Studies on identification and functional analysis of mutant alpha-galactosidase A

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A Dissertation

Submitted to the Department of Biomedical Laboratory Science and the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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July 2003

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July 2003

This dissertation is affectionately dedicated to my parents, my sisters, my wife and my son, who have encouraged me.

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ABBREVIATION

4-MU	: 4-methylumbelliferone
4MUG	: 4-methylumbelliferyl-a-D-galactoside
α-GAL A	: alpha-galactosidase A
BSA	: bovine serum albumin
DGJ	: 1-deoxygalactonojirimycin
EDTA	: ethylenediaminetetraacetic acid
EGTA	: ethyleneglycolbistetraacetic acid
FBS	: fetal bovine serum
LSD	: lysosomal storage disease
MEM	: minimal essential medium
PBS	: phosphate-buffered saline
PCR	: polymerase chain reaction
RFLP	: restricted fragment length polymorphism
SSCP	: single stranded conformation polymorphism

ABSTRACT

Studies on identification and functional analysis of mutant alpha-galactosidase A

Fabry disease is an X-linked recessively inherited metabolic disorder, which results from the deficient activity of the lysosomal hydrolase alpha-galactosidase A (α-GAL A) leading to the systemic deposition of glycosphingolipids with terminal alpha-galactosyl moieties. Single-stranded conformation polymorphism (SSCP) analysis was performed, followed by DNA sequencing of PCR amplified exons of the human α-GAL A gene in Korean patients with classic Fabry disease. Three different mutations were identified; missense mutation E66Q, a transition at position 5095 in exon 2 substituted a glutamic acid for a glutamine at codon 66, R112C, a transition at position 5233 in exon 2 substituted an arginine for an cysteine at codon 112, D266N, a transversion at position 10287 in exon 5 substituted an aspartate for an asparagine at codon 266.

To investigate whether these mutations in the human *α-GAL A* gene affected the function of α-GAL A protein, the full-length cDNA of α-GAL A wild type was isolated, cloned, and sequenced. The full-length α-GAL A

cDNA had seven exons, and encoded a precursor peptide of 429 amino acids including a signal peptide of 31 residues. The optimal pH of wild type α-GAL A enzyme was 4.6. Their enzymatic properties toward α-methylumbelliferyl α-glactopyranoside were same as previously reported wild type α-GAL A.

The intracellular α-GAL A activities in COS-7 cells transfected with E66Q, R112C, D266N mutant plasmid DNAs were markedly lowers than the wild type enzyme activity (about 35, 4, and 2 % of wild type α-GAL A, respectively).

In E66Q mutant form of α-GAL A, enzyme had a residual activity (about 30%). The pH range of E66Q mutant form was 4-6, same as the range of wild type. Experimental studies on residual activities of mutant α-GAL A showed that they had enzymatic properties similar to those of wild type α-GAL A, but was less stable.

Furthermore, the intracellular α-GAL A activities cotransfected with mutants and wild type cDNA showed a lower activity than those with wild type alone (~50% of wild type alone), which suggested these mutations have the dominant negative effect and implied these mutated sites were critical for its activity.

In conclusion, novel mutations of *α-GAL A* gene were identified in Korean patients with Fabry disease, subsequently expressed *in vitro*. It

elucidated the effect of mutations on the function of α-GAL A protein.

Furthermore, our understandings of functional basis for mutant α-GAL A will provide more information on the relationship between the amino-acid substitution and the pathophysiology of the disease.

Keywords: X-linked disorder, α-galactosidase A, Fabry disease, mutagenesis, mutation, E66Q, R112C, D266N

CHAPTER I

Detection of genetic mutation in Fabry patients

I. INTRODUCTION

Lysosomal storage disorders (LSD) are caused by an inherited deficiency of one or more of the several lysosomal enzymes that normally catalyze the metabolism of glycoproteins glycolipids, and other macromolecules (Meikle PJ *et al*., 1999). About 40 different genes have been identified as gene loci for mutations resulting in a lysosomal storage disease (LSD). A large number of mutations have been delineated for most disorders, and this contributes to the wide clinical spectra observed in patients with a deficiency of a necessary protein. As a group, LSDs occur in approximately 1 in 5000 to 8000 births in the United States, Europe, and Australia (Meikle PJ *et al,* 1999).

Therefore, about 500 to 800 people are born each year with a LSD in the United States. While some of these disorders result in purely nonneurologic manifestations (eg, Gaucher disease type 1), many others are characterized by a wide range of neurologic symptoms, with or without somatic features, presenting from birth to adulthood. Owing to the complexity of the storage products and differences in their tissue distribution and rates of accumulation, the disease can cause pathologic changes in multiple organ systems or can be confined to the nervous system.

Fabry disease is a rare X-linked inherited lysosomal storage disease,

which is caused by deficient enzyme activity of α-GAL A, a lysosomal hydrolase needed in the catabolism of glycosphingolipids with α-galactosyl moieties. This enzymatic defects lead to progressive accumulation of neutral glycosphingolipids in body fluids and lysosomes of vascular endothelium and in the various body tissues such as the heart, kidney, eyes, and brain (Desnick RJ *et al*,. 1995; deVeber GA & Schwarting GA *et al*. 1992).

In classical hemizygous males with no detectable enzyme activity, clinical symptoms usually begin in childhood and manifest as acroparesthesia, hypohidrosis, angiokeratoma, corneal opacities, and autonomic dysfunction. Progressive vasculopathy of heart, brain, and especially kidney leads to death in the second to fifth decade of their life. Atypical hemizygotes with residual α-GAL A activity may be asymptomatic or present with late-onset, mild disease manifestations primarily limited to the heart. Female heterozygotes are usually asymptomatic, but rare cases of heterozygous females affected as severely as typical hemizygotes have been reported (Ferrans VJ *et al,*. 1969; Desnick RJ *et al.,* 1972; Bird TD & Lagunoff D. 1978).

The gene encoding α-GAL A, located at Xq22, is a small gene of 12 kb, containing seven exons varying in size from 92 to 291 bp (Kornreich R *et al.,* 1989). The full-length cDNA (Bishop DF *et al.,* 1986) and the entire genomic

sequence (Kornreich R *et al.,* 1989) of the human α-GAL A gene have been characterized.

Since about 15% of obligate heterozygotes have normal enzyme activities, carrier detection by the enzymatic assay has been difficult (Bishop DF *et al.,* 1986). Although the diagnosis of affected males with Fabry disease can be reliably made by demonstrating markedly deficient α-GAL A activity in plasma, leukocytes, or cultured cells (Desnick RJ *et al.,* 1972), the enzymatic identification of carrier females, even obligate heterozygotes, is less reliable due to random X-chromosomal inactivation (Lyon M, 1961: Brown RM and Brown BK. 1993).

Recently, molecular approaches have made it possible to detect asymptomatic carriers and also allowed genotype-phenotype correlations. More than 300 mutations in the α-GAL A gene have been described so far, the vast majority being point mutations (Desnick RJ *et al*,. 1995; Eng CM & Desnick RJ. 1994; Shabbeer J *et al.,* 2002).

However, molecular defects of the Korean patients with Fabry disease have not been reported. This study was undertaken to identify the molecular defects in the α-GAL A gene of three unrelated Korean patients with classic Fabry disease

II. MATERIALS AND METHODS

Materials

PCR *Taq* polymerase and restriction enzyme were purchased from Promega (Medison, WI, USA). Silver stain solutions were purchased from Bio-Rad (Hercules, CA, USA). Sequenase version 2.0 Kit was purchased United State Biochemical (Cheveland, OH, USA). All other chemicals and solvents were reagent grade or better.

Subjects

All the probands of each family had classic type Fabry disease, diagnosed on the basis of clinical features and markedly reduced enzyme activity of α-GAL A. Patient presented with painful paresthesia in the extremities since childhood.

Genomic DNA isolation

Genomic DNA isolated from peripheral blood leukocytes of the patients. Samples were centrifuged at 1300 g for 10 min and discard the supernatant plasma. After the pellets suspended with 0.5% sodium dodecyl sulfate, added

proteinase K to final concentration of 100 ug/ml. And then the mixture placed the water for 3 h at 50℃. After the reaction was finished, genomic DNA was extracted using phenol, chloroform and isoamylalcohol (25:24:1) and centrifuged 14,000 rpm for 10 min. and next step, samples were precipitated with cold ethanol. Next, DNAs washed twice with 70% ethanol, and centrifuged. The samples dried and resuspended with TE buffer and measured at 260nm. (Aldridge J *et al.,* 1984).

Polymerase chain reaction

Seven exons of the α-GAL A gene and their flanking intronic sequences were amplified by PCR with seven sets of primers (Table I-1) previously described (Blanch LC *et al.,* 1996). The amplification was performed in 30 cycles, each cycle consisting of denaturation at 95℃ for 45 sec, annealing at 45-55℃ for 45 sec, and extension at 72℃ for 1 min. PCR was carried out in reaction volumes of 50 ul, containing 100 ng of genomic DNA template, 1 uM each primer, 200 uM each dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 units of *Taq* polymerase. Four microliter of PCR products were mixed with 4 ul of a loading buffer (0.25% bromophenol blue, 0.25% xylenecyanol, 30% glycerol in water), denatured to single-stranded

DNAs by heating at 95℃ for 5 min, and then immediately cooled on ice.

exon	Primer	Sequence	Tm
1	Forward	5-TGATTGGTTAGCGGAACGTCTTAC-3	45
	Reverse	5-AAGGGAGTACCCAATATCTGATAC-3	
2	Forward	5-AGGTGCCTAATAAATGGGAGGTAC-3	45
	Reverse	5-GTGCTTACAGTCCTCTGAATGAAC-3	
3	Forward	5-GCTACCTCACGATTGTGCTTCTAC-3	45
	Reverse	5-TCAGCTACCATGGCCTCAAAGTTC-3	
4	Forward	5-GGATGACAGACTGAACCCCATCTC-3	45
	Reverse	5-GGAGACCTTGGTTTCCTTTGTTGTC-3	
5	Forward	5-TCTCACAAGGATGTTAGTAGAAAG-3	50
	Reverse	5-CTTGAATGTCAAAATAGGAAACAA-3	
6	Forward	5-GTTTCTCCATATGGGTCATCTAGG-3	60
	Reverse	5-AGGCCCAAGACAAAGTTGGTATTG-3	
7	Forward	5-AACAGGGCCACTTATCACTAGTTG-3	50
	Reverse	5-TGAATGGAGAAAAAGGTGGACAGG-3	

Table I-1. Primers used for *α-GAL A* gene amplification.

SSCP analysis

Three microliter of this mixture were loaded on 20-5% gradient, non-denaturating polyacrylamide gel containing 10% glycerol. Electrophoresis was performed at 4℃ and run at 300 V for 3 h. Gels were visualized by silver staining (Bio-Rad).

