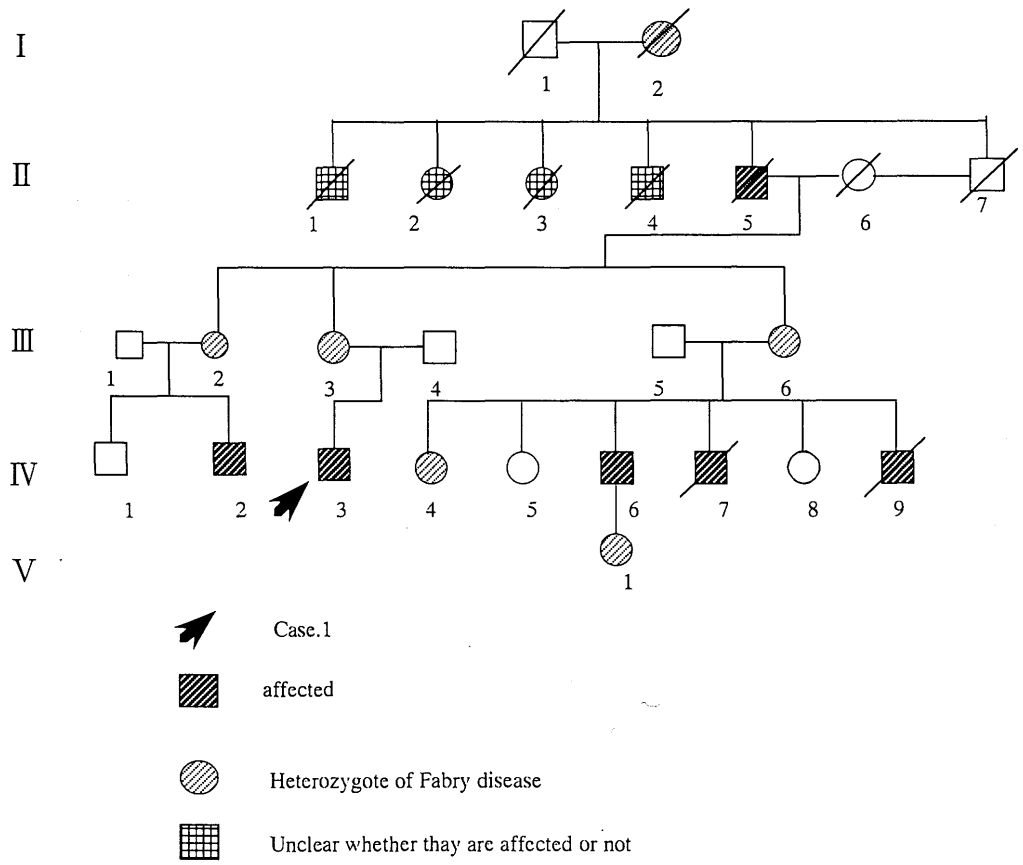

Fig. 1. Case 1 Lineage.

female carriers with this disease often show unreliable data. Among heterozygous, α-Gal A activities range from zero to the normal level, thus making it difficult to determine the heterozygous state. This variation is considered to be due to random X-chromosomal inactivation.[1,14] For genetic counseling, therefore, the identification of the heterozygous state is by no means required. Since there are many private mutations in Fabry disease as mentioned above, we need to elucidate the mutations for every patient. To quickly accomplish this aim we employed a simplified method of SSCP (Single Strand Conformation Polymorphism) analysis[15] and identified two novel mutations in human a-galactosidase gene.

MATERIALS AND METHODS

Patients

Case 1 (Fig. 1, IV-3). A 35-year-old Japanese male experiencing hypohidrosis and pain in the peripheral extremities from his childhood. A deficiency of α-Gal A activity (10% of normal range) was found and a diagnosis of Fabry disease was established. He has suffered from renal dysfunction since the age of 29, and he has been undergoing hemodialysis since 33 years of age. His mother and aunts were diagnosed as heterozygotes of Fabry disease based on clinical investigation. Three of four living male cousins had Fabry disease, all being diagnosed on the basis of enzyme activities of α-Gal A. In the present study, we studied Case 1 as well as one of his cousins (IV-6) and his cousin's daughter (V-1) (Fig. 1).

