

## Case Report

# Clinical and Molecular Characterization of an Extended Family with Fabry Disease

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**Objective:** To characterize clinical manifestations, biochemical changes, mutation of alpha-Galactosidase ( -Gal A) gene A (GLA), and functional capability of mutant protein.

**Material and Method:** Seventeen subjects from a family with a newly diagnosed patient with Fabry disease were enrolled in the present study. In each individual, clinical history, physical examination, leukocyte enzyme activity of -Gal A, and mutation analysis were performed. Those with a mutation were further investigated by ophthalmological and audiological evaluations, electrocardiography, echocardiogram, urinalysis, and blood tests to determine renal insufficiency. Expression study of the mutant protein was performed using a *Pichia pastoris* expression system.

**Results:** Four affected males and five symptomatic female carriers were identified. Clinical manifestations included severe neuropathic pain, acroparesthesia, hypo-/hyper-hidrosis, frequent syncope, ischemic stroke, cardiac hypertrophy, corneal dystrophy and cart-wheel cataract, high frequency sensorineural hearing loss, periorbital edema and subcutaneous edema over hands and interphalangeal joints. None had angiokeratoma or renal symptoms. The authors identified a novel mutation, p.L106R, in the GLA gene. Recombinant expression of the mutant protein gave little or no enzyme activity compared to the normal protein.

**Conclusion:** There were intrafamilial clinical variabilities, but consistent findings of the absence of angiokeratoma and renal symptoms, which could represent a unique feature of this particular mutation.

**Keywords:** Fabry disease, GLA gene, Alpha-galactosidase A, Neuropathic pain, Renal failure, Angiokeratoma

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Fabry disease (OMIM 301500) is an X-linked recessive disorder characterized by intermittent paresthesia and acroparesthesia, usually beginning in childhood, recurrent fever, heat/cold and exercise intolerance,

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angiokeratoma, corneal dystrophy and lens opacity, proteinuria, and microangiopathies progressing to cerebrovascular, cardiac, and renal ischemic diseases. It results from deficiency of lysosomal alpha-Galactosidase A ( -Gal A, or ceramide trihexosidase)<sup>(1)</sup> and leads to progressive accumulation of the sphingolipid, GLobotriaosylceramide (GL3) in the vascular endothelium and visceral tissue. Angiokeratoma usually

begins in childhood or adolescence, with frequencies of 70% in hemizygous males and 10-35% in heterozygous females<sup>(1-3)</sup>. Renal failure usually develops in the 4<sup>th</sup>-5<sup>th</sup> decade of life and is a common cause of death, if untreated<sup>(1)</sup>. Hypertension is common as a consequence of renal insufficiency. Treatment with recombinant  $\alpha$ -Gal A replacement therapy have been clearing microvascular endothelial deposit of GL3 from the kidneys, heart, skin, and preventing cerebrovascular and cardiac complications, and renal failure<sup>(4)</sup>.

Human alpha-galactosidase gene (*GLA*) is located on chromosome Xq22, and contains 7 exons<sup>(5)</sup>. Among over 360 mutations described, the majority are family-specific (The Human Gene Mutation Database, available at <http://www.hgmd.org/>). Although some mutations have been reported among East Asian cases<sup>(6-10)</sup>, few were studied at the level of expression. Clinical and mutational data in the Thai population are barely available<sup>(11)</sup>. In the present study, the authors performed a complete study of clinical, biochemical, genetic, and expression analysis of an extended family with Fabry disease.