Direct sequencing of double stranded PCR product

When a shifted band was detected by single-stranded conformation polymorphism (SSCP) analysis, the PCR product was subjected to direct double-stranded DNA sequencing using the same primers for PCR-SSCP analysis, and a Sequence version 2.0 kits according to manufacturer's instructions (ABI system, Forster city, CA, USA) with the following modifications. The original amount of template for individual sequencing reaction ranged from 5 to 10 ul of each PCR products. Sequenase (T7 DNA polymerase) and inorganic pyrophosphates were mixed together (1 volume of each) and diluted with 6 volumes of glycerol enzyme dilution buffer (20 mmole/L Tris-HCl pH 7.5; 2 mmol/L dithiothreitol; 0.1 mmol/l EDTA; 50% glycerol).

The reaction mixture was electrophoresed on 8% denaturing

polyacrylamide gel in 0.8X glycerol tolerant gel buffer (20X buffer is 216 g tris base, 72 g taurine, 4 g EDTA, in a liter of H_2O). Electrophoresis was performed at 1,600 V for 3 h. The gels were dried and exposed on X-ray film.

RFLP analysis

According to the recommendations of the supplier of the restriction enzymes, seven microliters of the PCR product was digested with restriction enzyme (*Hha* I) in a total volume of 20 ul at 37℃ for 2 h to monitor the inheritance of mutations in patients.

Electrophoresis was carried out with 7 ul of the digested DNA on 6% native polyacrylamide gels. Silver staining or EtBr staining visualized bands. A 100 bp ladder (from Promega) was used as size marker.

III. RESULTS

PCR results of Korean patients with Fabry disease

Seven exons of the α-GAL A gene and their flanking intronic sequences were PCR-amplified with seven sets of primers(Fig. I-1). Fabry disease results from mutations in α-GAL A that results in defective enzyme activity (Bardy RO *et al.,* 1967). The disease manifests severe and mild phenotypes, correlated with the amount of residual α-GAL A activity.

Shifted Bands detection in SSCP

Three microliter of this mixture were loaded on 20-5% gradient, non-denaturating polyacrylamide gel containing 10% glycerol, subsequently visualized by silver staining

Shifted bands were appeared in two exons with SSCP analysis with patient's PCR sample (Fig. I-2).

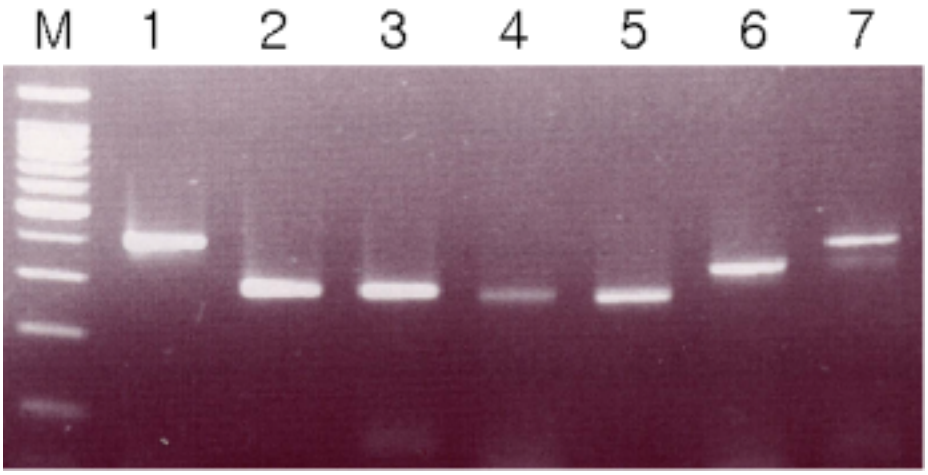

Figure I-1. Agarose gel electrophoresis of *α-GAL A* PCR products.

Lane M, 100 bp marker (Promega); lane 1-7, *α-GAL A* exon number.

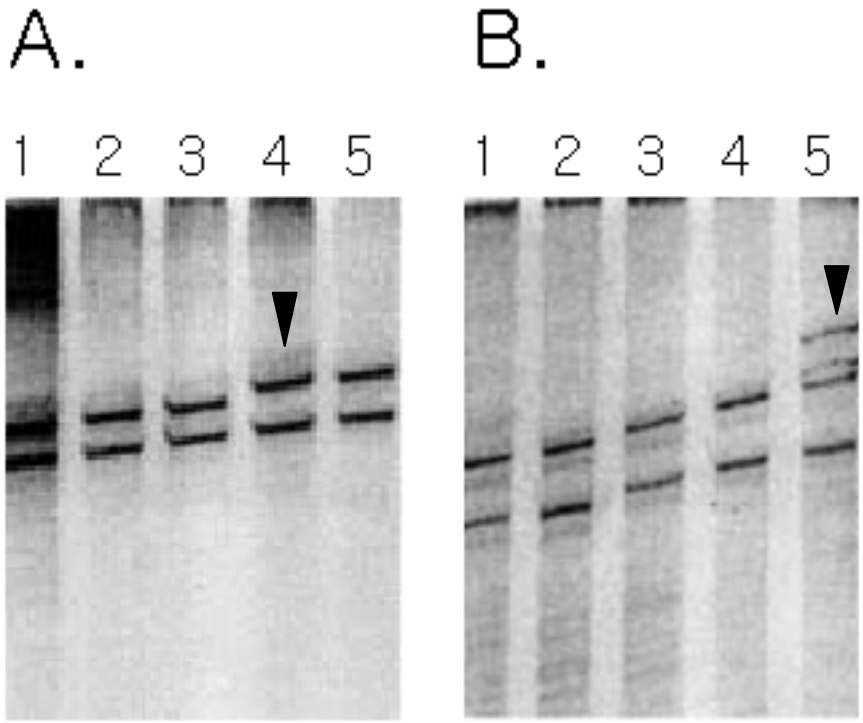

Figure I-2. Single Stranded Conformation Polymorphism of α-GAL A.

(A) SSCP analysis in exon 2. Lanes showed mobility shifts. Genomic DNA sequencing revealed a mutation in exon 2 for lane 4, but none for lane 5. (B) SSCP analysis in exon 5. Lane 5 shows an abnormal shift. Genomic DNA sequencing revealed a mutation in exon 5 for lane 5.

Identification of mutations in the *α-GAL A* gene in Fabry patients

When a shifted band was detected by single-stranded conformation polymorphism (SSCP) analysis, the PCR product was subjected to direct double-stranded DNA sequencing using the same primers for PCR analysis

Three different mutations were found, including two previously reported (Ishii *et al.,* 1993). Two kinds of single point mutation were found in exon 2.

Other mutation is a base change of C to T (CGC → TGC) in codon 112 of *α-GAL A* in exon-2 (Fig. I-3), resulting in a single amino acid change from arginine to cysteine (R112C).

The other patient had a G to A transversion at nucleotide position 10287 (GAT → AAT) in exon 5 which substituted an aspartate for an asparagine at codon 266 (D266N)(Fig. I-4). His mother and a daughter were heterozygotes for this mutation. His younger brother was unaffected.

Sequencing of the other PCR products revealed a substitution of G to C (GAG → CAG) in codon 66 (Fig. I-5), resulting in a single amino acid change from glutamic acid to glutamine (E66Q).

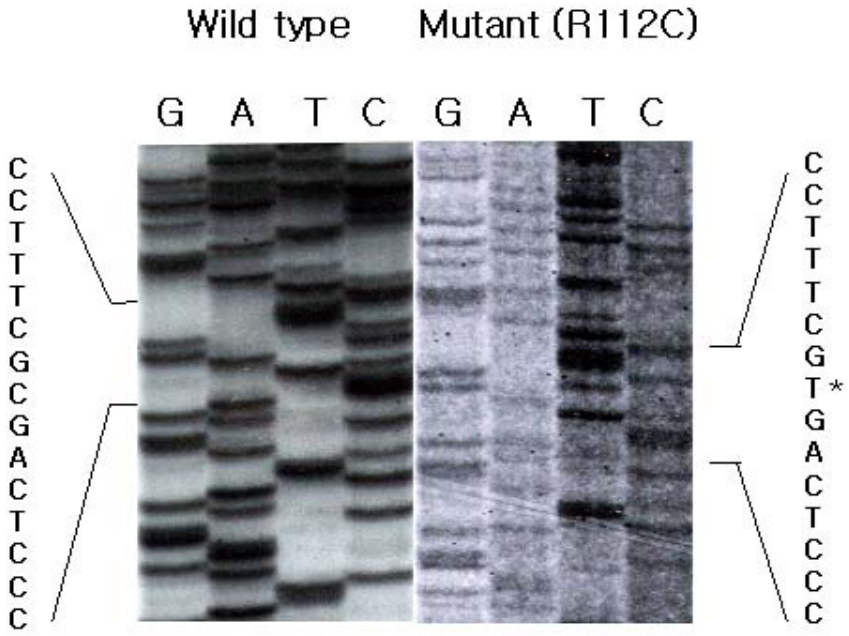

Figure I-3. Sequence analysis of the human *α-GAL A* gene of a Korean Fabry patient I (R112C).

Partial genomic DNA sequence analysis of the *α-GAL A* gene exon 2 showed a mutation. An asterisk indicates nucleotide substitution. R112C mutation is CGA →TGA.

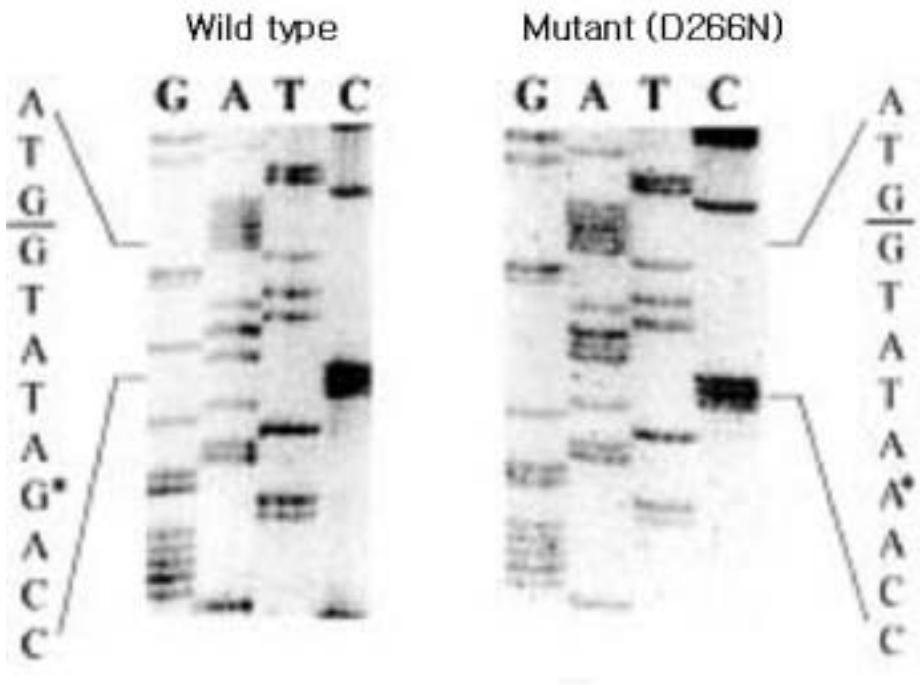

Figure I-4. Sequence analysis of the human *α-GAL A* gene of a Korean Fabry patient II (D266N).