Case 2. A 36-year-old man with no familial relationship to Case 1. He also had experienced pain in his extremities and hypohidrosis in his childhood, and was proven to have deficient lymphocyte α-Gal A activity (7% of the normal range). He currently is also undergoing hemodialysis.

Isolation of genomic DNA

Whole-blood samples were obtained from patients,

members of their families and healthy volunteers as normal controls with informed consent. High molecular weight genomic DNA was isolated from whole blood by standard techniques.[16]

Southern blot hybridization

Genomic DNA was digested with *Hind*III, *Msp*I, *Pvu*II, *Sac*I, *BamH*I, and *Taq*I (Takara Shuzo, Shiga, Japan), electrophoresed in 1% agarose gels, transferred to a nitrocellulose membrane by the Southern method, and hybridized with the ^{32}P-labeled α-Gal A cDNA (pcD-AG210)[5].

Single strand conformation polymorphism (SSCP) analysis

All exons as well as the flanking introns of the α-Gal A gene from the patients and normal controls were amplified by PCR with 14 sets of primers (Table 1). The size of each PCR product was designed to be approximately 200 bp. Fifty nanograms of DNA was amplified in 5 μl reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 25 μM dNTPs, 74 kBq ^{32}P-αdCTP, 0.025U *Taq* Polymerase with 2 μM each of PCR primers. Amplifications were performed for 35 cycles, with denaturation at 94°C for 30 sec., annealing at 60°C for 30 sec., and extension at 72°C for 60 sec. PCR products were mixed with nine volumes of 98.5% formamide solution after denaturation at 95°C for 5 min and then electrophoresed in 5% polyacrylamide gel containing 5% glycerol at 40 W for 3-4 h at a temperature of 20°C. In cases where PCR products showed patterns different from those of normal controls, direct sequencing analyses were performed.

Table 1. Sequences of the oligonucleotide primer

Exon		Sequence
1	S	5′AGTCATCGGTGATTGGTCCG3′
	A	5′ATTGTCCAGTGCTCTAGCCC3′
	S	5′TTCGCTTCCTGGCCCTCGTT3′
	A	5′GAAAAGCAAAGGGAAGGGAG3′
2	S	5′GGGAGGTACCTAAGTGTTCA3′
	A	5′AGGGTCTGCCTGAAGTCTGC3′
	S	5′GCTCATGGTCTCAGAAGGCT3′
	A	5′CAGAAGTGCTTACAGTCCTC3′
3	S	5′TCTGCTACCTCACGATTGTG3′
	A	5′AGATCTACTCCCCAGTCAGC3′
	S	5′ATAAAACCTGCGCAGGCTTC3′
	A	5′TCAGCTACCATGGCCTCAAA3′
4	S	5′ATAGCCCCAGCTGGAAATTC3′
	A	5′CTATTGGATTCTGGGCTCAC3′
5	S	5′GGCTACAAGTGCCTCCTTTA3′
	A	5′CTGGTCCAGCAACATCAACA3′
	S	5′CAGTACTGCAATCACTGGCG3′
	A	5′AAACAAGCCTACCGCAGGGT3′
6	S	5′CTCCATATGGGTCATCTAGG3′
	A	5′CCTTATCCTGAAGGAGAGCT3′
	S	5′GCAAGTAACTCAGATGGCCC3′
	A	5′GGCCCAAGACAAAGTTGGTA3′
7	S	5′GGGCCACTTATCACTAGTTG3′
	A	5′CAGGATTACAGGCCACTCCT3′
	S	5′GACCTCGCTCTTATACCATC3′
	A	5′AGCTGAAGCAAAACAGTGCC3′
	S	5′ACAGCTCCTCCCTGTGAAAA3′
	A	5′TGGAGAAAAAGGTGGACAGG3′

S represents "sense" primer, and A represents "antisense" primer.

RESULTS

Southern blot analysis

Southern blot analysis revealed no remarkable abnormal bands for any of the restriction enzymes tested.

