p.E66Q Mutation in the *GLA* Gene is Associated with a High Risk of Cerebral Small-Vessel Occlusion in Elderly Japanese Males

Katsuya Nakamura, MD, PhD[1],Yoshiki Sekijima, MD, PhD[1,2], Kimitoshi Nakamura MD, PhD[3], Kiyoko Hattori, MD, PhD[3], Kiyoshiro Nagamatsu, MD[1], Yusaku Shimizu, MD, PhD [4], Masahide Yazaki[1], Akihiro Sakurai, MD, PhD[2], Fumio Endo MD, PhD[3], Yoshimitsu Fukushima, MD, PhD[2,] Shu-ichi Ikeda MD, PhD[1]

[1]Department of Medicine (Neurology and Rheumatology), and [2]Division of Clinical and Molecular Genetics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

[3] Department of Pediatrics, Graduate School of Medical Science, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

[4] Department of Neurology, Ina Central Hospital, 1313-1 Koshiroukubo, Ina 395-8555, Japan

Correspondence:

Yoshiki Sekijima, Department of Medicine (Neurology and Rheumatology),

Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.

TEL +81-263-37-2673,   FAX +81-263-37-3427,   E-mail sekijima@shinshu-u.ac.jp

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Abstract

***Background and purpose*:** *GLA* is the causative gene of Fabry disease, an X-linked lysosomal

storage disorder resulting from α-galactosidase A (α-GAL) deficiency. Stroke is an important

manifestation of Fabry disease, and recent epidemiologiccal studies indicated that up to 4.9%

of young male cryptogenic stroke patients have *GLA* mutations. To determine the importance

of *GLA* mutations in the general stroke population, we measured the frequency of *GLA*

mutations in Japanese male ischemic stroke patients with various risk factors and ages.

***Methods*:** A total of 475 male ischemic stroke patients (average age 69.7±12.5 years), were

enrolled in this study. A blood sample was obtained to produce blood spots for measurement

of α-GAL activity. Blood samples with decreased enzymatic activity were reassayed and the

entire *GLA* gene was analyzed by direct DNA sequencing if α-Gal A activity was consistently

low.

***Results*:** α-Gal A activity was decreased in 10 men, 5 of whom (1.1%) had the *GLA* gene

mutation, p.E66Q. All ischemic stroke patients with p.E66Q mutation had substantial residual

α-Gal A activity, in contrast to patients with classic-type Fabry disease. Clinically, all patients

with p.E66Q mutation were > 50 years old and had multiple small-vessel occlusions (lacunar

infarctions). Statistical analysis using Fisher's exact test showed the allele frequency of *GLA*

p.E66Q in patients with small-vessel occlusion to be significantly higher than that in the

general Japanese population (odds ratio 3.34, *P*=0.025).

***Conclusions*:** *GLA* p.E66Q mutation is a genetic risk factor for cerebral small-vessel

occlusion in elderly Japanese males.

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Introduction

Fabry disease (MIM301500) is an X-linked lysosomal storage disorder resulting from deficiency of α-galactosidase A (α-Gal A) [1]. The enzymatic defect leads to progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids in the vascular endothelial lysosomes of the kidneys, heart, brain, and skin. Affected males who have little or no detectable α-Gal A activity exhibit the classic phenotype with onset of angiokeratoma, acroparesthesia, and hypohidrosis in childhood. With advancing age, the occurrence of renal failure, cardiac disease, and stroke lead to a decline in activities of daily living and premature death. Stroke is one of the major complications of classic Fabry disease [2] and has been described in 6.9%–24.2% of patients [3-5]. Estimates of the prevalence of classic Fabry disease vary from 1 in 40000 to 1 in 60000 [1, 6].