Partial genomic DNA sequence analysis of the *α-GAL A* gene exon 5 showed a mutation. An asterisk indicates nucleotide substitution. D266N mutation in exon 5 is GAC→ AAC . Horizontal bar represents the exon-intron boundary.

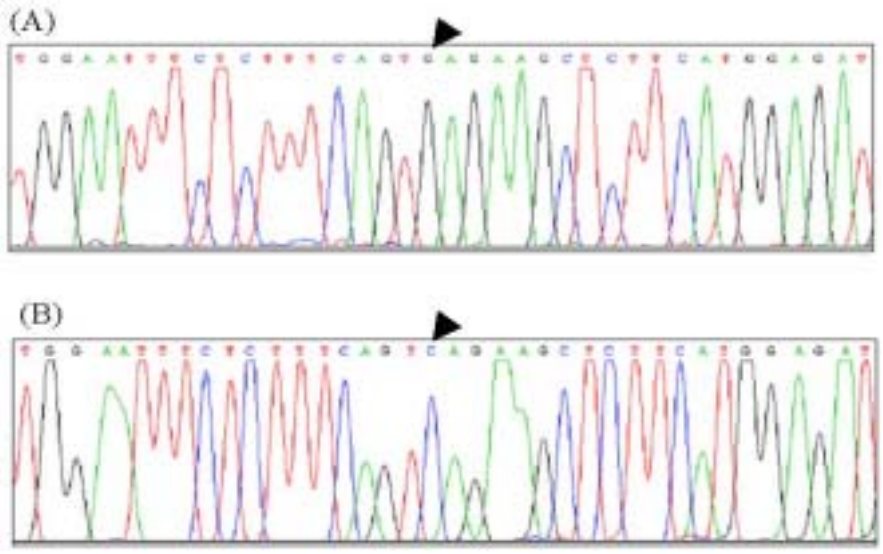

Figure I-5. Sequence analysis of the human *α-GAL A* gene of a Korean Fabry patient III (E66Q).

Partial genomic DNA sequence analysis of the exon 2 of *α-GAL A* gene showed a mutation. An arrow indicates nucleotide substitution. E66Q mutation in exon 2, is GAG→CAG..

RFLP of Fabry disease patients

To confirm the inheritance of mutations in patient, seven microliters of the PCR product (R112C) were digested with restriction enzyme (*Hha* I) in a total volume of 20 ul at 37℃ for 2 h. The mutation obliterates the specific digestion site of *Hha* I.

Therefore, the mutation can be detected with PCR-RFLP with *Hha* I digestion (Fig. I-6). This result confirmed that the patient was a hemizygote of R112C mutation.

Alignment of human α-Gal A protein and other homologous proteins in mutation sites

All the mutations identified in this study were located in base pairs highly conserved cross various species. These results showed that those sites E66 (Fig. I-7), R112 (Fig. I-8), and D266 (Fig. I-9) were critical sites in normal enzyme function.

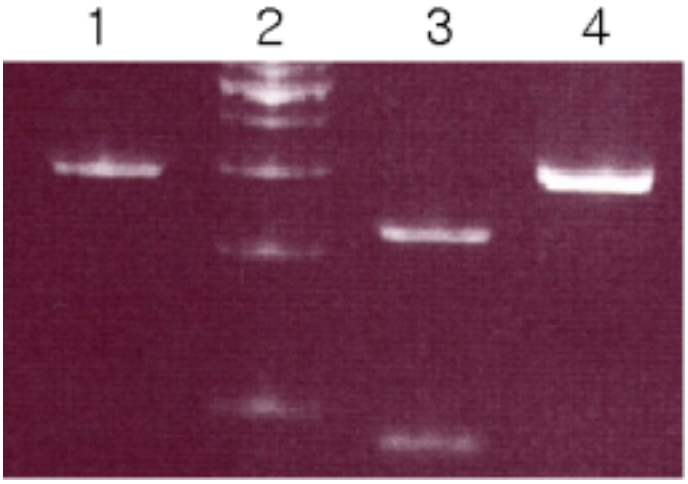

Figure I-6. PCR-RFLP analysis with restriction enzyme, *Hha* I for R112C mutation in a patient.

Lane 1, PCR product; lane 2, 100 bp marker (Promega); lane 3, wild type; lane 4, mutant type;

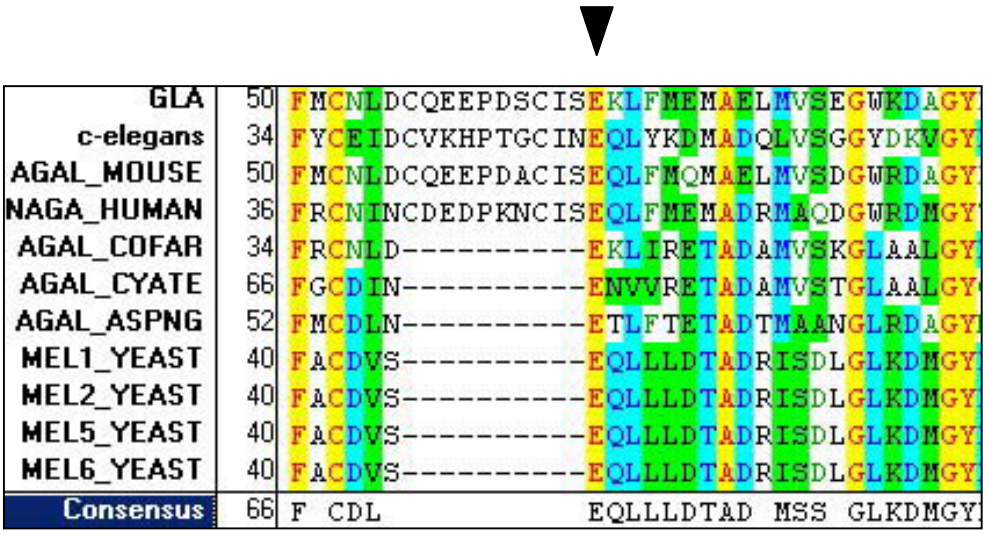

Figure I-7. Alignment of human α-GAL A protein and other homologous proteins of amino acid 66.

A glutamate residue, which is altered to a glutamine in E66Q, is arrowed

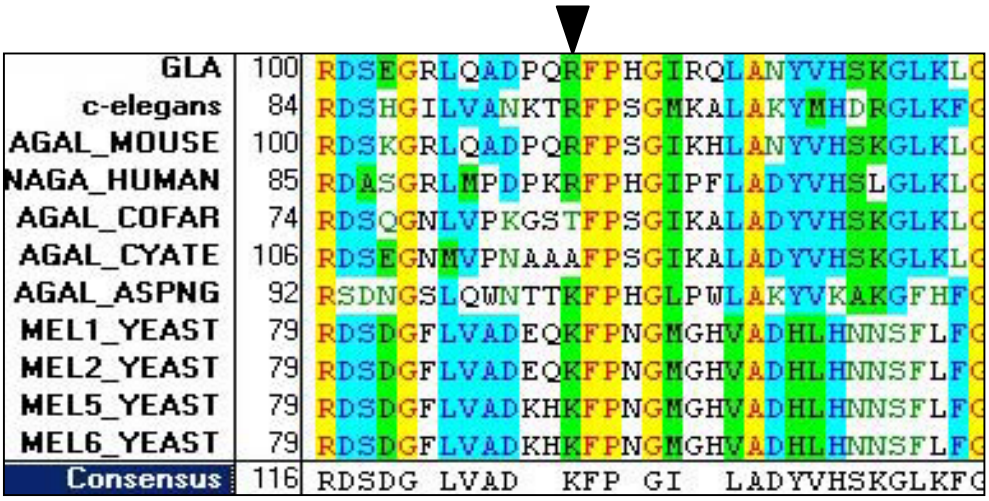

Figure I-8. Alignment of human α-GAL A protein and other homologous proteins of amino acid 112.

An arginine residue, which is altered to a cysteine in R112C, is arrowed

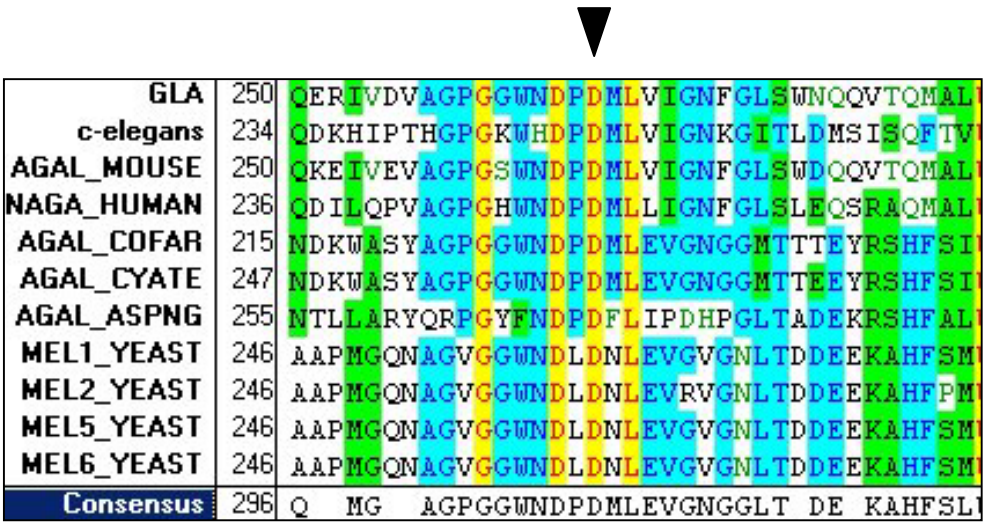

Figure I-9. Alignment of human α-GAL A protein and other homologous proteins of amino acid 266.

An aspartic acid residue, which is altered to an asparagine in D266N, is arrowed

IV. DISCUSSION

Fabry disease is characterized by a generalized vasculopathy, which in most cases appears in the second decade of life. Fabry disease results from decreased α-GAL A activity, which can be caused by perturbation of the active site, by reduced stability of the folded homodimer, and/or by defects in trafficking of the enzyme to the lysosome.

Because α-GAL A functions in the acidic lysosome, it requires considerable thermodynamic stability to remain folded, and mutations that produce less stable protein lead to disease symptoms. Thus, one of treatment modalities for the mild variant of Fabry disease uses galactose infusions, which presumably stabilizes the mutant α-GAL protein (Frustaci A *et al.,* 2001).

Since the cloning of full-length cDNA and genomic DNA encoding human α-GAL A, a variety of mutations causing the disease have been described, including partial gene rearrangements, small deletions and insertions (Bernstein HS *et al.* 1989; Ploor van Amstel JK *et al.,* 1994), splice-junction consensus site alterations (Sukuraba H *et al.,* 1992; Yokoi T *et al.,* 1991) and various coding region single-base substitutions (Blanch LC *et al.*1996; Chen CH *et al*., 1998; Davies J *et al*., 1994; Kawanishi C *et al*.,

1995; Miyazaki T *et al*., 1998; Okumiya T *et al*., 1995).

