AN ATYPICAL VARIANT OF FABRY'S DISEASE IN MEN WITH LEFT VENTRICULAR HYPERTROPHY

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Abstract *Background.* *Fabry's disease is an x-linked recessive disorder that results from a deficiency of α-galactosidase. Left ventricular hypertrophy is one of the common manifestations in men with classic hemizygous disease. Recently, several cases of an atypical variant of hemizygous Fabry's disease, with manifestations limited to the heart, have been reported. Therefore, we assessed the incidence of hemizygosity for Fabry's disease among male patients with left ventricular hypertrophy.*

Methods. *We measured plasma α-galactosidase activity in 230 consecutive male patients with left ventricular hypertrophy. Clinical manifestations were assessed, endomyocardial biopsies were performed, and the patients were screened for mutations in the α-galactosidase gene.*

Results. *Seven of the 230 patients with left ventricular hypertrophy (3 percent) had low plasma α-galactosidase activity (4 to 14 percent of the mean value in normal controls). These seven unrelated patients, ranging in age from 55 to 72 years, did not have angiokeratoma, acroparesthesias, hypohidrosis, or corneal opacities, which are typical manifestations of Fabry's disease. Endomyocardial biopsy was performed in five patients and revealed marked sarcoplasmic vacuolization in all five. Samples from four patients were examined by electron microscopy and revealed typical lysosomal inclusions with a concentric lamellar configuration in all four. Two patients had novel missense mutations in exon 1 and exon 6. The remaining five had no mutations in the coding region of the α-galactosidase gene, but the amounts of the α-galactosidase messenger RNA were markedly lower than normal.*

Conclusions. *Seven unrelated patients with atypical variants of hemizygous Fabry's disease were found among 230 men with left ventricular hypertrophy. Fabry's disease should be considered as a cause of unexplained left ventricular hypertrophy. (N Engl J Med 1995;333:288-93.)*

FABRY'S DISEASE is an X-linked recessive disease resulting from a deficiency of the lysosomal hydrolase α-galactosidase.[1-3] This enzymatic defect leads to the progressive accumulation of glycosphingolipids, predominantly globotriaosylceramide, throughout the body, particularly in the skin, kidney, nervous system, eye, and heart.[4] The clinical manifestations differ between the classic form and the atypical hemizygous form. In male patients with the classic hemizygous form, acroparesthesias, hypohidrosis, corneal opacities, and dysfunction of the kidney, brain, and heart are observed.[1] Various cardiac manifestations, including left ventricular hypertrophy, valvular involvement, and arrhythmias, have been reported.[4-21]

Recently, several cases of an atypical variant of Fabry's disease, with manifestations limited to the heart, have been reported.[16-19] In patients with this type of Fabry's disease, the diagnosis was made by the pathological study of endomyocardial-biopsy specimens or autopsy specimens of the heart. These patients had left ventricular hypertrophy as a result of the deposition of globotriaosylceramide in the cardiomyocytes. However, there has been only a single report of the incidence of Fabry's disease among patients with cardiac symptoms; in that study the disease was found by endomyocardial biopsy.[12] The purpose of the current study was to clarify the incidence of Fabry's disease among male patients with left ventricular hypertrophy, to examine the clinical characteristics of these patients, and to detect mutations in the α-galactosidase gene.

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METHODS

We prospectively studied male patients who were seen at the cardiovascular division of Kagoshima University Hospital from October 1992 to June 1993. All male patients who had cardiac symptoms, cardiac murmurs, arrhythmias, hypertension, abnormal electrocardiograms, or enlargement of the cardiac silhouette on chest radiography were examined by echocardiography. A total of 1603 male patients underwent echocardiography, and 230 (14 percent) were found to have left ventricular hypertrophy. The criterion for the diagnosis of left ventricular hypertrophy was a ventricular-septum or posterior-wall thickness of at least 13 mm (or both) on echocardiography.[22] The patients with left ventricular hypertrophy ranged in age from 16 to 87 years (mean [±SD], 62±13). Eighty-nine normal, healthy male subjects ranging from 14 to 80 years of age (mean, 52±19) were used as controls. The research protocol was reviewed and approved by the review board of Kagoshima University Hospital.