#### Material and Method

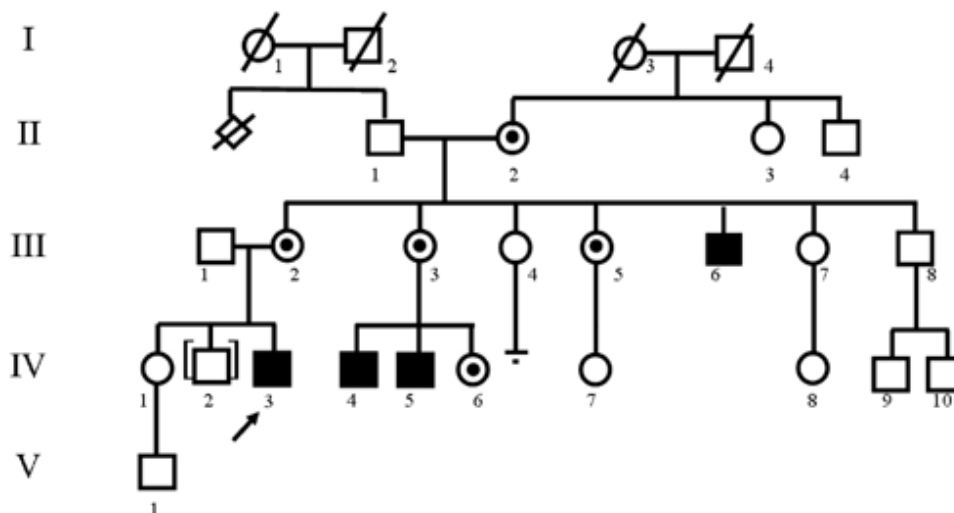
Seventeen subjects (II-2, III-2-8, IV-1, IV-3-7, IV-9, IV-10, V-1) from the family of a newly diagnosed patient with Fabry disease were enrolled (Fig. 1). Each individual (except II-2) was assessed by history taking, physical examination,  $\alpha$ -Gal A activity and mutation analysis. Those who were found to harbor a mutation

would have additional investigations including ophthalmological and audiological evaluations, electrocardiography (EKG), echocardiogram, urinalysis, and Blood Urea Nitrogen (BUN) and Creatinine (Cr).

Leukocytes were prepared from EDTA blood with HistopaqueR-1077 (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The cells were resuspended in 0.5 mL 0.9% (w/v) NaCl and disrupted by sonication on ice. The lysosomal  $\alpha$ -galactosidase enzyme was assayed with methylumbelliferyl  $\alpha$ -D-galactoside as substrate and N-acetylgalactosamine as inhibitor of Gal B, as described<sup>(12)</sup>, except that the reaction volume was reduced to 70  $\mu$ L to allow fluorescence measurement with a microtiter-plate reader. Protein was determined by the method of Bradford with bovine albumin as standard<sup>(13)</sup>.

Genomic DNA was isolated from peripheral blood following established protocol. Exons were amplified and sequenced with intron-flanking primers (Table 1). To detect the mutation in family members and the general population, the exon 2 amplicon was digested with AatII, when a site is created by the mutation.

The cDNA from the proband and a normal control were PCR amplified with the primers BamHI-GLA (CACCGGATCCCTGGACAATGGATTGGCAAG) and HindIII-GLA (CCCAAGCTTAAAGTAAGTCTTTAATGACATCTG), gel purified, and cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA). The cDNA were recombined with LR Clonase (Invitrogen)



**Fig. 1** Extended family with Fabry disease. ■ represents the hemizygous male, ⊙ heterozygous female, □ and ○ represent normal male and female, respectively, represents deceased individual. The arrow indicates the proband. Roman numerals represent each generation, with Arabic numerals representing individuals in each generation

**Table 1.** Primers used in amplification of each of the 7 exons of the GLA gene\*

Primer name and sequence		Product Size (bp)
Forward Primer (5' to 3')	Reverse Primer (5' to 3')	
F1-GATAACCGTCCCAGTTGCCAG	R1-GCTCAACTGTTCCCCTTGAGAC	418
F2-AGGTGCCTAATAAATGGGAGGTACC	R2-AGGGCTGTTTCTAAACAAGCTTCTG	328
F3-GGTTCTCTTTCTGCTACCTCACG	R3-CTCAGCTACCATGGCCTCAAAG	310
F4-ATATATAGCCCCAGCTGGAAATTCAT	R4-GGAACCTGGGAGAGATGGTAGG	281
F5-CTCAAGAGAAGGCTACAAGTGCCTC	R5-GTCAAAAATAGGAAACAAGCCTACCG	316
F6-GTGGTTTCTCCATATGGGTCATCTAG	R6-GGCCCAAGACAAAGTTGGTATTG	343
F7-AAGAATGAATGCCAAACTAACAGGG	R7-CTAGCCTTGAGCTTTTAAAGTGAATGG	436

\* reference sequence NT\_011651, annealing temperature is 60°C for all primer pairs

into the *Pichia pastoris* expression vector, pPICZ - BNH8/DEST, which contains a Gateway cloning cassette after the prepro-alpha-factor sequence of pPICZ (Cairns and Onkoksoong, unpublished). The plasmids were transformed into *P. pastoris* strain GS115, and selected with zeocin. Cultures of colonies containing the empty expression vectors, vectors with wild type GalA or L106R GalA cDNA, were induced to produce protein with 0.5% methanol according to the *Pichia* manual (<http://www.invitrogen.com>). Five day media from 3 normal cDNA and 8 mutant cDNA clones were assayed for  $\alpha$ -galactosidase A activity.