On the other hand, patients with substantial levels of residual α-Gal A activity have late-onset milder phenotypes, including renal [7] and cardiac [8] variants. Recent studies involving newborn screening for α-Gal A activity in Fabry disease showed surprisingly high incidences of mutations of 1 in 1250–3100 male infants [9, 10]. Most mutations found in newborn screening were associated with the late-onset variant phenotype, suggesting that many patients with these *GLA* mutations are underdiagnosed. Screening for Fabry disease in high risk populations identified previously undiagnosed Fabry patients in 0.2% to 1.2% of males undergoing hemodialysis [7, 11, 12] and 0.9% to 4% of males with left ventricular hypertrophy or hypertrophic cardiomyopathy [8, 13, 14]. Furthermore, the prevalence of unrecognized Fabry disease in young male patients with stroke was reported to be up to 4.9%

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[15-20]. However, most stroke patients are elderly and there has been only one

population-based study in unselected patients with stroke [21]. It is likely that *GLA* mutation

is itself a risk factor for accelerated atherosclerosis and cardiac and renal disease, which can

lead to emboli and hypertension, and therefore unrecognized Fabry patients may be found

among elderly stroke patients [22]. To determine the importance of *GLA* mutations in the

general stroke population, we measured the frequency of *GLA* mutations in Japanese male

ischemic stroke patients with various risk factors and ages.

Methods

Patients

Fifteen clinical neurology departments in Nagano prefecture, Japan, participated in this

prospective cross-sectional study. From August 2007 to December 2011, 475 male patients

aged 20–91 years (mean±SD: 69.7±12.5 years), presenting consecutively at a participating

neurology department with ischemic stroke, were enrolled in this study. Patients who were

unable to provide informed consent or who had already been diagnosed with Fabry disease

were excluded from the study. This study was approved by the Ethical Committee of Shinshu

University School of Medicine and the ethics committees of each of the participating clinical

neurology centers, and written informed consent was obtained from each patient prior to

enrollment. After informed consent was obtained, demographic data, cerebrovascular risk

factors, presence of signs and symptoms of Fabry disease, and clinical and neuroimaging data

were registered in a database using case report forms. Assessment of clinical symptoms and

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signs suggestive of Fabry disease was performed in all patients with *GLA* gene mutations.

Screening for angiokeratoma was performed by routine clinical examination, and the presence

of acroparesthesia and hypohidrosis was determined by anamnesis. In addition, cardiac

function tests, including serum brain natriuretic peptide (BNP) and human atrial natriuretic

peptide (hANP) concentrations, chest roentgenography, electrocardiography, and

echocardiography, and renal function tests, including routine urine test and determination of

serum creatinine and blood urea nitrogen (BUN) levels, were performed in all patients with

GLA mutation.

α-Gal A enzyme assay and mutation analysis

A blood sample was obtained for production of blood spots for measurement of α-Gal A

activity. α-Gal A activity was determined using a fluorescent substrate as described previously

[23]. Briefly, 40 μL of McIlvan buffer (0.1 M citrate, 0.2 M NaH_2PO_4, 36.8 : 63.2, pH 6.0)

was added to each well of 96-microwell plates. Three millimeter punch specimens of dried

blood spots were added to the buffer and processed for extraction at room temperature for 2 h.

Aliquots of 30 μL of blood extract were transferred to fresh 96-microwell plate. An aliquot of

100 μL of the reaction mixture (3.5 mM 4-MU galactosylpyranoside, 100 mM citrate, 200

mM phosphate, 100 mM N-acetylgalactosamine) was added to each well of the microwell

plates and incubated at 37°C for 24 h. The reaction was terminated with 150 μL of

termination solution (300 mM glycine, NaOH, pH 10.6) immediately after the reaction.

Fluorescence intensity from the 4-methylumbelliferones in the wells was measured with a

fluorescence plate reader (BIO-TEK) at 450 nm. One unit (AgalU) of enzymatic activity was equal to 0.34 pmol of 4-methylumbelliferyl-D-galactopyranoside cleaved/h/disc. Blood samples with decreased enzymatic activity (<17 AgalU) were reassayed.

If blood αGal A activity was consistently low, the entire *GLA* gene was analyzed. For DNA analysis, total genomic DNA was extracted from leukocytes of patients. All 7 exons and the flanking intronic sequences of the *GLA* gene were amplified by polymerase chain reaction (PCR), and the amplification products were analyzed by a direct sequencing (Figure 1).