Plasma α-galactosidase activity was measured in all patients with left ventricular hypertrophy and all normal subjects. The assay was performed with the fluorogenic substrate 4-methylumbelliferyl-α-D-galactopyranoside (Sigma Chemical, St. Louis), with *N*-acetyl-D-galactosamine (Nacalai Tesque, Kyoto, Japan) used as an inhibitor of α-*N*-acetylgalactosaminidase.[23] Seven patients who had no or very low plasma α-galactosidase activity were given a diagnosis of Fabry's disease.[3] Lymphocyte α-galactosidase activity was then measured in these patients and in 43 controls. Clinical manifestations of Fabry's disease, such as angiokeratoma, acroparesthesias, hypohidrosis, corneal opacities, albuminuria, and cerebrovascular damage, were also assessed.

Endomyocardial biopsies were performed in the patients given a diagnosis of Fabry's disease after written informed consent was obtained. The specimens were fixed in 10 percent formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin for light-microscopical analysis. For electron microscopy, the specimens were immersed in a fixative containing 3 percent glutaraldehyde and 2 percent paraformaldehyde buffered with 0.1 M phos-

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phate buffer (pH 7.4). They were then post-fixed with osmium tetroxide, dehydrated in a graded series of ethanol baths, and embedded in epoxy resin. Ultrathin sections were cut, double-stained with uranyl acetate and lead citrate, and examined with an electron microscope (model H-7100, Hitachi, Tokyo, Japan).

Heparin-treated blood samples were obtained from the seven patients given a diagnosis of Fabry's disease after they provided informed consent. Genomic DNA was isolated from the whole blood with a DNA extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Lymphoblast cell lines were established for six of the seven patients (Patients 2 through 7), with use of the Epstein–Barr virus.[24] The cell lines were maintained in RPMI-1640 medium supplemented with 10 percent fetal-calf serum and 1 percent penicillin–streptomycin (GIBCO, Grand Island, N.Y.) at 37°C in 5 percent carbon dioxide. A lymphoblast cell line for Patient 1 could not be established. Total RNA was prepared from the cultured lymphoblasts by the acid guanidium thiocyanate–phenol–chloroform extraction method.[25] Poly(A)$^+$RNA was purified by oligodeoxythymidine–cellulose column chromatography.[25]

Northern hybridization analysis was performed with poly(A)$^+$RNA as a sample according to the standard method.[26] Poly(A)$^+$RNA was electrophoretically separated in formaldehyde–agarose gel, transferred by capillary blotting to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, United Kingdom), and hybridized with a full-length α-galactosidase complementary DNA (cDNA) probe labeled with [α-^{32}P]deoxycytidine triphosphate according to standard techniques.[26] After autoradiography, the filter was washed with 0.1 percent sodium dodecyl sulfate in water at 95°C for 10 minutes, and a second hybridization was performed with an [α-^{32}P]deoxycytidine triphosphate–labeled β-actin cDNA as a control.

Four biotinylated sense oligonucleotide primers and four antisense oligonucleotide primers were used to amplify exons 1 through 7.[27] The polymerase chain reaction (PCR) was used to amplify genomic DNA from each of the seven patients with Fabry's disease with the respective primer sets. The double-stranded PCR products were denatured, and the biotinylated single strands were isolated by affinity capture with streptavidin-coated magnetic beads (Dynabeads M-280; Dynal, Norway). The biotinylated single-stranded PCR products were then subjected to dideoxy-chain-termination sequencing with α-galactosidase–specific sequencing primers.[27]

A study of the transient expression of α-galactosidase was done with COS-1 cells.[28] We transfected COS-1 cells in 60-mm tissue-culture dishes with 20 μg of plasmid DNA per dish that contained either normal or mutant α-galactosidase cDNA, using the calcium phosphate–glycerol shock technique.[29] The transfected cells were incubated in Ham's F10 medium supplemented with 10 percent fetal-calf serum, harvested in phosphate-buffered saline after 72 hours, and used for the α-galactosidase assay and immunoblotting. For immunoblotting, 25 μg of protein from cell lysates was subjected to sodium dodecyl sulfate–polyacrylamide-gel electrophoresis on a 12.5 percent slab gel, transferred to a nylon membrane (Hybond-N), and allowed to react with an anti–α-galactosidase antibody.[30]

RESULTS

Of the 230 patients who had left ventricular hypertrophy, 121 had systemic hypertension, 27 had asymmetric septal hypertrophy, 8 had apical hypertrophy, 9 had aortic valvular stenosis, and 7 had aortic valvular regurgitation.[31,32] Five of the 27 patients with asymmetric septal hypertrophy and 5 of the 8 patients with apical hypertrophy also had systemic hypertension. The remaining 58 patients did not have any findings indicating systemic hypertension, asymmetric septal hypertrophy, apical hypertrophy, aortic valvular stenosis, or aortic valvular regurgitation.