## Results

Four male patients and five female carriers were identified. Another eight individuals, III-1, III-4, III-7, III-8, IV-1, IV-7, IV-9, and IV-10, were found asymptomatic. They had wild-type sequences and normal levels of enzyme (data not shown).

Proband, individual IV-3, experienced acroparesthesia and episodic pain in the lower extremities since age 12. The pain characteristics were preceding low-grade fever and occurred every 2-3 months. At age 13, hypertension, BP 160/110 mm Hg, was noted and thought to be secondary to thyrotoxicosis, based on abnormal thyroid function tests (free-T4 31.7 ng/dl, free-T3 1.51 ng/dl, TSH 1.75 IU/ml. Goiter, exophthalmos, thyroid and microsomal antibodies were absent. Antithyroid drugs, furosemide and calcium-channel blocker were given, but hypertension was poorly controlled.

At age 14, pain increased in intensity and occurred every 1-2 weeks. The patient suffered hypertensive encephalopathy with transient cortical blindness at BP 190/120 mm Hg, necessitating nitroprusside infusion. Hypertensive and atherosclerotic retinopathy

grade I was noted, which resolved following control of blood pressure. He was in a hypothyroid state at the time. Investigations revealed normal findings of protein C, protein S, antithrombin III and fibrinogen, ESR and CRP, abdominal CT and MBIG ( $I^{131}$ -metaiodobenzylguanidine) scan, urinary VMA, voiding cystourethrography, renal and femoral angiogram, and nerve conduction velocity. DiMercapto Succinic Acid (DMSA) scan indicated cortical defect of the left kidney, representing a renal scar, believed to cause the hypertension. Angiotensin-converting enzyme inhibitors were started.

During severe pain, narcotics injection and epidural anesthesia were required. The diagnosis of Fabry disease was made at age 16 (Table 2). Due to the lack of enzyme therapy, the patient was put on carbamazepine. This substantially alleviated the pain intensity during a 4-month follow-up. The blood pressure normalized without the need for antihypertensive drugs during such a period.

Individual III-6 was diagnosed with rheumatoid arthritis, since age 30, despite negative rheumatoid factor and normal hand x-ray. Individual IV-5, had periorbital edema worsening in the morning. He was worked up for possible nephrotic syndrome and allergic conjunctivitis, with negative results.

Other clinical findings are detailed in Table 2. None of the patients and carriers had angiokeratoma, renal insufficiency or proteinuria (determined by BUN and Cr, and by urinary dipstick). Quantitative measurement of albumin in 24-hr-urine indicated absent microalbuminuria, a very sensitive marker of endothelial dysfunction (12.3 in the proband and < 8 in individual IV-5; normal range < 20 mg/L)<sup>(14)</sup>. Glomerular filtration rate (GFR) was normal (proband: 111, and patient IV-5: 164 ml/min/1.73m<sup>2</sup>).

**Table 2.** Clinical manifestation and correlated enzyme activity in 4 hemizygous mutant male and 5 heterozygous female carriers

Individual	Age (yr)	$\alpha$ -Gal A activity (% of normal control) <sup>a</sup>	Neurological manifestations					Cardiac abnormality (echocardiogram and ECG)	Corneal dystrophy/ Cataract	Audiologic abnormality <sup>b</sup>	Subcutaneous edema <sup>c</sup> (onset, yr)	
			Painful extremities (onset, yr)	Acropar esthesia	Abnormal sweating	Syncope attacks (onset, yr)	Blood pressure (mm Hg)					Ischemic stroke
<i>Hemizygous males</i>												
<i>(L106R/-)</i>												
III-6	35	3.13 (7.5)	++ (8)	+++	hypohidrosis	++ (8)	140/80	-	LVH, mild MR, slightly enlarged aortic root diameter	+/-	high frequency SNHL	++ (8)
IV-3	16	2.5 (6.0)	+++ with pain crisis (12)	++	-	-	episodic hypertension	-	-	+/+	-	+ (12)
IV-4	19	2.79 (6.7)	++	++	hypohidrosis	-	120/80	-	trivial MR	+/+	-	+ (9)
IV-5	12	2.34 (5.6)	+	+	hyperhidrosis	++ (10)	100/60	-	-	+/+	-	++ (10)
<i>Heterozygous females</i>												
<i>(L106R/wt)</i>												
II-2	65	NA	-	NA	-	NA	NA (at 50 yr)	+	NA	NA	NA	-
III-2	42	13.7 (32.9)	-	++ (since childhood)	-	-	100/60	-	mild MR	+/-	-	-
III-3	40	13.4 (32.0)	-	+	-	-	130/80	-	NA	+/-	-	-
III-5	39	12.5 (30.0)	-	-	-	+	120/80	-	trivial MR	+/-	high frequency SNHL, tinnitus	-
IV-6	5	10.00 (24.0)	-	-	-	-	NA	-	NA	+/-	-	-