In general, mutations causing the severe phenotype of Fabry disease tend to be buried in the interior of the α-GAL A, while mutations causing the mild phenotype tends to be less disruptive to the hydrophobic core of the protein.

These recent molecular investigations on α-GAL A have proven the existence of atypical variants in Fabry disease with manifestations confined to the heart and kidney.

Mutations involving charged residues are common in the database of point mutations. Of 114 residues in the point mutation database, 60 (53%) represent changes to or from the ionizable residues arginine, lysine, histidine, glutamic acid, and aspartic acid. The point mutation database contains 24 residues mutated from charges and 46 residues mutated into charges. Charged residues found in ion pairs aid the correct folding of proteins, and a mutation of one-half of an ion pair introduces an unpaired charge, which is highly energetically unfavorable in the interior of a protein. Point mutation leading to an unpaired or misplaced charge can destabilize a protein's folded conformation (Garman S *et al*., 2002).

Three amino acid substitution cases were observed. One of the cases revealed a substitution of C for T in codon 112 of α-GAL A, resulting in a

single amino acid change from an arginine to a cysteine (Fig. I-3).

Another revealed a substitution of G for C in codon 66 of α-GAL A, resulting in a single amino acid change from a glutamic acid to a glutamine (Fig. I-5).

These normal amino acid all were highly conserved in eukaryotic orthologs including human, mouse, C.elegans, and in the related sequence of human (Wang AM *et al*., 1990), mouse (Wang AM *et al.,* 1998) (Fig. I-7, I-8, I-9).

Many mutations reported are located in amino acid residues buried in the hydrophobic core of α-GAL A (Garman SC and Garboczi DN. 2002). These mutations create a polypeptide with folding defects, where the hydrophobic core of the protein is disrupted and the enzyme fails to fold or to remain folded in the acidic environment of the lysosome.

Patient with a D266N mutation (Fig. I-4) has a full-length α-GAL A molecules with one amino acid being changed from acidic to polar type. This is presumed to be the responsible mutation for causing the disease on the basis of the following: first, this is the only mutation found throughout the entire exons and their intronic junctions, as well as the promoter regions. Second, aspartate residue at codon 266 is highly conserved in various species (Fig. I-9),

suggesting its critical role in normal enzyme function. Acidic to polar amino acid alteration may have changed the secondary structure of the protein.

The arginine side chain, with both an aliphatic portion and a planar charged guanidine group, can make simultaneous hydrophobic and ionic interactions. The preponderance of arginine mutations contrasts with the dearth of mutations in the chemically similar lysine, indicative of the particular importance of arginine for the folding of proteins (Borders Jr. Cl *et al.,* 1994).

The frequency of gene rearrangements in the α-GAL A gene causing Fabry disease is known to be about 3% (Bernstein HS *et al*., 1989), which is similar to that reported in other X-linked diseases (Kornreich R *et al*., 1990).

Fabry disease now enters the realm of molecular disease, where the specific mutation responsible for disease is understood at the molecular level. Fabry disease can be caused either by perturbation of the active site of α-GAL A or by destabilizing the α-GAL A protein.

In summary, three different mutations were identified in three unrelated Korean families with classic Fabry disease probands. Two of them were previously reported. This result demonstrates the well-known genetic heterogeneity in the α-GAL A gene of patients with Fabry disease. The

present results strongly suggest that the missense mutations, E66Q, R112C, and D266N in the α-GAL A gene cause an extremely low activity and a typical form of Fabry disease

CHAPTER II

Expression and characterization of wild type and mutant alpha-galactosidase A

I. INTRODUCTION

Two major acids, α-GALs are present in human tissues that will hydrolyze the artificial substrate, 4-methylumbelliferyl-α-D-galactopyronoside. These two enzymes have been designated α-GAL A and α-GAL B.

Human α-GAL A is a lysosomal hydrolase that catalyzes the cleavage of the terminal α-galactose of ceramide trihexoside and other glycosphingolipids (Brady RO *et al.,* 1967). The enzyme is synthesized as a precursor that is processed to a mature form by specific N-glycosylation and proteolytic cleavage of a signal peptide (LeDonne NC Jr *et al*., 1983)

In contrast to α-GAL A, α-GAL B is moderately active as a α-GAL with both the natural (ceramide trihexoside) and artificial (4-methylumbelliferyl-a-D-galactopyronoside) substrates and the α-GAL B enzyme also has a-N-acetylgalactosaminidase activity. Therefore, the hydrolysis of 4-methylumbelliferyl-α-D-galactopyranoside by the α-GAL B enzyme is strongly inhibited by α-N-acetylgalactosaminidase while the hydrolysis by the α-GAL A enzyme is not inhibited (Kusiak JW *et al*., 1978).

The degradation of macromolecules, including glycopeptides and glycolipids, occurs in the lysosome via catabolic enzymes. For example,

glycosidases cleave the oligosaccharides from glycoproteins and glycolipids into smaller components used by the cell. In humans, defects in these enzymes lead to lysosomal storage diseases, in which a genetic defect results in the loss of a functional enzyme and the accumulation of excess substrate.

A large number of mutations in α-GAL A have been identified in individuals suffering from Fabry disease.

Human α-GAL A is a homodimeric glycoprotein with a mature subunit molecular weight of 46 KDa (Mayes JS and Beutler E. 1977; Kusiak JW *et al.*, 1978; Dean KJ and Sweeley CC, 1979; Bishop DF and Desnick RJ, 1981). The gene structure of α-GAL A has been determined (Calhoun DH *et al.*, 1985) and subsequent studies on unrelated families with Fabry disease have shown a variety of molecular pathology. The 12 kb genomic sequences contain 7 exons encoding the 429 amino acid α-GAL A polypeptide, including an NH2-terminal 31 residue signal peptide.

Biosynthetic studies using polyclonal antibodies produced against the purified human enzyme indicated that the α-GAL A subunit is normally synthesized as a precursor glycopeptide of 50 kDa. After cleavage of the signal peptide and carbohydrate modification in the Golgi complex and lysosomes, mature enzyme subunits of 46 KDa form the active, homodimeric

enzyme (LeDonne NC, 1983; Lemansky P, 1987).

The enzyme is targeted to its lysosomal site of action by mannose-6-phosphate (M6P) residues on the α-GAL A molecule. The M6P moiety binds to a specific receptor in the Golgi complex and thus is directed to prelysosomal compartments. Enzymes that escape this routing system are secreted by the cell via the constitutive secretory pathway and often are recaptured by cell surface M6P receptors that return α-GAL A to endocytic pathway (Kornfeld S *et al.*, 1989).

Although various mutations have been identified from the gene analysis of Fabry patients, including missense and nonsense mutation, splicing defects, small deletions and insertions, and larger gene rearrangements, functional studies on amino acid substitutions are limited.

In order to characterize enzymatic properties of these mutant enzymes, *α-GAL A* mutant cDNAs of E66Q, R112C, and D266N were constructed by site-directed mutagenesis. In addition, transient expression of these mutants in COS-7 cells was also performed to evaluate the behavior of the mutant enzyme in mammalian cell.

II. MATERIALS AND METHODS

Materials

Minimum essential medium (MEM), and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Gaithersburg, MD, USA). 4-methylumbelliferyl-*a*-D-galactopyronoside and *a*-N-acetylgalactodiaminidase were purchased from Sigma-Aldrich (St.Louis, MO, USA). Some lots of *a*-N-acetylgalactodiaminidase had a large amount of fluorescence and had to be crystallized from ethanol. All other chemicals and solvents were reagent grade or better.

Cell culture

Stable COS-7 cells were grown in MEM supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and maintained in a controlled atmosphere (5% CO_2) incubator at 37℃.

Cloning the wild-type α-GAL A cDNA and construction of the expression plasmid

For RT-PCR, total RNA was prepared according to the TRIZOL Reagent (Roche, Mannhein, Germany) manufacturer's protocols for isolation of RNA from small quantities of cell. Total RNA was reverse transcribed using Reverse Transcription System (Promega) according to manufacturer's protocol, except all reactions were double to 40 ul of final volume. Reverse transcription reactions were performed on a half or one third of the total RNA isolated form cells, respectively.

Two overlapping *a*-GAL A cDNA fragments, one 950-bp fragment covering nt 1-843, and another 700-bp fragment covering nt 844-1347, both with an additional *Eco*RI and *Xho*I sites at the 5' and 3'end, respectively, were synthesized by the RT-PCR method using 3 ug of total RNA prepared from unaffected hemizygote. Each amplified fragment was reamplified with primer, cDNA-making primer (Table II-1).

A 950-bp fragment was isolated and cleaved with *Eco*RI and *Bst*XI, and ligated into the *Eco*RI and *Bst*XI site of pUC19. A 700-bp fragment was isolated and cleaved with *Bst*XI and *Xho*I, and ligated into the *Bst*XI and *Xho*I site of pUC19. The sequence of each fragment was confirmed by sequencing

method. Thus, 1.3 Kb fragment, which contained the entire coding region of the wild type cDNA, was inserted into the cloning site of *Eco*RI and *Xho*I of the expression plasmid pDNA3. The plasmid was designated as pCDNA3-α-GAL A.

CFab-*Eco*RI	ACT ACT GAA TTC ATG CAG CTG AGG AAC
Frag1-AS	GAC ATG AAT AAA GGA GCA GC
CFab-*Xho*I	ACT ACT TCT AGA AAA GTA AGT CTT TTA ATG ACA TCT
Frag2-S	CTT TAT ATG TGG CCC TTT CA

Table II-1. PCR primers used to make wild type *α-GAL A* cDNA coding sequence.

Transient expression of α-GAL A

COS-7 cells were grown in MEM with 10% heat-inactivated fetal bovine serum. One day after seeding cells into 60 mm dishes (2 X 10^5 cells), cells were transfected with 2 μg of mammalian expression vectors using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmid pCMV/β-gal was included to monitor the transfection efficiency.

The total amount of DNA was 1 μg with an empty vector pcDNA3.0. Twelve hours after transfection, cells were harvested and protein was extracted in PBS. One to five μg of protein was assayed for β-galactosidase activities.

Enzyme activity assay of α–GAL A

The α-GAL A activity was assayed with an artificial substrate, 4-methylumbelliferyl *a*-D-galactosidase (4-MN-*a*-gal), as described by Desnick RJ *et al.* with the following modification. The reaction mixture (60 ul) contained the following constituents; 5 mM 4MU-galactopyranoside in 0.1 M sodium citrate buffer, pH 4.6, 75 mM N-acetylgalactosamine (from Sigma) as an inhibitor of α-GAL B, and the enzyme source.

It was incubated at 37℃ for 30 min. The reaction was terminated with 0.7 ml of 0.17 M glycine-carbonate buffer, pH 10.6. The fluorescence of each sample was measured at an excitation wavelength of 365 nm and emission wavelength of 450 nm.