The values for plasma α-galactosidase activity in the 89 controls ranged from 4.8 to 17.6 nmol per hour per milliliter (mean [±SD], 8.4±2.4). Very low levels of plasma α-galactosidase activity were found in 7 of the 230 patients with left ventricular hypertrophy (3 percent). These values ranged from 0.4 to 1.2 nmol per hour per milliliter, approximately 4 to 14 percent of the mean value in the controls. The values for α-galactosidase activity in the remaining 223 patients ranged from 4.5 to 17.8 nmol per hour per milliliter (mean, 8.5±2.4) (Fig. 1). The seven patients with low α-galactosidase activity were diagnosed as having hemizygous Fabry's disease. The lymphocyte α-galactosidase activity in Patients 2 through 7 ranged from 1.6 to 8.4 nmol per hour per milligram of protein (Table 1). These values were approximately 3 to 18 percent of the mean values in 43 controls (mean, 46.4±6.5; range, 33.4 to 60.9).

Endomyocardial biopsies were performed in five of the seven patients with hemizygous Fabry's disease. The myocardial cells showed marked sarcoplasmic vacuolization, leaving large clear spaces in the paraffin-embedded sections stained with hematoxylin and eosin.

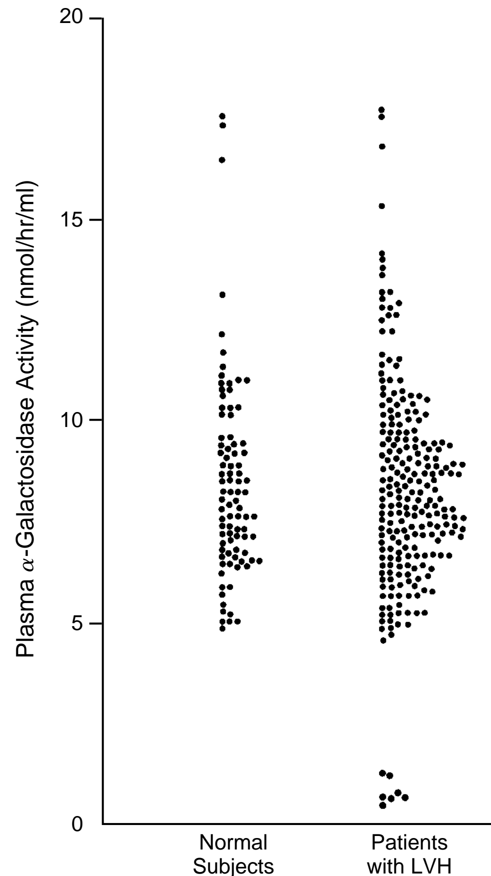

Figure 1. Plasma α-Galactosidase Activity in 89 Normal Male Subjects and 230 Male Patients with Left Ventricular Hypertrophy (LVH).

The values in the normal subjects ranged from 4.8 to 17.6 nmol per hour per milliliter (mean, 8.4±2.4). In seven patients with left ventricular hypertrophy, the values ranged from 0.4 to 1.2 nmol per hour per milliliter. In the remaining 223 patients with left ventricular hypertrophy, the values ranged from 4.5 to 17.8 nmol per hour per milliliter (mean, 8.5±2.4).