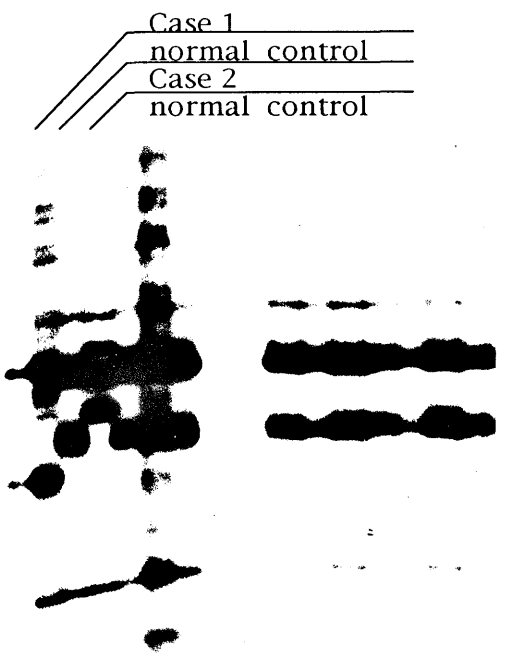

Fig. 2. Single strand conformation polymorphism (SSCP). ^{32}P-labeled PCR products derived from 3′ half of α-Gal A exon 7 of genomic DNA from normal subjects and the patients were denatured and electrophoresed through 5% polyacrylamide gel containing 5% glycerol. The lanes from the patients reveal patterns different from those of normal subjects.