Results

Clinical data of enrolled patients

Table 1 summarizes the demographic characteristics of the enrolled patients. The mean age (±SD) in the cohort of 475 patients participating in this study was 69.7±12.5 years. Stroke etiology was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [24] as large-artery atherosclerosis in 114 patients (24.0%), cardioembolism in 79 (16.6%), and small-vessel occlusion (lacunar infarction) in 240 (50.5%). Stroke of other determined etiology was present in 12 patients (2.5%): cervicocephalic arterial dissection (*n*=4) and paraneoplastic coagulopathy (*n*=8). Stroke of undermined etiology was present in 61 patients (12.8%). Subtypes of ischemic stroke overlapped in 31 patients, as they had histories of multiple ischemic strokes with different subtypes.

Diagnostic test results

The average α-Gal A activity of the study population was 27.7±10.7 AgalU. The distribution

of α-Gal A activity in the whole study population is shown in Figure 2. Initial screening for

α-Gal A activity in blood spots from 475 male patients with stroke detected 28 (5.9%) patients

with enzyme level below the normal cut-off value of 17.0 AgalU. A repeat blood spot was

obtained from 20 patients. When retested, 10 (2.1%) patients had α-Gal A activities <17.0

AgalU, whereas the other 10 (2.1%) had normal enzyme activities (≥17 AgalU). DNA

sequencing of *GLA* was performed in these 10 doubly screened-positive patients with low

α-Gal A activity, and *GLA* gene mutation was identified in 5 (1.1%) patients. All 5 patients

had the same missense mutation, a single base sequence change (c.196G>C) causing

substitution of a glutamate reside with glutamine at codon 66 (p.E66Q). The average α-Gal A

activity of the stroke patients with p.E66Q mutation was 11.3±1.6 AgalU (range: 9.8–13.5

AgalU), which was relatively high compared to patients with classic type Fabry disease.

Clinical data of patients with *GLA* p.E66Q mutation

The clinical, biochemical, and molecular features of the patients with *GLA* p.E66Q mutation

are summarized in Table 2. All patients with p.E66Q mutation were older than 50 years with a

mean age of 69.6±12.5 years. All patients had multiple small-vessel occlusions (Figure 3),

which were accompanied by white matter lesions (leukoaraiosis) in 3 patients (Figure 3C, E,

G). Intracerebral hemorrhage was observed in 4 patients. Three patients had a history of

symptomatic intracerebral hemorrhage (Figure 3B) and the other patient had asymptomatic

multiple microbleeds detected by T2*-weighted MRI (Figure 3F). Two patients took low

doses of aspirin when they developed cerebral hemorrhage. Vertebrobasilar dolichoectasia

was observed in one patient (Figure 3D). Increased signal intensity in the pulvinar region on

T1-weighted MRI was not observed in any patients. Two patients had a history of

hypertension; however, their blood pressures were well controlled by antihypertensive drugs.

None of the patients had other common risk factors for stroke, including dyslipidemia,

diabetes mellitus, hyperuricemia, and smoking. No patients with *GLA* p.E66Q mutation

showed characteristic symptoms of Fabry disease, such as renal dysfunction, cardiomyopathy,

acroparesthesia, hypohidrosis, and angiokeratoma. None of patient with p.E66Q mutation had

a family history of Fabry disease, although an uncle of patient 1 and mothers of patient 2 and

3 had histories of cerebral infarction, and a brother of patient 2 had a history of chronic renal

failure (Supplementary figure 1).