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*Table 1. Characteristics of Seven Patients with Hemizygous Fabry's Disease.**

CHARACTERISTIC	PATIENT NO.						
	1	2	3	4	5	6	7
Age (yr)	66	69	62	62	55	70	72
Left ventricular wall thickness (mm)							
Interventricular septum	20	20	13	13	16	15	15
Posterior wall	20	17	13	12	16	15	14
α-Galactosidase activity†							
In plasma (nmol/hr/ml)	1.2	0.6	1.2	0.6	0.4	0.7	0.6
In lymphocytes (nmol/hr/mg of protein)	NE	8.4	6.1	1.7	1.6	4.1	4.2
Endomyocardial-biopsy findings							
Light microscopy (vacuolization)	+	+	+	+	NE	+	NE
Electron microscopy (inclusions)	+	NE	+	+	NE	+	NE
Coronary angiogram	Normal	Normal	Normal	Normal	NE	Normal	Normal
Blood pressure (mm Hg)	130/80	124/60	108/60	170/110	106/72	130/60	104/60
Albuminuria	–	–	–	+	–	–	–
Serum creatinine (mg/dl)‡	1.2	1.1	0.9	1.3	1.1	1.2	0.9
Cerebrovascular damage	–	–	–	Bleeding	–	Infarction	–
Angiokeratoma	–	–	–	–	–	–	–
Acroparesthesias	–	–	–	–	–	–	–
Hypohidrosis	–	–	–	–	–	–	–
Corneal opacities	–	–	–	–	–	–	–

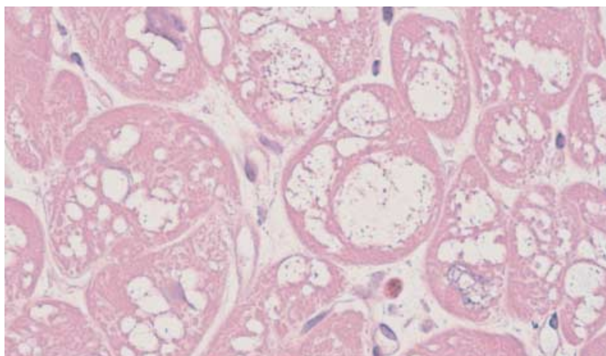
*NE denotes not examined. A plus sign indicates the presence of a finding, and a minus sign its absence.

†Values in normal subjects ranged from 4.8 to 17.6 nmol per hour per milliliter (mean, 8.4±2.4; n=89) in plasma and from 33.4 to 60.9 nmol per hour per milligram of protein (46.4±6.5; n=43) in lymphocytes.

‡To convert values for serum creatinine to micromoles per liter, multiply by 88.4.

Specimens from four of the patients were examined by electron microscopy, and typical lysosomal inclusions with a concentric lamellar configuration were observed in the sarcoplasm of myocardial cells in all four (Fig. 2).

Table 1 shows the clinical characteristics of the seven patients with hemizygous Fabry's disease. The patients ranged in age from 55 to 72 years and were not related. Left ventricular wall thickness ranged from 13 to 20 mm. None of the patients had asymmetric septal hypertrophy, apical hypertrophy, obstruction of the left ventricular outflow tract, or aortic valvular disease. Coronary angiograms showed normal coronary arteries in all six patients studied (Patients 1, 2, 3, 4, 6, and 7). Two had histories of a cerebrovascular accident without paresis. Computed tomography revealed cerebral hemorrhage (Patient 4) and infarction (Patient 6) in two patients. One of these patients (Patient 4) had systemic hypertension (blood pressure, 170/110



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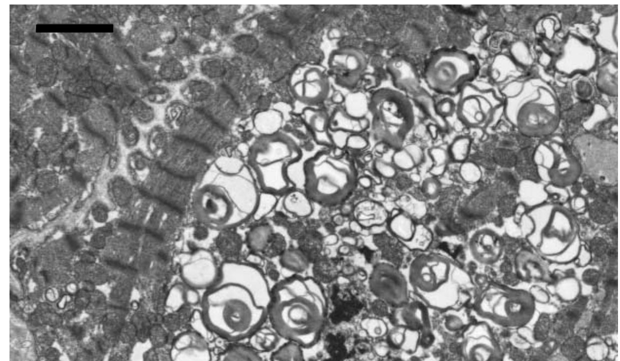

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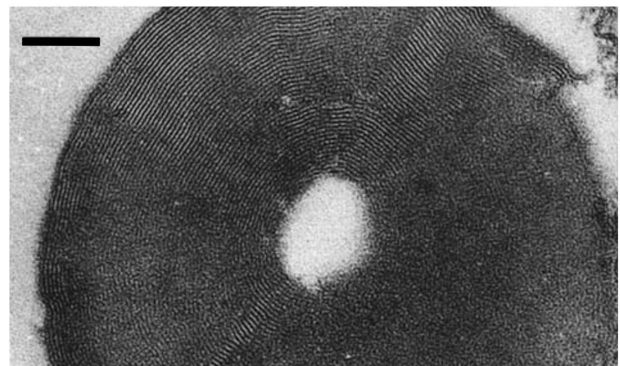

C

Figure 2. Photomicrographs of Myocardial Cells in an Endomyocardial-Biopsy Specimen from Patient 4.