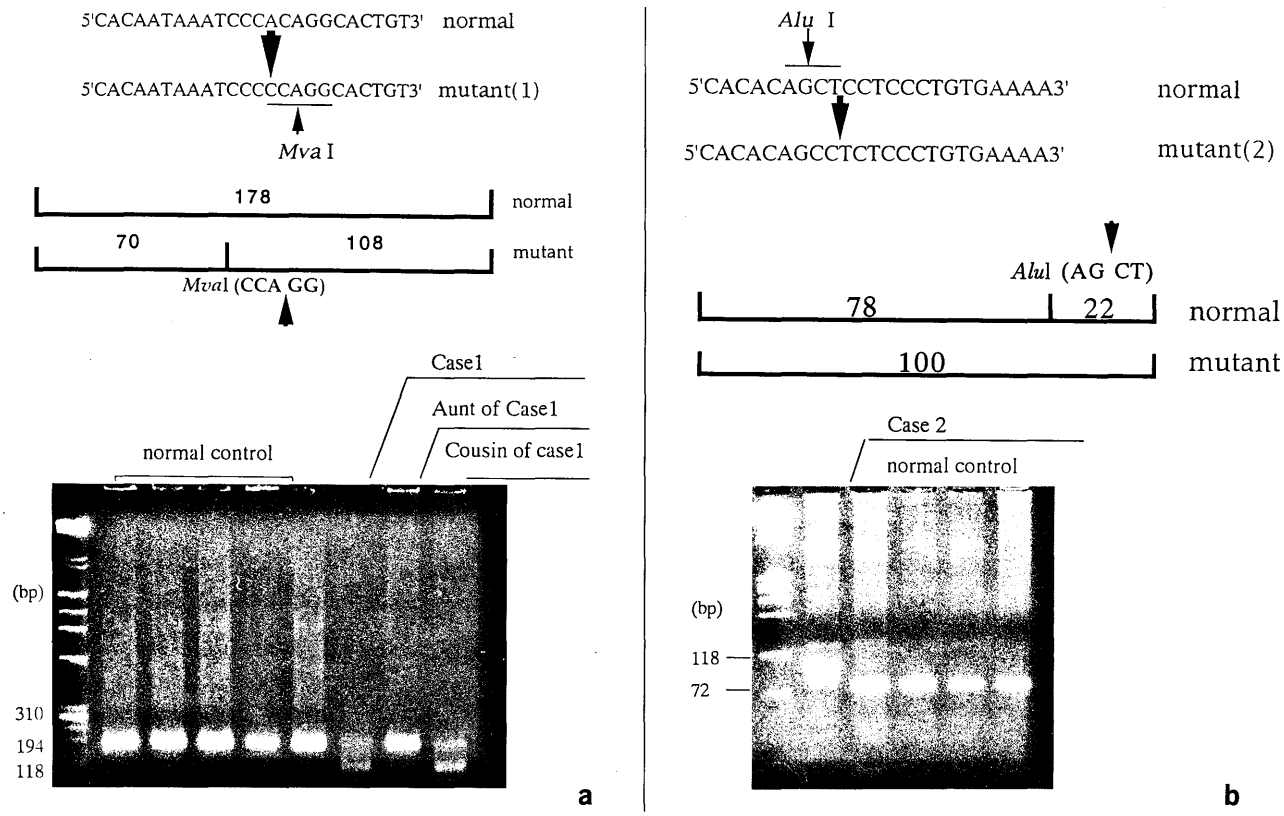

Fig. 3. RFLP analysis of the mutations. **a.** Direct sequencing analysis reveals a single base substitution at codon 410. This mutation creates an *Mva*I site. The PCR products were derived from exon 7 flanking the mutation. The PCR products from genomic DNA of normal controls were 178 bp in size and were not cut by restriction enzyme *Mva*I, whereas the products fron the mutant were digested to 70 bp and 108 bp fragments by *Mva*I. This analysis showed that Case 1 and his cousin carry the mutant gene. An aunt of Case 1 had both normal and mutant fragments, and thus is a heterozygote for this mutation. **b.** The single base substitution at codon 388 removes an *Alu*I site. PCR products derived from the normal allele (100 bp in length) have been cleaved into 78 and 22 bp fragments by *Alu*I digestion, whereas only a 100 bp fragment can be observed for the mutant allele.

There were neither large deletions nor rearrangements in the α-Gal A gene in these patients judged from the Southern blot hybridization analysis (data not shown).

SSCP analysis and nucleotide sequence analysis

Of 14 species of PCR products, 13 PCR products derived from the patients genomic DNA revealed identical electrophoretic patterns as those of the normal controls. The patients' PCR products derived from the 3' portion of exon 7, however, revealed different patterns as compared with those obtained from the normal controls (Fig. 2). These differences suggested the presence of sequence variations in this region. More interestingly, the PCR products from the two patients showed different patterns, suggesting that they possessed different mutations in the exon 7.

To confirm the presence of mutations in genomic DNA in these two patients, we undertook direct sequencing analyses in this region. Through this analysis, two different types of new point mutations were disclosed. Case 1 had an A-to-C transition in codon 410 which changed a threonine codon (ACA) to a proline codon (CCA). This mutation created an *Mva*I restriction site (Fig. 3a). We analyzed family members of Case 1 to determine if they carried mutant alleles at codon 410 using *Mva*I RFLP analysis. The analysis revealed that his cousin had the same mutation. The cousin's daughter was also shown to be a heterozygote carrying the mutant allele at codon 410 and a normal allele as shown in Figure 3a.