One unit of enzyme activity was defined as nmloe of 4-methylumbelliferone released per hour at 37℃.

Mutagenesis of α-GAL A using wild-type plasmid

pCDNA3-α-GAL A wild-type plasmid was used as DNA template in PCR amplification with primer (Table II-2) designed to introduce the E66Q, R112C, and D266N mutations. The resulting fragments were degested with *Eco*RI and *Xho*I, cloned into the pUC19 vector (Invitrogen). Replaced sequences were confirmed by using 3100 ABI Sequence analyzer (ABI system, Foster CA, USA).

Measurement of thermal stability of enzymes

Wild type and the mutant α-GAL A, 1.7-2.0 g, were incubated at 37℃ in 0.1 M sodium-citrate buffer, pH 4.4, the final volume was 100 ul. After various time intervals, samples were incubated with 5 mM 4MU-

galactopyranoside in 0.1 M sodium citrate buffer, pH 4.6, 75 mM N-acetylgalactosamine and assayed for residual activities toward 4MU-galactopyranoside.

Mutation	Sequence
E66Q-S	5- GCC AGA TTC CTG CAT CAG TCA GAA GCT CTT CAT GGA GAT G –3
E66Q-AS	5- CAT CTC CAT GAA GAG CTT CTG ACT GAT GCA GGA ATC TGG C-3
R112C-S	5- CTT CAG GCA GAC CCT CAG TGC TTT CCT CAT GGG ATT CGC C –3
R112C-AS	5- GGC GAA TCC CAT GAG GAA AGC ACT GAG GGT CTG CCT GAA G –3
D266N-S	5- AGG GGG TTG GAA TGA CCC AAA TAT GTT AGT GAT TGG CAA-3
D266N-AS	5- TTG CCA ATC ACT AAC ATA TTT GGG TCA TTC CAA CCC CCT-3

Table II-2. Oligonucleotides used in mutagenesis of *α-GAL A*

Measurement of optimal pH for enzymes

Wild type and mutant type enzymes (10 ug/ml) were incubated at 37℃ for 30 min in 0.17 M citrate-phosphate buffer containing 1mg/ml BSA at various pH values. After incubation, an aliquot was diluted with a 4-fold volume of 0.2 M sodium citrate buffer (pH 4.0), and then α-GAL A activity was assayed immediately.

Cotransfection of wild type and mutant of α-GAL A

Since it is known that a-GAL A forms a homodimer in lysosome, we investigated whether mutant type forms have the dominant negative effect in the heterozygous genotype.

To examine this possibility, 1 ug of plasmid DNA containing a 1:1 mixture of pCDNA3-α-GAL A wild type and vector vehicle only or pCDNA3-α-GAL A mutant were cotransfected into COS-7 cells. The experiments were performed in triplicate for each cotransfection.

Enzyme activity assay of *a*-GAL A treated with DGJ

COS-7 cells were grown in MEM with 10% heat-inactivated fetal bovine serum. One day after seeding cells into 60 mm dishes (2 X 10^5 cells), cells were transfected with 2 μg of mammalian expression vectors using Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer's instructions.

Twenty uM of DGJ was administrated to cells. Next day, cells were harvested and enzyme activity was assayed. Plasmid pCMV/β-gal was included to monitor the transfection efficiency.

III. RESULTS

Construction of the expression vector of α-Gal A

The cDNA PCR primers were designated to amplify the desired regions from cDNAs, and ligate the two PCR products (Fig. II-1) at the restriction enzyme BstXI site generated an in-frame fusion (Fig.II-2). Restriction enzymes digested the PCR product and subsequently the digested PCR products were inserted plasmid pUC19.

The expression plasmids, pCDNA3-α-GAL A, which contain the entire coding region of the wild type of α-GAL A, respectively, were generated as indicated in Fig.II-3. There was not any missense or nonsense point mutation in full sequence (Fig.II-4).

Production of recombinant α-GAL A

The total amount of DNA was maintained at 1 μg with an empty vector pCDNA3.0. Twelve hours after transfection, cells were harvested and protein extracts were performed in PBS. The level of recombinant α-GAL A activity was assayed with the artificial substrate 4-MUG.

Expression of the transfected α-GAL A cDNA was examined by enzyme assay. Both the cells without tranfection and with mock transfection

(transfected with vector alone) showed a very low activity, which was considered to be an endogeneous product of COS-7 cells.

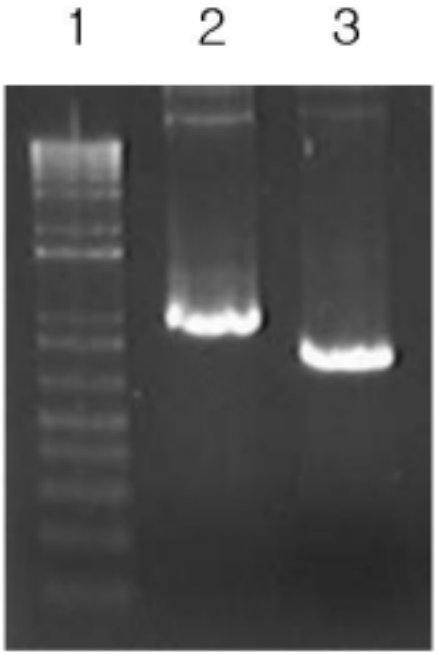

Figure II-1. Gel electrophoresis in wild type *α-GAL A* cDNA 1st PCR product.

Lane 1, 100 bp marker; lane 2: PCR product of fragment-1 of wild type α-GAL A; lane 3: PCR product of fagment-2 of wild type α-GAL A.

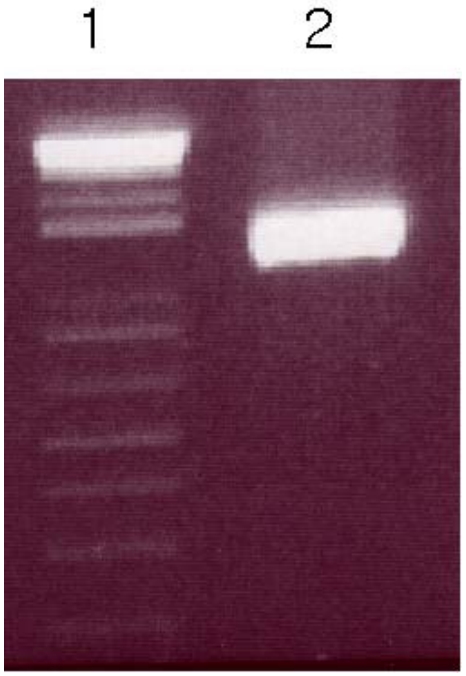

Figure II-2. Gel electrophoresis in wild type *α-GAL A* cDNA 2nd PCR product.

Lane 1: 100 bp marker; lane 2: 2nd PCR product of wild type α-GAL A**.**

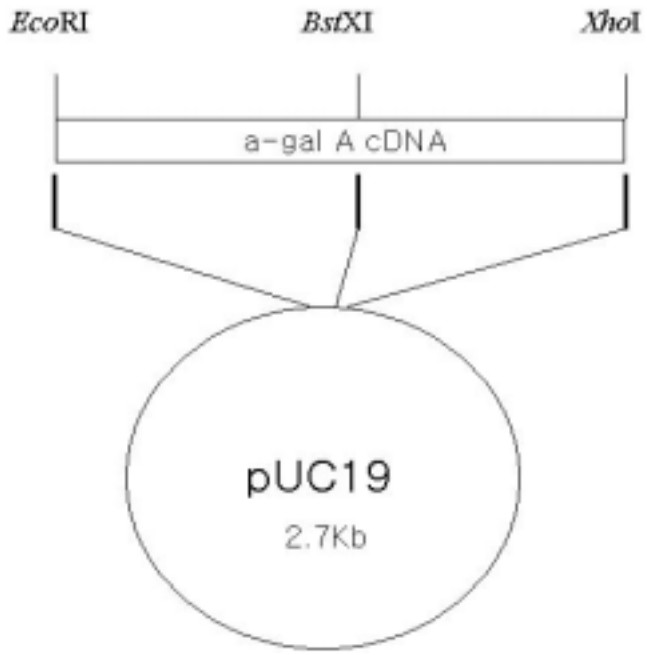

Figure II-3. The construction of α-GAL A cloning plasmids.

Wild type α-GAL A cDNA was inserted into the *Eco*RI and *Xho*I cloning site of pUC19 vector

Figure II-4. Entire DNA sequence of wild type *α-GAL A* cDNA.

Enzyme activity assay of wild type α-GAL A

To determine whether the α-GAL A constructs were expressed and the encoded proteins had enzyme activity, these constructs were expressed in COS-7 cells. Wild type α-GAL A enzyme was expressed in COS-7 cells by transfection with purified plasmids DNAs. Cells transfected with pCDNA3-a-gal A showed distinct α-GAL A enzyme activity compared to these with vector only. Enzyme activity was calculated as nmoles of 4-methylumbelliferone (fluorescent cleavage product of 4-methylumbelliferyl-*a*-D-galactoside) / mg of total protein/h (nmoles/mg/h). Lysates of COS-7 cells, transfected with the cDNA of the α-GAL A wild type, contained an activity of 440.5 ± 15 nmoles/mg/h (Fig. II-5). It showed a 200-fold increase of α-GAL A activity when it compared with those with a mock transfection. We have showed that a wild type α-GAL A has normal enzyme properties.

Optimal pH range for wild type α-Gal A

The pH stability was studied by incubation of enzyme at 37℃ for 30min at different pH. The reaction was terminated with 0.7 ml of 0.17 M glycine-carbonate buffer, pH 10.6. The fluorescence of each sample was measured.

The wild type α-GAL A was stable at pH range between 4.0 and 5.0 (Fig. II-9). The optimal pH for the wild type enzymes was 4.6.

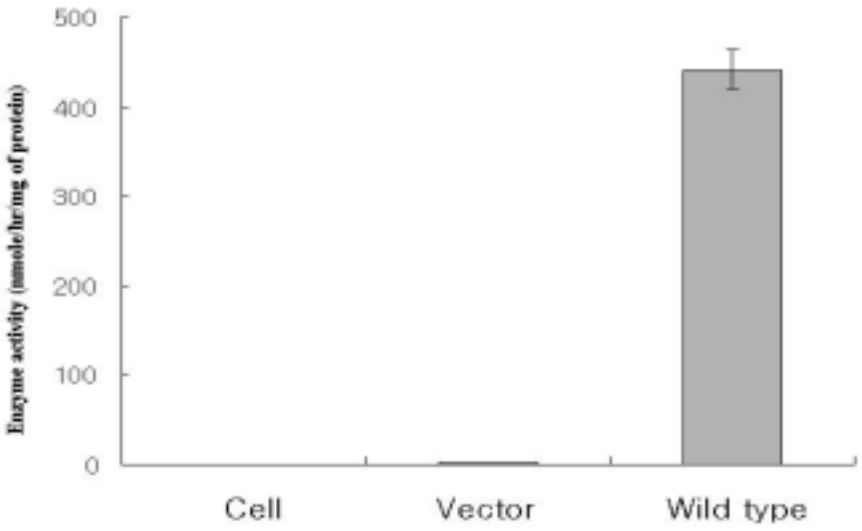

Figure II-5. Enzyme activity assay of wild type α-GAL A.