Panel A shows marked sarcoplasmic vacuolization, leaving large clear spaces in the myocardial cells (hematoxylin and eosin, ×460). Panels B and C show typical lysosomal inclusions with a concentric lamellar configuration on electron microscopy. The black bar indicates 3 μm in Panel B, and 0.1 μm in Panel C.

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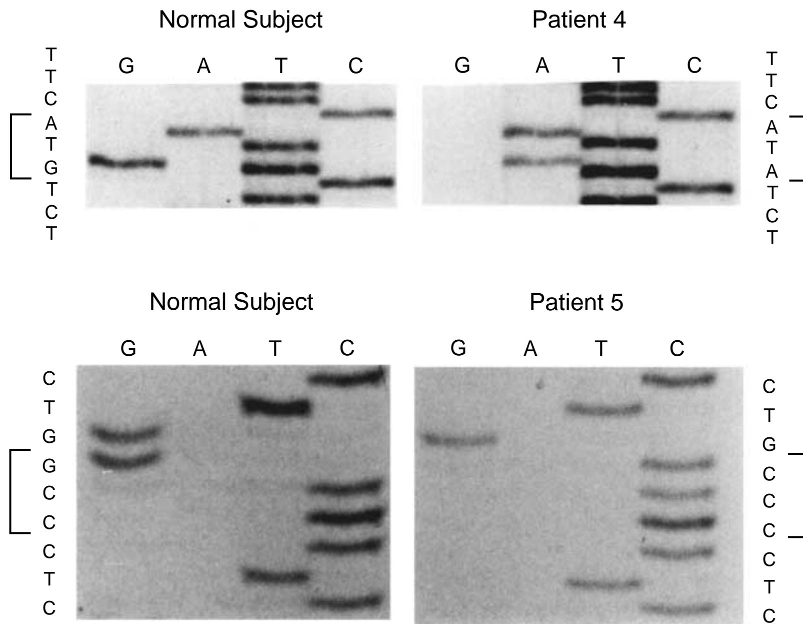

Figure 3. Partial DNA Sequence of α-Galactosidase Exon 6 (Upper Panel), Indicating the G-to-A Shift at Nucleotide 888 (Met296Ile) in Patient 4, and α-Galactosidase Exon 1 (Lower Panel), Indicating the G-to-C Shift at Nucleotide 58 (Ala20Pro) in Patient 5.

mm Hg), albuminuria, and a serum creatinine concentration of 1.3 mg per deciliter (115 μmol per liter). Angiokeratoma, acroparesthesias, hypohidrosis, or corneal opacities were not observed in any of the seven patients.

Three of the seven patients had cardiac symptoms. Two had dyspnea (Patients 1 and 5), and one had palpitations (Patient 2). The reason for echocardiographic examination was dyspnea in Patients 1 and 5, palpitations in Patient 2, premature ventricular contractions in Patients 3 and 6, systemic hypertension in Patient 4, and the finding of left ventricular hypertrophy on electrocardiography in Patient 7. Three patients (Patients 1, 3, and 6) had ventricular arrhythmias, and two (Patients 2 and 5) had supraventricular arrhythmias. None of the patients had a short PR interval or mitral-valve disease, including mitral-valve prolapse.