Allele frequencies of the *GLA* p.E66Q mutation in the Japanese population and

statistical analysis

To estimate the frequency in the general Japanese population of the *GLA* p.E66Q mutation

found in the ischemic stroke patient cohort, we utilized the data from newborn screening for

Fabry disease performed in Kumamoto prefecture from October 2009 to May 2010. In this

screening, we tested 5051 consecutive male neonates (5051 alleles). This study was approved

by the Kumamoto University Ethics Committee and written informed consent was obtained

from each parent prior to enrollment. Genomic DNAs were isolated from whole blood, and

exon 2 of the *GLA* gene was amplified by PCR. The amplification product was analyzed by

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direct sequencing. We identified 32 hemizygous male neonates, and the allele frequency of the *GLA* p.E66Q in the Japanese population was thus determined to be 0.637%. Statistical analysis using the Fisher's exact test indicated that the allele frequency of *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (odds ratio 3.34, *P*=0.025) (Table 3). However, in all ischemic stroke, large-artery atherosclerosis, cardioembolism, and non-cardioembolism patients, the odds ratios were 1.67, 1.39, 0, and 2.01, respectively; the differences were not statistically significant (Table 3).

Discussion

Screening for Fabry disease in high-risk populations became an important concern when enzyme replacement therapy became available [25]. Studies performed in different settings indicated severe complications of Fabry disease, including left ventricular hypertrophy/hypertrophic cardiomyopathy [8, 13, 14], renal insufficiency [7, 11, 12], and stroke [15-21]. The prevalence of unrecognized Fabry disease in young male patients with stroke was first reported in 2005 [15]. Since then, several studies have estimated the prevalence of Fabry disease in young male patients with stroke as ranging from 0% to 4.9% [15-20]. Recently, Rolfs et al. [19] reported the results of the largest screening for Fabry disease in young patients with acute cerebrovascular disease. They enrolled 5023 patients from 15 European countries and found 27 patients (0.54%) with definite Fabry disease and 18 patients (0.36%) with probable Fabry disease. However, most stroke patients are elderly and there has been only one population-based study in unselected patients with stroke [21].

In the present study, 5 patients were identified as having *GLA* mutation and all of them had the same missense mutation, c.196G>C (p.E66Q). All patients with p.E66Q mutation showed similar clinical pictures, i.e., multiple small-vessel occlusions with a high frequency of intracerebral hemorrhage. Interestingly, most patients with this mutation lacked common risk factors for stroke. Only two patients had hypertension and their blood pressures were well controlled by antihypertensive drugs (Table 2). The p.E66Q mutation was first identified in a male patient with classic-type Fabry disease. However, he also had another *GLA* missense mutation, p.R112C, in the same allele, which was predicted to cause a large structural change in the α-Gal A protein that leads to classic type Fabry disease [26]. Subsequently, 26 patients with *GLA* p.E66Q mutation who developed adult-onset left ventricular hypertrophy or renal insufficiency were identified [7, 12, 27-29], and therefore this mutation was considered to be pathogenic, causing late-onset variant Fabry disease. However, none of these studies provided histological evidence confirming the diagnosis of Fabry disease in such cases. Recently, subjects harboring the p.E66Q mutation in the *GLA* gene have been found at unexpectedly high frequencies among Korean [30] and Japanese [31] populations, which has raised interest regarding whether p.E66Q is a disease-causing mutation or a functional polymorphism.

Recent studies showed that the structure of α-Gal A protein with the p.E66Q amino acid substitution was less stable than that of the wild-type protein [31], and plasma and white blood cell α-Gal A activities in male subjects harboring the p.E66Q mutation were 13%−26% and 19%−65% of the normal mean α-Gal A activity, respectively [30, 31], compatible with our dried blood spot enzymatic assay results (approximately 40% of the normal mean) (Figure

2). The levels of these residual α-Gal A activities of the individuals with p.E66Q mutation were almost the same as those of male stroke patients with p.R118C and p.D313Y mutations identified in a Portuguese screen (PORTYSTROKE study) [17]. *GLA* p.D313Y mutation was found in 0.45% of normal X chromosomes in the Caucasian population [32]. In the PORTYSTROKE study [17], the allele frequency of p.D313Y in the stroke population was higher than that in normal controls, although the difference was not statistically significant. In this study, we showed that the allele frequency of the *GLA* p.E66Q in patients with small-vessel occlusion was significantly higher than that in the general Japanese population (odds ratio 3.34, *P*=0.025) (Table 3), indicating that this mutation confers a high risk of small-vessel occlusion in Japanese males.