Values of α-GAL A enzyme activity represent as a unit (nmole/h/mg protein) control of the activity in cells transfected with wild type plasmids (n=3).

Thermal stability of wild type α-GAL A

At different time intervals, samples were incubated with 5 mM 4MU-galactopyranoside in 0.1 M sodium citrate buffer, pH 4.6, 75 mM N-acetylgalactosamine and assayed for residual activities toward 4MU-galactopyranoside. Then the sample assayed for residual activities toward 4MU-galactopyranoside. The wild type α-GAL A was maintained more 40% enzyme activity to 1 h. (Fig. II-10).

Mutagenesis of *α-Gal A*

Construction of plasmids carrying the substitution mutation of human *a*-GAL A was performed as described above materials and methods. The point mutations were confirmed by sequencing analysis. The mutant constructs are shown in Fig. II-6. The expression plasmids and pCDNA3-α-GAL A-MUT, which contain the entire coding region of the mutant form of α-GAL A, were constructed as described (Fig. II-7).

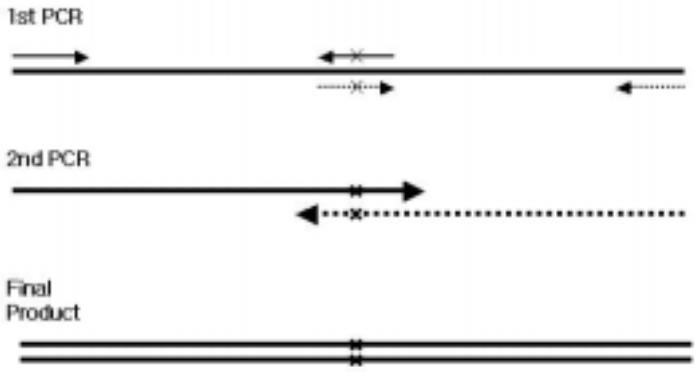

Figure II-6. Diagram of site-directed mutagenesis for construction of α-GAL A mutants. 1st PCR performed with mutated primer, then 2nd PCR used with product of 1st PCR as a template and primer.

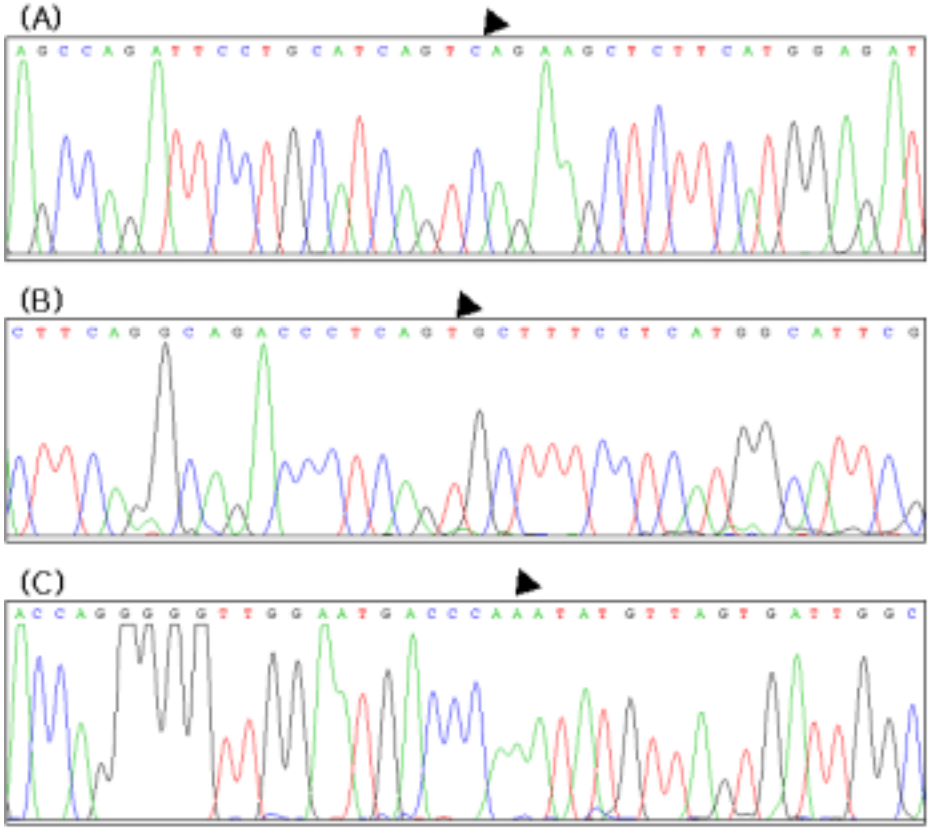

Figure II-7. Sequencing data of α-GAL A mutants form.

DNA sequence analysis of the mutant α-GAL A gene showed mutation. The arrows indicate nucleotide substitution. (A) E66Q, (B) R112C, (C) D266N

Enzyme activity assay of mutant α-GAL As

Cells transfected with pCDNA3-α-GAL A-R112C and pCDNA3-α-GAL A-D266N showed distinct α-GAL A enzyme activities (Fig. II-8). It showed complete loss of α-GAL A activity when it compared with those with a wild type α-GAL A transfection. The intracellular enzyme activities of R112C and D266N were 2.2% and 1.5% of normal, respectively. But E66Q mutant type did not showed complete loss of α-GAL A activity. This mutant had a low residual activity (~30%).

Optimal pH range for E66Q mutant α-Gal A

The pH stability assay of mutant protein was studied by incubation of enzyme at 37℃ for 30min as wild type enzyme. E66Q mutant α-GAL A was stable at pH range between 4.0 and 5.0 (Fig. II-9).

The optimal pH for the mutant enzymes was 4.6.

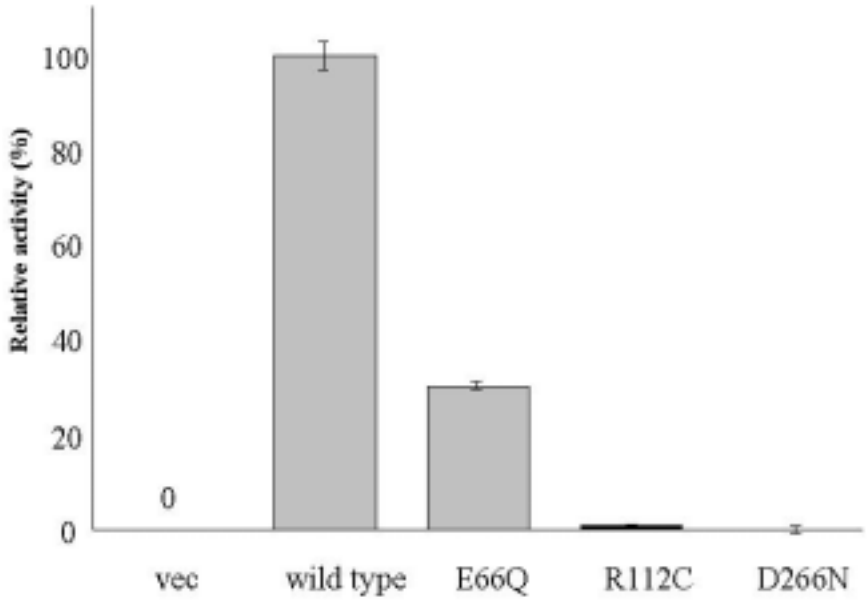

Figure II-8. Enzyme activity assay of mutant α-GAL As.

α-GAL A activities of the cell lysate were assayed by standard methods. Values of α-GAL A enzyme activity represent as percent control of the activity in cells transfected with wild type plasmids (n=3).

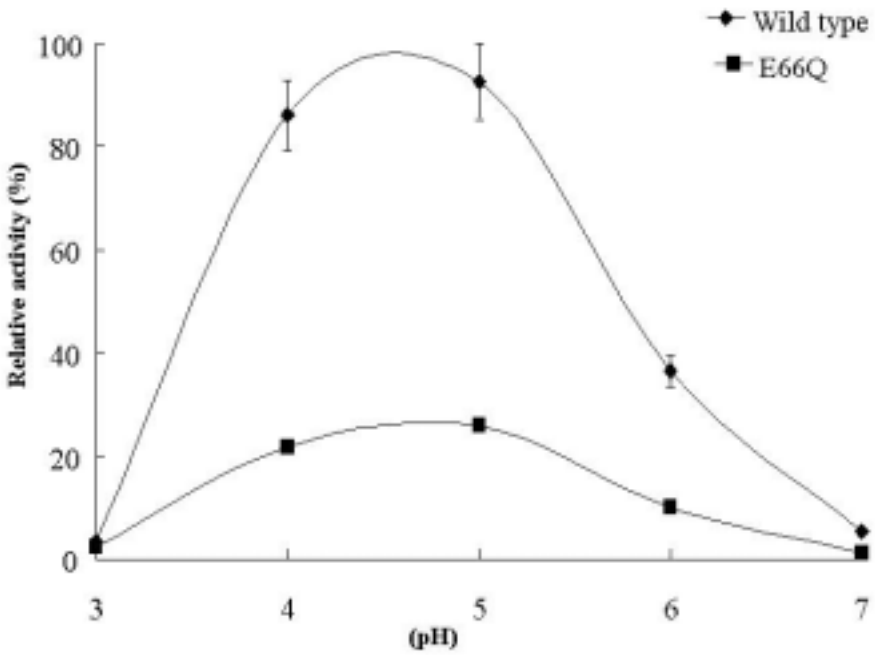

Figure II-9. Determination of pH range for E66Q mutant α-GAL A.

Values of α-GAL A enzyme activity represent as relative control of the high-activity in cells transfected with mutant plasmids (n=3).

Thermal stability of E66Q mutant α-GAL A

At various time intervals, samples were incubated and assayed for residual activities toward 4MU-galactopyranoside.

The E66Q mutant type α-GAL A result in lower stability compared to its wild type α-GAL A. The enzyme activities of the wild type α-GAL A after 5, 15 30, and 60 min of incubation at 37℃ maintained higher, than the E66Q α-GAL A (Fig. II-10).

Cotransfection of wild type and mutant of α-GAL A

To investigated possibility that α-GAL A mutant type have the dominant negative effect in the heterozygous genotype, pCDNA3-α-GAL A wild type and vector vehicle only or mutant pCDNA3-α-GAL A were cotransfected into COS-7 cells.

The experiments were performed in triplicate for each cotransfection. There was a significant decrease (~60%) in the α-GAL A activity of cells cotransfected with WT / mutant mixture compared with the wild type / vector only mixture (Fig. II-11).

This suggested that the mutant α-GAL A had a dominant negative effect in the heterozygous genotype.