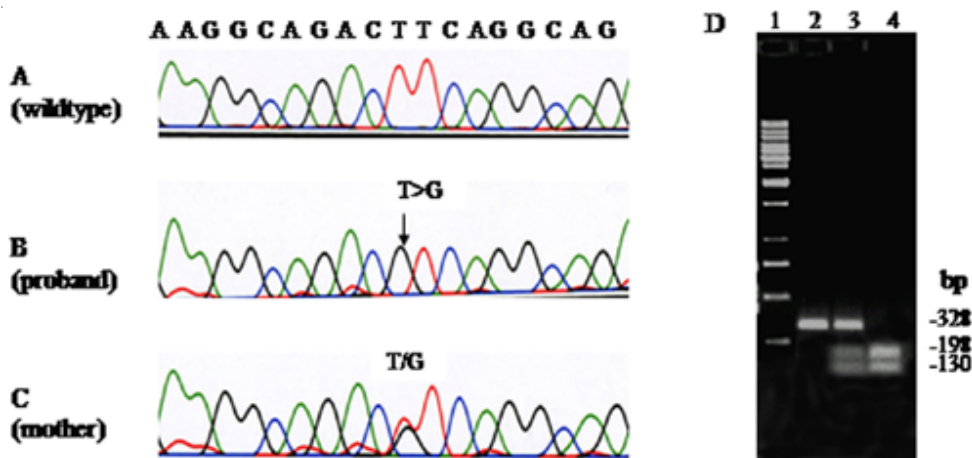
<sup>a</sup> measurement of leukocyte enzyme activity. Normal range in control individuals 21.0-58.4 nmol/mg protein/hr (n = 28), mean 41.7 ± 10.3

<sup>b</sup> determined by using otoacoustic emission, tympanography, and pure tone (air and bone) audiometry

<sup>c</sup> involving hands, feet, and in some cases (individual IV-4, IV-5) periorbital edema included

ECG, electrocardiogram; LVH, left ventricular hypertrophy; MR, mitral valve regurgitation; SNHL, sensorineural hearing loss; NA, not available; wt, wild type or normal sequence

Symbols: (+) means present, (-) means absent; the more numbers of (+), the more severity and/or frequency of such presentation



**Fig. 2** Identification of the L106R mutation in affected individuals. (A) Sequenogram of control DNA, which shows T at nucleotide 377. (B) Sequenogram of the proband (patient IV4-3) with the hemizygous T > G mutation at the same position (C) Sequenogram of the mother with heterozygous pattern. (D) Agarose gel electrophoresis of the exon 2 amplicon following *Aat*II digests of normal control (lane 2), mother (lane 3), and proband (lane 4), with the marker ladder in lane 1. Note undigested 328 bp product of the wildtype alleles in lanes 2 and 3, and 198 and 130 bp restriction fragments of the mutant in lanes 3 and 4

The genomic DNA and cDNA sequencing showed only one mutation in the proband and his mother, a T-to-G transversion at nucleotide 377 (c.377T > G) in exon 2 resulting in substitution of arginine for leucine at codon 106, p.L106R (Fig. 2). The four hemizygous males had enzyme levels of 5-8% of normal, while the four heterozygous females had enzyme levels of 24-33% of normal (Table 1). Individual II-2 was an obligate heterozygous carrier according to information from family members and pedigree analysis. Out of 100 chromosomes from normal Thai individuals (44 males and 28 females) tested, none carried the mutation.

When mutant and normal cDNA were used to express protein in *Pichia pastoris*, three normal clones were all found to express > 2200 nmoles/h/mg protein of  $\alpha$ -galactosidase activity in 5 day media, but of eight mutant clones studied, seven showed no  $\alpha$ -galactosidase on 5 day, and one showed much lower activity than normal (39 nmoles/h/mg protein).