Genomic amplification and solid-phase direct sequencing analysis of single-stranded genomic DNA from the seven patients identified two missense mutations, in Patients 4 and 5 (Fig. 3 and Table 2). The missense mutation in Patient 4 was a G-to-A transition at nucleotide 888 in exon 6 of the coding sequence, which predicted the substitution of isoleucine for methionine at residue 296 (Met296Ile). The mutant cDNA expressed low α-galactosidase activity in COS-1 cells in the subsequent expression study. The activity of the mutant cDNA was 24 percent that of the wild-type cDNA. The enzyme activity was proportional to the amount of enzyme protein, as estimated by Western blotting (Fig. 4). The mutant product expressed in Patient 4 therefore appears to maintain a normal level of catalytic activity per molecule. The missense mutation in Patient 5 was a G-to-C transition at nucleotide 58 in exon 1 of the coding sequence, which predicted the substitution of proline for alanine at residue 20 (Ala20Pro). This missense mutation is in the coding region for the signal peptide of α-galactosidase.[33]

Northern analysis revealed moderate decreases in the amounts of α-galactosidase messenger RNA in these two patients (Patients 4 and 5), as compared with the control. In the other patients (Patients 2, 3, 6, and 7), no gene mutations were found in the full coding region for α-galactosidase, but the amounts of α-galactosidase messenger RNA were markedly decreased (Fig. 5 and Table 2).

Discussion

We identified 7 patients with hemizygous Fabry's disease by measuring plasma α-galactosidase in 230 unselected men with left ventricular hypertrophy. This biochemical examination has been reported to be a reliable method of identifying hemizygotes.[3,23] Most cases of the atypical variant of hemizygous Fabry's disease with left ventricular hypertrophy have been identified by biopsy of the heart or at autopsy.[16-19] We believe that the measurement of plasma α-galactosidase activity may be clinically useful for screening and identifying patients with Fabry's disease.

Fabry's disease is thought to be rare, and the esti-

Table 2. α-Galactosidase Gene Analyses in Seven Patients with Hemizygous Fabry's Disease.

Variable	Patient No.						
	1	2	3	4	5	6	7
Sequence of the coding region	Normal	Normal	Normal	G→A at nucleotide 888 (Met296Ile)	G→C at nucleotide 58 (Ala20Pro)	Normal	Normal
Amount of α-galactosidase messenger RNA	NE*	Markedly decreased	Markedly decreased	Moderately decreased	Moderately decreased	Markedly decreased	Markedly decreased

*NE denotes not examined.

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mated incidence of classic hemizygous disease is approximately 1 in 40,000.[1] The frequency of the atypical variant has not been determined. In the present study, 230 of the 1603 male patients (14 percent) referred to the cardiology section of the Kagoshima University Hospital for the workup of cardiac symptoms, hypertension, or abnormal findings on electrocardiography or chest radiography had evidence of left ventricular hypertrophy on echocardiography. Seven of these patients (3 percent) were given a diagnosis of hemizygous Fabry's disease. We could find only one other report on the incidence of atypical Fabry's disease.[12] In that report, endomyocardial biopsy revealed that 2 of the 22 patients with hypertrophic nonobstructive cardiomyopathy (9 percent) had Fabry's disease. On the basis of the current study and the earlier report, atypical Fabry's disease in patients with left ventricular hypertrophy may not be as rare as previously thought.

Recently, several patients with atypical variants of Fabry's disease, in which manifestations were limited to the heart, have been described.[16-19] In our study, all seven of the hemizygotes had residual plasma α-galactosidase activity, ranging from 4 to 14 percent of the value in normal male controls. Clinical findings in five of the seven patients were unremarkable except for left ventricular hypertrophy. Six of the patients with Fabry's disease were found in a subgroup of 58 patients with otherwise unexplained left ventricular hypertrophy.

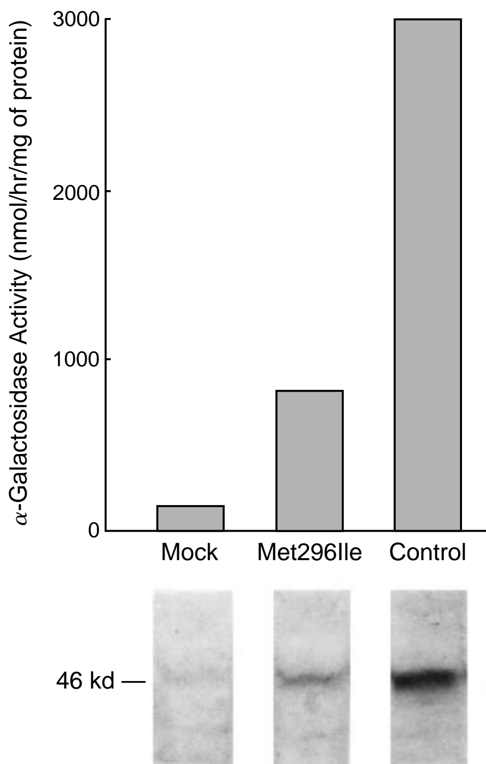

Figure 4. α-Galactosidase Activity (Upper Panel) and Immunoblotting (Lower Panel) in COS-1 Cells Transiently Expressing α-Galactosidase cDNA from Patient 4 (with the Met296Ile Mutation), a Normal Control Subject, and Cells Transfected with Expression Vector Only (Mock).