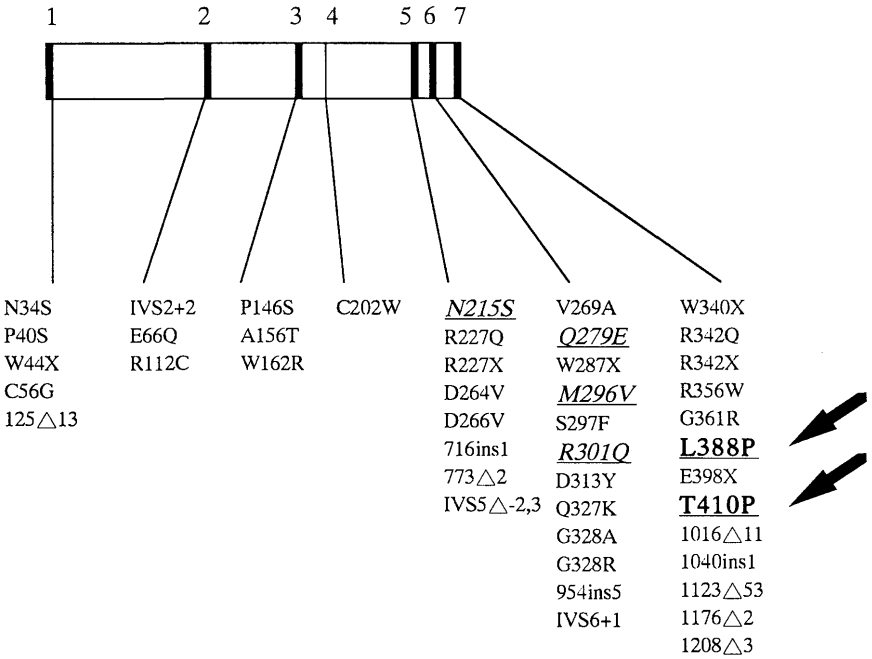

Fig. 4. Schematic presentation of the α-galactosidase A gene showing the relative position of the seven exons. Mutations which have been reported in the literature, along with the two mutations identified in the present study are shown. Abbreviations: P40S, proline to seline substitution at codon 40; ins, insertion; △, deletion; IVS, intervening sequence. Numbers for deletions of insertions refer to nucleotide position in the α-galactosidase A cDNA sequence. The mutations written in bold letters are the mutations found in the present study. The mutations written in italic letters are the mutations of atypical hemizygotes (refered by Eng 1994).

Case 2 had a T-to-C and C-to-T transitions in codon 388 that destroyed an *Alu*I site (Fig. 3-2). This transition resulted in a leucine (CTC)-to-proline (CCT) substitution.

These two mutations were not found in 12 normal controls.

DISCUSSION

In the present study, we employed the PCR-SSCP method to rapidly identify mutations. Since it has been demonstrated that the sensitivity to detect nucleotide substitutions reaches up to 100% in PCR products with the size of approximately 200 bp, and to 80% when the fragment size is approximately 400 bp,[15,17] we designed the primer pairs to obtain the PCR products with the size around 200 bp. Once the exons giving aberrant electophoretic patterns were identified, we determined the nucleotide sequences. As shown in the present study, this strategy has proved to be a highly efficient method for the identification of mutations, which can then be applied for the analysis of a large number of patients.

In this report, we identified two novel amino-acid substitutions in exon 7 which have not been described elsewhere (Fig. 4). These two mutations were not found in 12 normal controls. Furthermore, since these mutations involve substitutions of proline for threonine (Case 1) or leucine (Case 2), these two mutations are presumed to bring about considerable changes in the secondary structures of the α-galactosidase molecule. Taken together, these two mutations are considered to be the causative mutations in the two Fabry patients.

In patients with Fabry disease, frequencies of large rearrangements in the α-Gal A gene, as revealed by Southern blot analysis, were only 5%[18] even including either deletions[19–22] or splicing junction mutations.[19,23] The majority seemed to be caused by single base substitutions. So far six small deletions, three small duplications, and 31 point mutations have been described (Fig. 4). Interestingly, there seem to be few common mutations in Fabry's disease,[13,24] which indicates that there are many private mutations, and that extensive analysis of molecular lesions is required for

each case of Fabry disease. We have described two novel amino-acid substitutions in the present study (Fig. 4).

From the view point of clinical phenotypes, our patients belong to the classical type, which usually does not show apparent cardiac involvement. The atypical phenotype of Fabry disease which exclusively affects the cardiac organ has been recognized and molecular lesions in the atypical phenotype have recently been disclosed.[8,11,24] While classically affected hemizygotes have a variety of molecular lesions including large gene rearrangement, splicing junction mutations, and nonsense or missense mutations, asymptomatic or mildly affected hemizygotes have been demonstrated to have only missense mutations. Although mutations of mildly affected Fabry patients (for example, Q279E, M296V, R301Q) are all located in exon 5 or 6, the presumption of a clinical phenotype based on mutational location is still imperfect because mutations associated with a severe phenotype were also found in exon 5 and 6, and we still do not have complete lists of mutations responsible for various clinical phenotypes. The two mutations identified in exon 7, which are associated with the classical phenotype, would certainly extend our understanding of the correlation of genotypes with phenotypes and be of help for genetic counseling for those carrying the mutations described in the present study.

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