In this study, the frequency of p.E66Q mutation in control subjects was determined by DNA sequencing, while that in stroke patients was determined by enzymatic screening followed by DNA sequencing. Therefore, the frequency of p.E66Q in stroke patients is likely to have been underestimated. Fuji et al. [29] analyzed the prevalence of Fabry disease in Japanese hemodialysis patients using dried blood spot screening followed by DNA sequencing, which was identical to the method used in the present study. They found only one patient with p.E66Q mutation among 625 Japanese male hemodialysis patients (prevalence of p.E66Q mutation was 0.16%), which was much lower than that in our newborn DNA screen (Table 3), suggesting that we may have missed a substantial number of patients with this mutation in dried blood spot enzymatic screening. In addition, we could not follow-up 8 patients whose α-Gal A activities were below the cutoff value at the initial screening because

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they moved to other hospitals or clinics. Therefore, there may have been additional patients with *GLA* mutations among those who dropped out, and therefore the frequency of p.E66Q in stroke patients may have been underestimated in this study. Another point to be taken into consideration is patient selection bias, as we enrolled patients only from selected neurology departments, while a substantial number of stroke patients may be managed by neurosurgeons. In addition, we could not obtain informed consent from some of severe stroke patients. These may explain why the proportion of small-vessel occlusion was more than 50% in this study. Although DNA sequencing of the *GLA* gene in all patients is necessary to determine the precise frequency of p.E66Q mutation in stroke patients, it is clear that p.E66Q mutation in the *GLA* gene is an important genetic risk factor for small-vessel occlusion in elderly Japanese males.

The precise pathomechanism by which *GLA* p.E66Q mutation increases the risk of lacunar infarction remains unknown. Recently, it was reported that cerebral small-vessel disease, which is known to be associated with lacunar infarction, white matter lesions (leukoaraiosis), and cerebral hemorrhage, rather than large-artery stroke, is frequently observed in Fabry disease [4, 5, 33, 34]. These observations are compatible with clinical findings of our patients with p.E66Q mutation who developed multiple small-vessel occlusions and cerebral hemorrhage. Our findings suggest that *GLA* mutations associated with relatively high residual α-Gal A activity may add to the risk of cerebral small-vessel disease, possibly by contributing to the underlying multifactorial pathogenesis rather than through a classic Mendelian effect.

Enzyme replacement therapy (ERT) is currently the only approved therapy for Fabry disease. However, patients with *GLA* p.E66Q are not considered to be candidates for ERT, since p.E66Q is not a causative mutation for classic-type or variant-type Fabry disease [30, 31]. On the other hand, Shimotori et al. [28] reported that 1-deoxygalactonojirimycin, an active site specific pharmacological chaperone (ASSC), significantly increased α-Gal A activity of COS-7 cells with *GLA* p.E66Q mutation, suggesting that ASSC may be a potential therapeutic option for patients with this mutation.

Disclosure of conflict of interest   The authors report no disclosures relevant to the manuscript.

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Figure legends

Figure 1. Flowchart for the present study.

Figure 2. The distribution of α-Gal A activity in all of the patients included in this study. The x-axis indicates age (years old) and the y-axis indicates α-Gal A activity (AgalIU). The cut-off α-Gal A activity was 17 AgalIU. Filled diamonds, open triangles, open squares, and open circles indicate individuals with normal enzymatic activity in dried blood spot screening, low enzymatic activity without *GLA* gene mutations, low enzymatic activity without DNA analysis, and low enzymatic activity with *GLA* p.E66Q mutation, respectively.