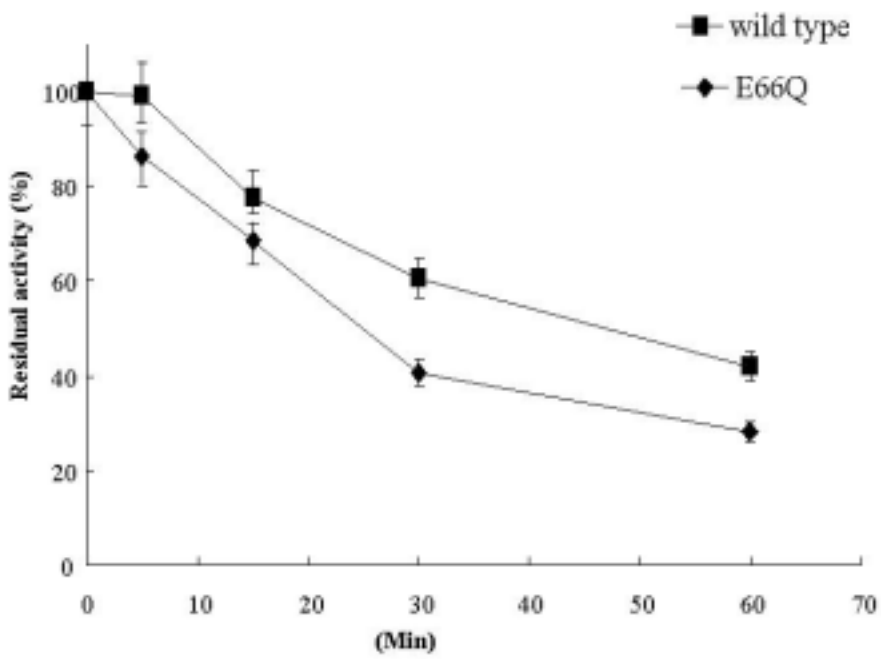

Figure II-10. Thermal stability of E66Q mutant α-GAL A.

Sample were incubated at 37℃ as the same condition of wild type α-GAL A. Values of α-GAL A enzyme activity represent as percent control of the activity in cells transfected with wild type or mutant type plasmids (n=3).

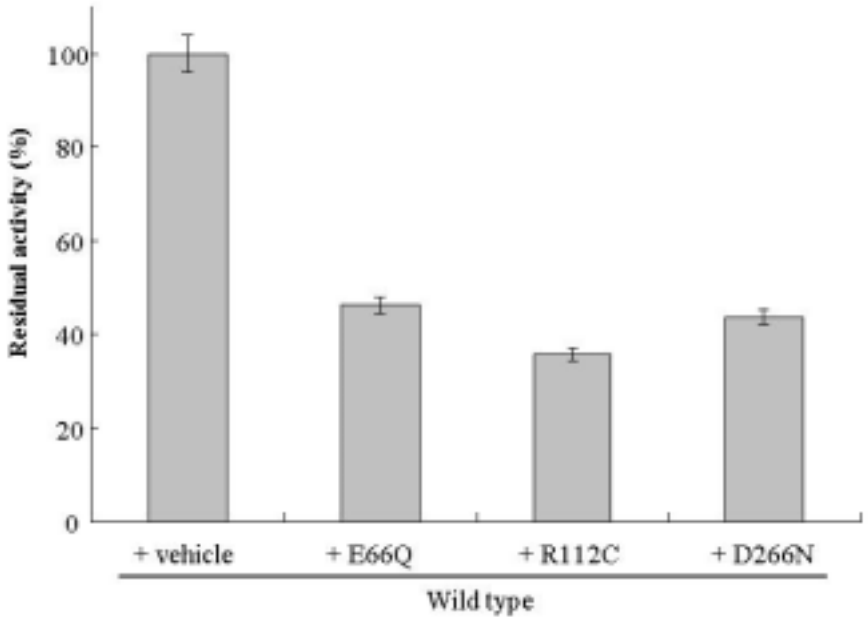

Figure II-11. α-GAL A enzyme assay of cotransfected plasmid in COS-7 cells.

Obtained data in each experiment was subtracted with that of vector/vector type transfection. Values of α-GAL A enzyme activity represent as percent control of the activity in cells transfected with vector / wild type mixtures plasmids (n=3).

Enzyme activity of E66Q mutant α-GAL A treated with DGJ

In some Fabry patients, a mutation in α-GAL A causes imperfect but flexible folding of the enzyme, whereas the catalytic center remains intact. DGJ acts as a chaperon to force the mutant enzyme to assume the proper conformation. We proposed the concept of chemical chaperon may assist a protein to fold properly and put into normal processing pathway.

However, E66Q mutant type α-GAL A did not show an increased enzyme activity compared with wild type α-GAL A (Fig. II-12). Our result indicates that chemical chaperon cannot be utilized as a therapy for this mutation.

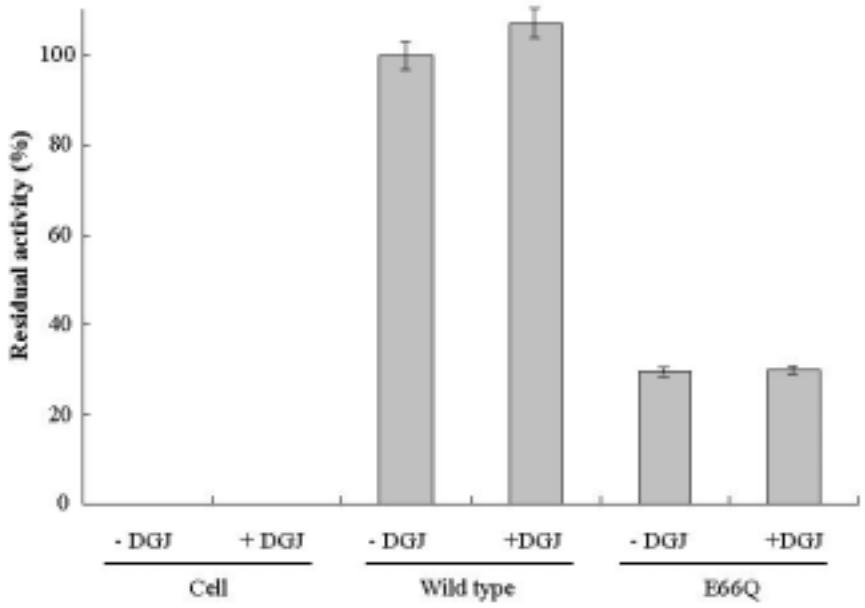

Figure II-12. α-GAL A enzyme assay in 20 uM DGJ treated COS-7 cells.

Obtained data in each experiment was subtracted with that of vector-DGJ treated cell. Values of α-GAL A enzyme activity represent as percent control of the activity in cells transfected with only wild type plasmids (n=3).

IV. DISCUSSION

Fabry disease is an X-linked recessive disorder caused by a deficiency of α-Gal A, which affects about 1 in 40,000 people (Desnick RJ and Bishop DF, 1989) and characterized by a generalized vasculopathy which in most cases appears in the second decade of life.

After the isolation and characterization of the cDNA and genomic sequences encoding human α-Gal A, a variety of molecular lesions causing Fabry disease has been demonstrated, as reviewed by Desnick (Christine ME and Desnick RJ, 1994).

A full-length cDNA has been cloned (Bishop DF. *et al.,* 1986; Bishop DF *et al.,* 1988) and its genomic structural organization analyzed (Kornreich R *et al.,* 1989). The 12 Kb genomic sequence has seven exons and the α-GAL A cDNA encodes a precursor peptide of 429 amino acids include a 31-residual signal peptide (Fig. II-4). Southern hybridization analysis has revealed that the frequency of large α-GAL A gene rearrangements in patients with Fabry disease is only 5-10% (Bernstein HS *et al.,* 1989; Sakuraba H, 1989).

In the present study, it was identified mutations of the human α-Gal A

gene E66Q, which resulted from a substitution of G for C, R112C, which resulted from a substitution of C for T and D266N, which resulted from a substitution of G for A (Fig. II-7).

Of the 19 arginines in the protein, 10 (53%) are found in the mutations of α-Gal A. The arginine side chain, with both an aliphatic portion and a planar charged guanidium group, can make simultaneous hydrophobic and ionic interactions. The preponderance of arginine mutations contrasts with the dearth of mutations in the chemically similar lysine, indicative of its particular importance of arginine to the folding of proteins (Borders CL *et al*., 1994). It had been reported that the recombinant mutant enzyme (Q279E and R301Q) did not significantly affect the enzymatic activity (Ryoichi K *et al.,* 2000). Other study also reported that amino acid substitutions (W277L, N278E, and V281R) did not affect the enzyme activity (Ishii S *et al*., 1995).

Among the more than 300 mutations described in unrelated patients with Fabry disease, a mutation encoding the substitution of arginine for histidine at position 112 (R112H), R301Q, and G328R have been identified in patients with either the classic phenotype or the cardiac-variant phenotype (Ishii S *et al*., 1994; Ashton-Prolla P *et al*., 2000; Eng CM *et al*., 1994; Sakuraba H *et al*., 1990). These missense mutations presumably allow extremely low levels of

residual activity that can be stabilized by certain substrates.

In our *in vitro* assays of α-Gal A activity, the R112C and D266N mutations caused almost complete loss of the enzymatic activity (Fig. II-8), while the E66Q mutant type showed approximately 30% of the residual α-Gal A activity with kinetic properties similar to the wild type of α-Gal A (Fig. II-9).

It had been reported that the recombinant mutant enzyme (Q279E) had 15% of enzyme activity (Ishii S *et al*., 1993). Other report described that amino acid substitutions (W277L, N278E, and V281R) did not affect the enzyme activity (Ishii S *et al*., 1995). However, Q279E and R301Q mutant types did not significantly affect the enzyme activity in atypical variant, but the mutant protein levels were decreased presumably in the ER of the cells (Ryoichi K *et al.,* 2000).

Our mutant type E66Q enzyme did not show any alteration in thermostability (Fig. II-10).

A few heterozygotes have previously been reported in whom the expression of the disease was comparable to that observed in severely affected hemizygous males (Ferrans VJ *et al*., 1969; Desnick RJ *et al.,* 1972; Rietra PJGM et al., 1976).

In contrast, obligate heterozygotes without any clinical manifestations and with normal levels of leukocyte α-Gal A have been reported (Rietra PJGM *et al*., 1976; Avila JL *et al*., 1973). Such a markedly variable expression is expected in female heterozygous for X-linked disease due to random X inactivation (Romeo G *et al*., 1983). At the cellular level, carriers of Fabry disease have been shown to have two distinct clonal populations of cells, one with normal and the other with defective α-Gal A activity, providing evidence of random X inactivation (Romeo G and Migeon BR. 1983).

To investigate whether there might be some interaction between the normal and mutant α-GAL A proteins such as heterodimerization, since this enzyme is known to exist as a homodimer (Kornreich R *et al*., 1989), we cotransfected equal amounts of wild type and mutant cDNAs into COS-7 cells for transient expression. The resultant α-Gal A activity was 50% of that of cells transfected with wild type cDNA alone (Fig. II-11). This could be due to an interaction between the wild type and mutant α-Gal A proteins.