## Discussion

Despite classical presentation of severe neuropathic pain in the proband, the diagnosis of Fabry disease was delayed owing to absence of angiokeratoma, coinciding with thyrotoxicosis, and presence of renal cortical defect believed to cause hypertension. In retrospect, the authors believed that the proband's severe hypertension was probably induced by the agonizing pain, the focal renal defect, autonomic dys-

regulation related to Fabry disease, or combination of the three factors. Without histopathological proof of the kidney, it is impossible to conclude whether or not the focal renal defect represents GL3-occluded renal arterioles as a result of Fabry disease. Normalization of the blood pressure and fair control of pain following treatment with carbamazepine which is an antiautonomic dysfunction was perhaps an indirect evidence supporting the authors' assumption. Occlusion of central retinal artery, a rare complication of Fabry disease as a cause of sudden loss of vision, was excluded, based on the ophthalmologic findings. Familial hypertension was not a case owing to normal blood pressure in the other family members who did not have Fabry disease (data not shown).

Pain and paresthesia in Fabry disease is caused by severely reduced small myelinated (A-delta) and unmyelinated (C) nerve fibers<sup>(1-3, 15)</sup>, which carry temperature and pain sensations and autonomic functions<sup>(16)</sup>. The authors assumed that unmyelinated (C) nerve fiber and autonomic ganglia may be preferentially deposited with the sphingolipid, and there is relatively less involvement in the endothelium of vessels of skin and kidneys. This may lead to obvious autonomic presentations, absence of typical skin lesions, and delayed-onset of renal impairment in the present family. Theoretically, this hypothesis may be tested by comparative histopathological studies of the according tissues, but a larger clinical cohort will also be required.

Corneal dystrophy was present as early as in the 5-year-old female carrier, individual IV-6, who may be the youngest female carrier reported with Fabry's cornea. Subclinical sensorineural hearing loss in one patient and one female carrier is consistent with the frequency reported recently<sup>(2,3)</sup>. Left ventricular hypertrophy was not yet identified in female carriers of this family but this will need regular follow-up. It is likely that the stroke in the carrier II-2 represents cerebrovascular complication of Fabry disease. Cerebro- and cardio-vascular ischemia in the other patients and carriers need to be monitored.

The other family members experienced common manifestations of Fabry's disease, namely hypo- and hyper-hidrosis, frequent syncopal attack, acroparesthesia, yet all had difficulty in describing their symptoms to the physician and remained undiagnosed prior to the present study. None of the patients and carriers had angiokeratoma, which could represent a unique feature of the mutation L106R. Plausible explanations for the absence or very late onset of renal insufficiency in individual III-6 is may be due to residual enzyme activity (defined as  $\geq 1\%$  of normal control  $\alpha$ -gal A activity) in affected males<sup>(3)</sup> or some special characteristics of this particular mutation.

This is the first time the p.L106R mutation of GLA gene has been reported. From the recent structure of human  $\alpha$ -galactosidase, Leu 106 lies on the inside of a loop on the outside of the protein, approximately 15 Å from the active site<sup>(17)</sup>, and within 3 Å of the side-chain of Arg 100, which forms a salt-bridge with Asp 155. Conversion of the non-polar Leu residue to a positively charged Arg may lead to repulsion that destabilizes the protein. The fact that L106R Gal A expressed in *Pichia pastoris* showed little or no activity supports this idea. In addition, leucine at codon 106 is conserved among the  $\alpha$ -Gal A gene of several species including mouse (*Mus musculus*, NM 013463), chimpanzee (predicted sequences of *Pan troglodytes*, XM 521181), and cow (predicted sequences of *Bos taurus*, XM 521181). In summary, the authors have described an extended family with Fabry disease in which there were intra-familial clinical variabilities but with consistent findings of the absence of angiokeratoma and renal symptoms. This perhaps represents the genotype-phenotype correlation with L106R. The p.L106R mutant showed very little or no  $\alpha$ -galactosidase activity in expression studies. Despite the clinical hallmark of neuropathic pain, children with Fabry disease are often undiagnosed because physical examination may not provide clues for diagnosis, especially if typical skin lesions

are absent.