Figure 3. Brain MRI and CT findings of patients with *GLA* p.E66Q. (A, B) Patient 1. T2-weighted MRI showed small-vessel occlusions in the right cerebellar hemisphere and pons (A). Brain CT showed large left thalamic hematoma with rapture into the lateral ventricle (B). (C, D) Patients 2. Fluid attenuated inversion recovery (FLAIR) MRI showed multiple small-vessel occlusions accompanied by marked leukoaraiosis (C). MR angiography (MRA) showed moderate dolichoectasia of the basilar and vertebral arteries (D). (E, F) Patient 3. T2- (E) and T2*-weighted (F) MRI showed multiple small-vessel occlusions and microbleeds in the cerebral white matter and basal ganglia. Microbleeds were also observed in the dentate nucleus. (G, H) FLAIR MRI of patient 4 (G) and patient 5 (H) showed multiple small-vessel occlusions in the cerebral white matter. Leukoaraiosis was also observed in patient 4 (G).

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Supplementary figure 1. Pedigrees of patients with p.E66Q mutation. (A) Patient 1. (B)

Patient 2. (C) Patient 3. (D) Patient 5. We could not obtain detailed information on patient 4's

family history.

Table 1. Demographic and characteristics of the patient population

	All ischemic stroke	Subtypes of ischemic stroke*				
		Large-artery atherosclerosis	Cardio-embolism	Small-vessel occlusion	Stroke of other determined etiology	Stroke of undetermined etiology
Number of patients	475	114	79	240	12	61
Age (mean $\pm$ SD)	69.7 $\pm$ 12.5	70.4 $\pm$ 11.0	74.1 $\pm$ 12.9	68.5 $\pm$ 12.1	67.3 $\pm$ 17.2	73.6 $\pm$ 13.2

*Subtypes of ischemic stroke overlapped in 31 patients, as they had episodes of multiple ischemic stroke with different subtypes.

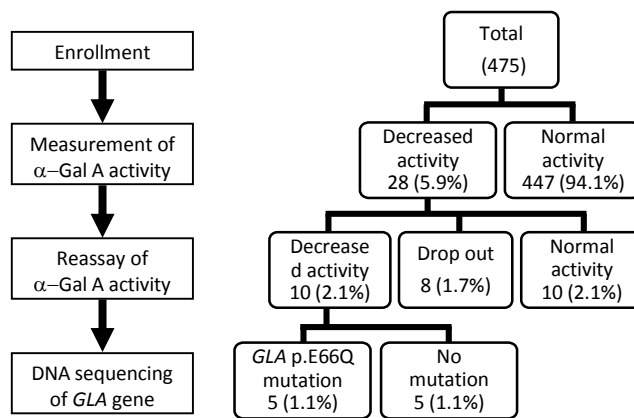**Table 2. Clinical features of patients with *GLA* gene mutation**

Patient	Age	Sex	αGalA activity (AGalU) (normal > 17)	*GLA* gene mutation	Type of cerebral infarction		Cerebral hemorrhage	Risk factor for stroke	Other complications of Fabry disease
1	70	M	11.6	p.E66Q (c.196G>C)	multiple	lacunar	thalamic hemorrhage	–	–
2	83	M	12.0	p.E66Q (c.196G>C)	multiple	lacunar / atherothrombotic / leukoaraiosis	putaminal hemorrhage	Hypertension (good control)	–
3	76	M	13.5	p.E66Q (c.196G>C)	multiple	Lacunar / leukoaraiosis	multiple microbleeds	–	–
4	66	M	9.8	p.E66Q (c.196G>C)	multiple	Lacunar / leukoaraiosis	symptomatic hemorrhage*	–	–
5	50	M	9.8	p.E66Q (c.196G>C)	multiple	lacunar	–	Hypertension (good control)	–