Studies on different mutant α-Gal A enzymes have shown that the residual enzymatic activity encoded by two other mutations in the cardiac variant of Fabry disease-one encoding the substitution of glutamine for glutamic acid at position 279 (Q279E) and one encoding the substitution of arginine for

glutamine at position 301 (R301Q) is markedly increased by galactose (Okumiya T *et al*., 1995) and 1-deoxygalactonejirimycin (another reversible competitive inhibitor of the enzyme)(Fan JQ *et al*., 1999; Asano N *et al*., 2000), whereas the activity of the G328R mutant enzyme was minimally enhanced. Such *in vitro* experiments may be used to predict which patient with a cardiac variant of Fabry disease (or even the classic disease) will benefit from galactose therapy.

To investigate that chemical chaperon therapy is applicable to E66Q mutant, we transfected E66Q mutant and cultured with 20 uM DGJ for 1 day in COS-7 cell. Our results were that there was no increased enzyme activity by treatment with DGJ (Fig. II-12), indicating chemical chaperon cannot be used as a therapy for this mutation.

In summary, by the expression study of E66Q, R112C and D266N mutant α-GAL A, we could demonstrate that these mutations led to a complete depletion of enzyme activity, except E66Q, and the mutant *a*-GAL A had a dominant negative effect in heterozygous type.

CONCLUSIONS

In this study, α-GAL A mutations were identified in patients with Fabry disease and experiments were carried out to investigate effects of α-GAL A mutant types on protein expression patterns, enzyme activity and their functions in COS-7 cells

1. The present results strongly suggested that the missense mutations, E66Q, R112C, and D266N in the α-GAL A gene caused an extremely low enzyme activity in patients and gave rise to a typical form of Fabry disease.
2. A 1392-base pair gene that encodes human α-GAL A has been cloned and sequenced to know whether there was any mutation in full sequence.
3. Recombinant of wild type α-GAL A had normal kinetic and functional activity. Its enzyme activity was about 400nmole/h/mg protein in COS-7 cells
4. Three mutant α-GAL A (E66Q, R112C, and D266N) plasmids were constructed by site-directed mutagenesis.
5. R112C and D266N mutant types led to the complete loss of α-GAL A

activity in transfected COS-7 cells.

6. E66Q α-GAL A mutant showed a residual activity. It showed about 30% of wild type α-GAL A .
7. Studies on residual activities of mutant α-GAL A showed that they had kinetic properties similar to those of wild type α-GAL A, but was less stable
8. Enzyme activity had a significant decrease (~50%) in the α-GAL A in cells cotransfected with wild type / mutant mixture compared with the wild type / vector only mixture.
9. When E66Q α-GAL A mutant type was transiently expressed in cos-7 cells treated with DGJ, it did not increased enzyme activity compared wild type α-GAL A. This result indicates that chemical chaperon can not be used as a therapy for this mutation.

In conclusion, three mutations were identified in Korean patients with Fabry disease, subsequently all the mutants were transiently expressed in cos-7 cells to elucidate the effect of genotype on the structure and function of the α-GAL A protein. However, there are some issues that have to be addressed.

First, in most patients, there is complete loss of enzyme activity. But, in case of E66Q mutant type, transient expression showed some residual activity of *a*-GAL A enzyme. However, phenotype of a patient carrying this mutation is severe and early onset. Therefore, there is no genotype-phenotype correlation in this mutation. Modifying gene or modifying SNPs might be involved for the development of severe phenotype in this case.

Second, there was no, if any, investigation about mutant enzyme structure and domain. Mutated proteins were not purified in this study, which hampered the characterization of properties of mutant protein in detail.

To solve these problems, mutated proteins have to be purified, and kinetic studies showed be done. Further experiments will be needed to clarify genotype-phenotype correlation, such as investigation of modifying gene or SNPs.

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국문 요약

Alpha-galactosidase A 의 유전적 돌연변이 확인 및 변이 단백질 기능에 대한 연구

페브리병은 X-염색체 관련질환으로 열성으로 유전되는 라이소자임 대사질환이다. 이 질환은 라이소자임의 α-GAL A 라고 불리는 효소의 결핍으로 나타나는데 말단에 a-galactosyl 부분을 가지고 있는 글리코스핑고리피드(glycosphingolipid)의 체내 축적을 야기한다. 한국인에서 페브리 질환의 경우 유전적 돌연변이는 다양하게 보고가 되어있지만, 이러한 변이는 인종간에 많은 차이를 보이고, 질환에서 결핍되어 나타나는 효소의 변이와 연관된 단백질의 기능에 대한 연구는 미비한 실정이다.

우리는 페브리 질환이 의심되는 환자의 유전자를 이용하여 이 실험을 진행하였다. 먼저 환자의 말초혈액에서 DNA 을 분리 후 중합효소연쇄반응을 이용하여 DNA를 증폭 후 SSCP 를 이용하여 변이가 의심되는 엑손을 찾아내고, 이 시료를 가지고 DNA 염기서열 분석을 시행하였다.

염기서열 분석 결과 엑손 2번에서 2종류의 변이, E66Q, R112C 를 알아낼 수 있었고, 엑손 5번에서 D266N 등, 3종류의 유전자 변이를 발견할 수 있었다. D266N 변이의 경우는 처음으로 알아낸 유전적 돌연변이였다.

다음으로 이러한 환자에서 나타나는 돌연변이가 페브리 질환에서 효소의 정상적인 기능에 어떠한 영향을 주는지 알기 위해 페브리 질환의 결핍 효소인 α-GAL A의 정상유전자를 클로닝하여 이렇게 만들어진 정상유전자와 변이된 유전자의 세포내 발현을 통해 효소의 특징을 확인하고자 하였다.

먼저 정상인의 유전자에서 mRNA를 분리 후 이것을 이용하여 정상적인 α-GAL A 단백발현백터를 만들고, 이것을 가지고 COS-7 원숭이의 신장세포에서 발현을 유도하여 정상의 α-GAL A 효소 단백질과 같은 기능을 하는 것을 확인하였다. 이렇게 만들어진 효소는 효소 활성화에서 약 440 nmole/h/mg prot. 의 수치를 보였고, 가장 높은 활성화를 나타내는 pH 는 4.6을 보였다.

E66Q, R112C, 그리고 D266N 등의 변이된 효소는 site-directed mutagenesis 방법을 이용하여 만들어 내고, 변이된 유전자의 효소 기능 차이를 알아보고자 하였다.

먼저 효소의 활성화에 대한 차이로 정상적인 유전자의 경우와

비교하여 볼 때, R112C, D266N 의 경우에는 효소의 활성화가 거의 나타나지 않는 것을 알 수가 있었다. E66Q의 경우는 효소의 활성화가 약 30% 정도 나타났지만 pH 에 따라 바뀌는 활성도의 변이는 거의 일치하였고, 효소 활성도의 안정성은 정상의 경우에 비교하여 변이된 단백질의 경우 약 20% 정도 낮게 나타나는 것을 보임으로 열에 대해서는 상대적으로 약한 것을 알 수 있었다.

정상적인 효소는 라이소자임에서 동종이합체로 존재를 하는데 정상적인 유전자와 함께 존재하는 경우 변이된 유전자에서 만들어지는 단백질이 정상적인 단백질의 효소 기능에도 영향을 주는 지에 대해 두 종류의 유전자를 동시에 발현시킨 결과, 이러한 변이된 유전자의 효소가 정상적인 효소의 기능을 방해하는 것으로 나타났다. 또한 최근 효소의 구조의 안정을 유도하여 활성도를 증가시키는 것으로 알려진 DGJ의 처리를 한 경우, 활성도에는 큰 변이는 보이지 않았다.

본 실험을 통하여 페브리 질환에서 나타나는 유전적인 변이를 찾아서 확인하고, 이러한 변이가 정상적인 효소의 기능과 비교하여 어떠한 차이를 나타내는지 확인할 수가 있었으며 이러한 변이 효소가 정상적인 효소의 기능에도 또한 억제하는 기능을 보이고 있음을 알 수가 있었다.

핵심어: 페브리 질환, 유전적 변이, X-염색체 질환, α-GAL A

감사하는 마음으로

참으로 긴 시간이 지나갔습니다.

이제야 춥고 긴 겨울을 지나와 따스한 봄날을 맞이하는 기분입니다. 돌이켜 보면 세상을 다 가진 듯 행복한 시간도 있었고, 가슴이 터질 것 같아 잠을 이루지 못할 만큼 아픈 시간도 있었습니다. 하지만 전 이제 하나의 일을 마치고 새로운 시작을 하려 합니다.

제가 지금이 저로 만들어주신 김태우 선생님, 양용석 선생님, 오옥두 선생님. 김종배 선생님, 그리고 지금은 미국에 계시지만 많은 도움을 주신 박용석 선생님 이 모든 분들에게 다시 한번 머리 숙여 깊이 감사의 마음을 전하고자 합니다.

전혀 모르는 유전질환에 대해 가르쳐 주시고 많은 지도를 해 주신 아산 병원에 유한욱 선생님, 서을주 선생님께 깊은 감사의 마음을 전합니다. 늘 함께하며 같이 한 아산병원에 김구한 선생님, 그리고 유전한 클리닉에 많은 선생님들에게도 감사드리고, 우리 유전체 연구센터 식구들에게도 감사드립니다.

늘 옆에서 힘이되주는 윤석이 형, 먼저 박사가 된 인식, 늘 구박을 받으면서도 잘 따라준 우리 대학원 후배, 승주, 인수, 호중, 그 외에도 열거할 수 없을 만큼 많은 후배들에게 많은 고마움을 표합니다.

그리고 강한 아들이 되길 바라시는 아버지, 늘 자상하신 어머니, 그리고 이제는 시집을 가서 아기 엄마가 된 두 동생, 성희와 현희에게도 옆에서 늘 힘이 되어주어 고맙다는 말을 전합니다. 학생인 사위를 두셔서 늘 걱정하시는 우리 장인, 장모님과 두 처제, 그리고 이제 공부를 다시 시작하는 처남에게도 늘 옆에 있어서 힘이 되어 준 것을 감사드립니다.

이제는 멀리 미국에 가 있는 석준 형, 선희 누나에게 한결 같은 마음으로 대해 주신것에 고마운 마음을 전합니다.

이렇게 글을 쓰다 보니 너무나 많은 분들에게 신세만 지고 살아오게 되어 죄송한 마음을 나타낼 수 조차 없습니다.

이제는 다시 시작을 하려 합니다. 겨울을 지나 새봄을 맞이하고, 이제는 뜨거운 여름을 준비해야 하는 위치에 서있습니다.

새로운 출발을 하는 저에게 항상 여러분들이 함께 하고 있다는 믿음을 주시고 힘을 주시어, 작은 힘을 가지고 시작하는 저의 앞에 등불이 되어 주십시오.

마지막으로 이 세상을 함께 할 동반자, 남화경과 우리 아들 영빈이에게 이 고마움을 전합니다.

다시 시작하는 마음으로