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## การวิเคราะห์ลักษณะทางคลินิกและระดับโมเลกุลของครอบครัวใหญ่ที่เป็นโรคฟาเบอร์

ดวงฤดี วัฒนศิริชัยกุล, ม.ร.ว. ชัยนุสรณ์ สวัสดิวัตน์, เจมส์ เกตุทัต คาร์นส์, กาญจนา ตั้งนารักษ์กิจ, อนันนิตย์ วิสุทธิพันธ์, ศิริพร กิรติจำเริญ, ลักษณ์า จิวสระ, พงษ์ศักดิ์ โค้วสฤติย์, ทัศนีย์ อ่อนโคกสูง, อภัทธสา เล็กสกุล, ดาวเรือง มงคลศิริ, จันทรชัย เจริญประเสริฐ, เจียมจิต ถวิล, สุวิมล รื่นเจริญ

**วัตถุประสงค์:** เพื่อวิเคราะห์ลักษณะทางคลินิก ระดับเอนไซม์ การกลายพันธุ์ของยีนแอลฟาแกแล็กโตไซด์-เอ และศึกษาการแสดงออกของโปรตีนกลายพันธุ์ ในครอบครัวใหญ่ครอบครัวหนึ่งที่เป็นโรคฟาเบอร์

**วัสดุและวิธีการ:** ผู้ป่วยโรคฟาเบอร์ซึ่งเพิ่งได้รับการวินิจฉัยและสมาชิกครอบครัว รวมทั้งสิ้น 17 ราย ได้รับการชักประวัติ ตรวจร่างกายโดยละเอียด ตรวจเลือดเพื่อวัดระดับเอนไซม์แอลฟาแกแล็กโตไซด์-เอ ในเม็ดเลือดขาว วิเคราะห์ลำดับเบสเพื่อหาการกลายพันธุ์ของยีนแอลฟาแกแล็กโตไซด์-เอ (GLA) ผู้ซึ่งผลตรวจเอนไซม์และการกลายพันธุ์บ่งชี้ว่าเป็นโรคหรือเป็นพาหะ จะได้รับการตรวจเพิ่มเติมคือ ตรวจตา ตรวจการได้ยิน ตรวจคลื่นหัวใจ คลื่นเสียงสะท้อนความถี่สูงหัวใจ ตรวจเลือดดูหน้าที่ไตและตรวจปัสสาวะ การศึกษาแสดงออกของโปรตีนกลายพันธุ์ทำโดยใช้เวคเตอร์ *Pichia pastoris*

**ผลการศึกษา:** มีผู้ป่วยชายทั้งสิ้น 4 ราย และหญิงที่เป็นพาหะ 5 ราย อาการและอาการแสดงที่พบ ได้แก่ ปวดตามแขนขาอย่างรุนแรง ปวดแสบปวดร้อนที่ฝ่ามือและฝ่าเท้า เหงื่อออกมากหรือน้อยผิดปกติ เป็นลมบ่อย เป็นอัมพาต หลอดเลือดในสมองอุดตัน หัวใจโต มีการเปลี่ยนแปลงที่กระจกตาและพบต่อกระจกที่มีลักษณะจำเพาะต่อโรค สูญเสียการได้ยินที่ความถี่สูง อาการบวมตึงที่หนังตาและผิวหนังที่มือและข้อนิ้วมือ ไม่มีรายใดมีรอยโรคของหลอดเลือดขนาดเล็กที่ผิวหนังที่เป็นลักษณะจำเพาะของโรค (แองจิโอเคอราโตมา) ยังไม่มีรายใดแสดงอาการทางไตหรือไตวาย พบการกลายพันธุ์ชนิดใหม่ของยีนแอลฟาแกแล็กโตไซด์-เอ ซึ่งทำให้กรดอะมิโนลูซีนที่ตำแหน่ง 106 ถูกแทนที่ด้วยกรดอะมิโนอาร์จินีน ซึ่งยืนยันทั้งในระดับดีเอ็นเอ และอาร์เอ็นเอ การศึกษาหน้าที่ของโปรตีนพบว่าโปรตีนที่กลายพันธุ์มีการทำงานลดลงอย่างมากเมื่อเทียบกับโปรตีนปกติหรือแทบไม่มีการทำงานเลย

**สรุป:** ผู้ป่วยและพาหะในครอบครัวนี้ มีอาการและอาการแสดงที่ตรวจพบได้รุนแรงมากน้อยต่างกันไปทุกรายไม่มีอาการแสดงรอยโรคของหลอดเลือดขนาดเล็กที่ผิวหนังและไม่มีอาการทางไต ซึ่งเป็นไปได้ว่าลักษณะสองประการหลังนี้เป็นลักษณะเฉพาะของการกลายพันธุ์ชนิดใหม่ที่พบ

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