*Location of the hemorrhage was unknown

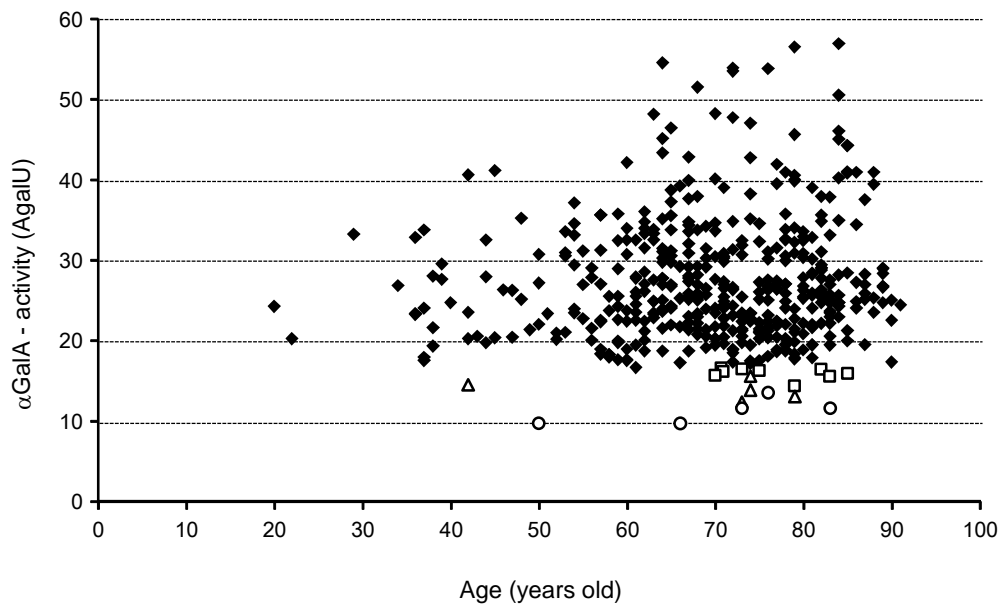**Table 3. Allele frequencies of the *GLA* p.E66Q mutation in the Japanese men**

	Control (newborn screening)*	All ischemic stroke patients**	Subtypes of ischemic stroke			
			Large-artery atherosclerosis**	Cardio-embolism**	Small-vessel occlusion**	Non-cardioembolism**
Number of subjects	5051	475	114	79	240	396
Number of subjects with p.E66Q	32	5	1	0	5	5
p.E66Q allele frequency (%)	0.64	1.05	0.88	0	2.08	1.41
Odds ratio (vs. control)		1.67	1.39	0	3.34	2.01
P-value (vs. control)		0.244	0.522	1	0.025	0.188