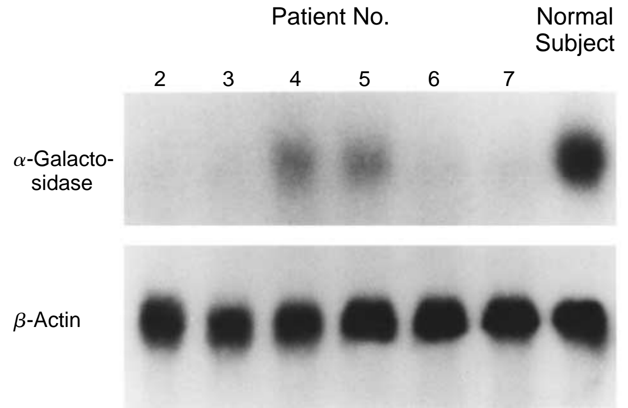

Figure 5. Northern Blot of α-Galactosidase Messenger RNA and β-Actin Messenger RNA in Patients 2 through 7 and a Normal Subject.

The amount of messenger RNA was moderately decreased in Patients 4 and 5 and markedly decreased in Patients 2, 3, 6, and 7.

Previously, four kinds of missense mutations in exon 5 (Asn215Ser) and exon 6 (Met296Val, Gln279Glu, and Arg301Gln) have been reported in atypical variants of Fabry's disease with manifestations confined to the heart.[19,27,28,34] We found two novel mutations, Met296Ile (in Patient 4) and Ala20Pro (in Patient 5), that differ from the two mutations — Gln279Glu and Arg301Gln — that have previously been found in Japanese patients.

In Patient 4, a G-to-A transition at nucleotide 888 was identified that predicted the substitution of isoleucine for methionine at residue 296. Von Scheidt et al.[19] reported a missense mutation at the same codon in a variant form of Fabry's disease: an A-to-G transition at nucleotide 886, causing the substitution of valine for methionine (Met296Val). They predicted that the Met296Val mutation would replace a region of random coil with a β-pleated–sheet motif in the secondary structure of the enzyme. Both isoleucine and valine, which take the place of methionine in the mutant forms, are hydrophobic amino acids, and the mutant proteins expressed as a result of the Met296Ile and Met296Val mutations are probably structurally similar to each other. The results of the Northern blotting and expression analyses of Met296Ile revealed that the amount of the α-galactosidase messenger RNA was moderately decreased and the product expressed by the residual messenger RNA seemed to retain the normal level of catalytic activity per molecule but might be degraded more quickly than normal.

In Patient 5, a G-to-C transition at nucleotide 58 was identified. This mutation resulted in the substitution of proline for alanine in the coding region for the signal peptide. Because the signal peptide is known to be important in the intracellular transport of lysosomal matrix enzymes, including α-galactosidase, a structural

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change in the signal peptide may interfere with the expression of α-galactosidase activity.

We could not find any mutations in the coding regions of either the signal peptide or the enzyme subunit in the other five patients. However, the results of the Northern blot analysis revealed that the amounts of the α-galactosidase messenger RNA were markedly decreased in the four other patients in whom it was measured. In these patients, there are presumably some mutations outside the coding region that involve the transcription of α-galactosidase. Further genetic analysis should clarify the pathogenesis of Fabry's disease in these patients.

In conclusion, we detected Fabry's disease in 3 percent of unselected male patients with left ventricular hypertrophy who were referred to a cardiology clinic in Japan. Fabry's disease was found in 10 percent of the patients who had no other underlying cause of left ventricular hypertrophy. This atypical variant of Fabry's disease, with clinical manifestations limited to the heart, may be more common than previously believed. Fabry's disease should be considered in the differential diagnosis of male patients with unexplained left ventricular hypertrophy.

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