* Frequency of p.E66Q mutation was determined by DNA sequencing. ** Frequency of p.E66Q mutation was determined by enzymatic screening followed by DNA sequencing.
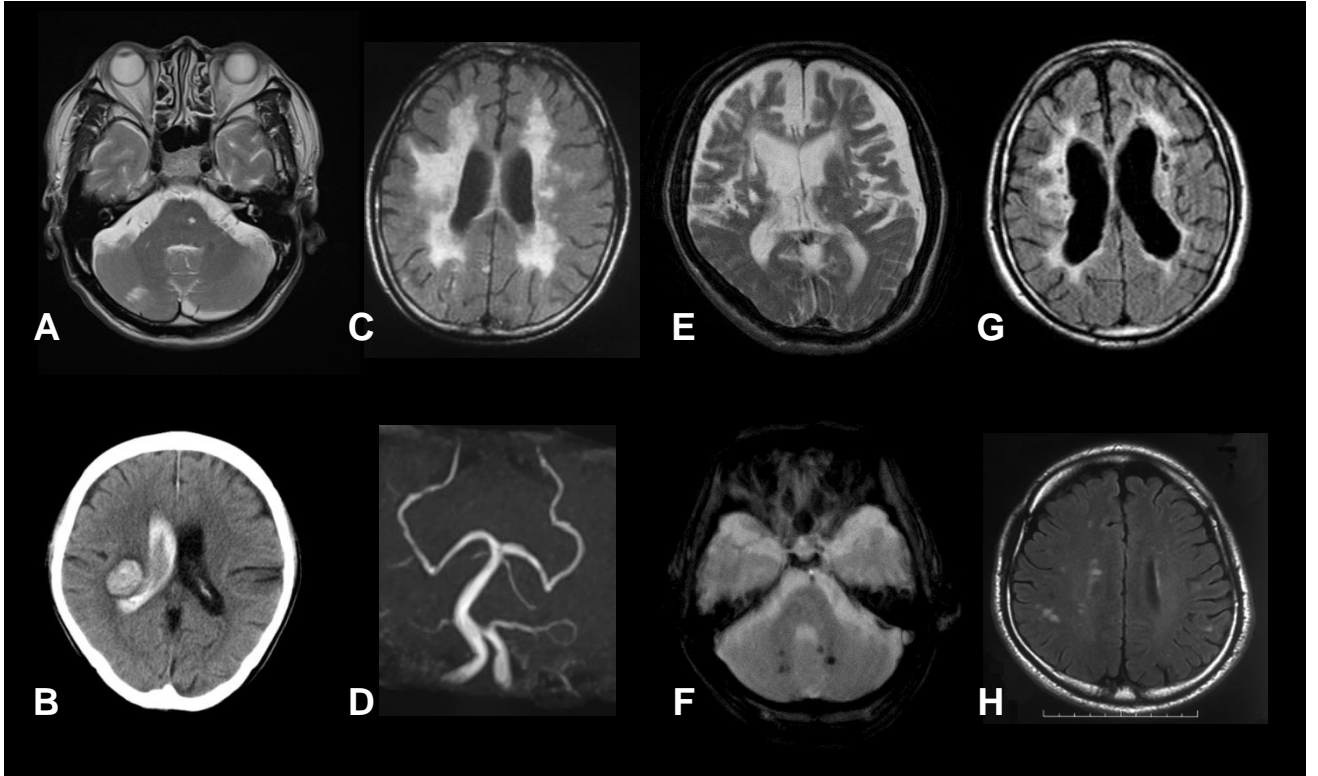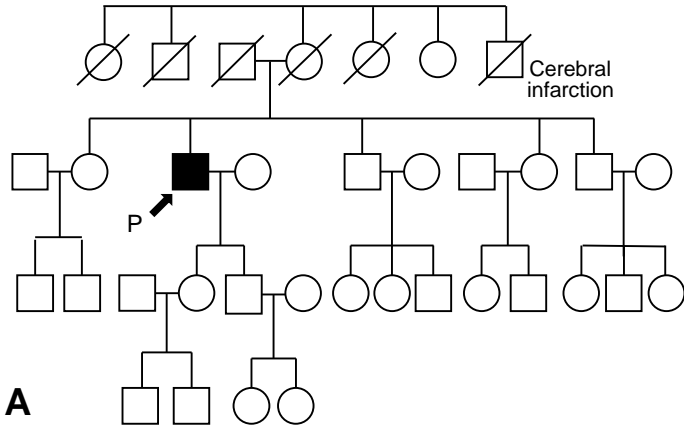
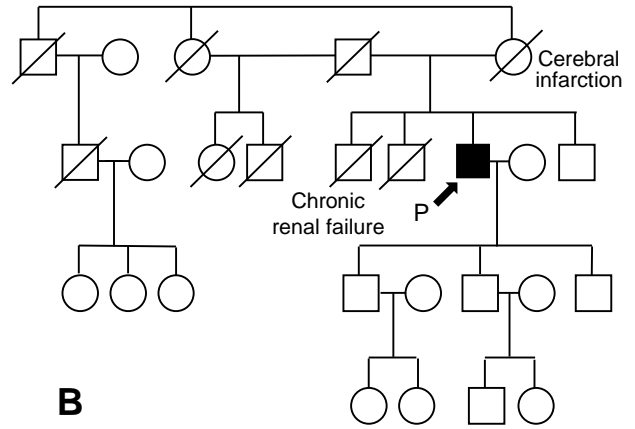Nakamura et al. Figure 1

Nakamura et al. Figure 2

Nakamura et al. Figure 3

Patient 1.

Cerebral
infarction

P

A

Patient 2.

Cerebral
infarction

Chronic
renal failure

P

B

Patient 3.

Cerebral
infarction

P

C

Patient 5.

P

D

Nakamura et al. Supplementary Figure 1