Prevalence of Fabry Disease and *GLA* c.196G>C Variant in Japanese Stroke Patients

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Running title: Prevalence of Fabry Disease in Stroke Patients

Abstract

***Background and purpose*:** Fabry disease is an important underlying disease in young cryptogenic stroke patients. However, little is known regarding the frequency of Fabry disease in the general stroke population, especially in elderly patients.

***Methods*:** A total of 588 stroke patients (61.7% men; average age 74.1 ± 12.5 years) were enrolled in this prospective study. Blood samples were obtained to produce blood spots to determine α-galactosidase A (α-GalA) activity and for *GLA* gene analysis.

***Results*:** One 65-year-old female patient had a known *GLA* gene mutation, c.2T>C (p.M1T), causing Fabry disease. Five male patients and two female patients had *GLA* c.196G>C (p.E66Q) variant, which is not associated with the full clinical manifestations of Fabry disease. The allele frequency of *GLA* c.196G>C was significantly higher in male patients with small-vessel occlusion (odds ratio 3.95, $P = 0.048$) and non-cardioembolism (odds ratio 4.09, $P = 0.012$) than that in the general Japanese population.

***Conclusions*:** Fabry disease is rare in the general Japanese stroke population. However, screening identified one elderly female patient with Fabry disease. *GLA* c.196G>C variant is a genetic risk factor for cerebral small vessel occlusion and non-cardioembolism in Japanese males, but not in females.

Introduction

Fabry disease (MIM301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene encoding the lysosomal enzyme, α-galactosidase A (α-Gal A, EC3.2.1.22) [1]. α-Gal A hydrolyses the terminal α-galactosyl moieties from glycolipids and glycoproteins, and therefore its deficiency results in progressive accumulation of globotriaosylceramide (GL-3) and related glycosphingolipids in the vascular endothelial lysosomes of the kidneys, heart, brain, and skin. Fabry disease is classified into three major subtypes, i.e., classic, late-onset, and female Fabry disease. The classic phenotype develops in hemizygous males that have little or no detectable α-Gal A activity with childhood-onset of acroparesthesia, hypohidrosis, and angiokeratoma. The occurrence of renal failure, cardiac disease, and stroke with advancing age lead to a decline in activities of daily living and premature death. On the other hand, the late-onset phenotype develops in hemizygous males with substantial levels of residual α-Gal A activity [2, 3]. Heterozygous females with *GLA* mutation were initially thought to be clinically unaffected. However, a large-scale study showed that 69.4% of such females have symptoms and signs of Fabry disease [4]. As most patients with late-onset and female Fabry disease have adult onset and frequently lack a relevant family history, there is a concern that many Fabry patients may be underdiagnosed and classified as having other more common diseases. Indeed, considerable numbers of adult patients with Fabry disease were found through screening of high-risk populations, including those with chronic kidney disease, left ventricular hypertrophy/ hypertrophic cardiomyopathy, and young stroke [2, 3, 5-14]. Recently,

we analyzed the frequency of *GLA* mutations in male Japanese ischemic stroke patients and showed that *GLA* c.196G>C (p.E66Q) variant is a genetic risk factor for cerebral small-vessel occlusion in elderly Japanese men [15]. In this previous study, the frequency of c.196G>C variant may had been underestimated as DNA sequencing was performed only in patients with reduced α-Gal A activity [15] and individuals with this variant occasionally showed blood α-Gal A activity within the normal range. In addition, female and hemorrhagic stroke patients have yet to be analyzed. In the present study, we performed additional screening for *GLA* gene mutation in a new set of Japanese stroke patients, including female and hemorrhagic stroke patients, with screening for *GLA* c.196G>C variant in all patients.

Materials and Methods

Patients

Six clinical neurology departments in Nagano prefecture, Japan, participated in this prospective study. From May 2012 to March 2016, a total of 588 stroke patients (61.7% men; average age 74.1 ± 12.5 years), were enrolled in this study (Table 1). The etiology of ischemic stroke was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [16] as large-artery atherosclerosis in 113 patients, cardioembolism in 88, and small-vessel occlusion (lacunar infarction) in 179. Eight patients showed ischemic stroke of other determined etiology: cervicocephalic arterial dissection (n = 5), patent foramen ovale plus venous thrombosis of lower extremity (n = 1), paraneoplastic coagulopathy (n = 1), and

sinus thrombosis (n = 1). Ischemic stroke of undetermined etiology was present in 88 patients. Cerebral hemorrhage was present in 126 patients. Subtypes of stroke overlapped in 14 patients, as they had histories of multiple strokes with different subtypes. Patients that were unable to provide informed consent or that had already been diagnosed with Fabry disease were excluded from the study. After obtaining informed consent, 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.

α-Gal A enzyme assay and *GLA* gene 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 [15, 17].

If blood αGal A activity was decreased (<10 AgalU), the entire *GLA* gene was analyzed. For DNA analysis, total genomic DNA was extracted from leukocytes of patients. All seven exons and the flanking intronic sequences of the *GLA* gene were amplified by polymerase chain reaction (PCR), and the amplification products were analyzed by direct sequencing. All patients were screened for *GLA* c.196G>C (p.E66Q) variant (rs104894833), as individuals with this variant occasionally showed blood αGal A activity within the normal range. Genotyping of rs104894833 was performed using the ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) in

accordance with the manufacturer's instructions. If *GLA* c.196G>C was detected, whole *GLA* gene was analyzed by PCR and direct sequencing as described above.

Statistical analysis

To analyze the impact of *GLA* c.196G>C variant on the onset of stroke, we compared allele frequencies of *GLA* c.196G>C variant in stroke patients with that in the general Japanese population; we utilized the data from newborn screening for Fabry disease [15] as a control (general Japanese population). We also compared blood α-Gal A activity of patients with c.196G>C variant with that of patients without the variant. Statistical comparisons were performed using Fisher's exact test for binary outcomes, and Mann–Whitney U test for continuous variables. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

Protocol approvals and patient consent

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. Written informed consent was obtained from each patient prior to enrollment.

Results

α-Gal A enzyme assay and *GLA* gene analysis

The average blood α-Gal A activities of the male and female study populations were 25.0 ± 8.8 AgalU and 25.5 ± 11.0 AgalU, respectively (Figure 1). We performed whole *GLA* gene

analysis in two female patients with low blood α-Gal A activity (< 10 AgalU), and the results indicated c.2T>C (p.M1T) mutation in one patient. The other patient showed no mutations or polymorphisms. Screening for c.196G>C variant in the whole study population detected five male patients and two female patients with this variant (Table 2). Whole *GLA* gene analysis of the seven patients detected no mutations or polymorphisms in coding regions other than c.196G>C. One female patient (patient 7, Table 2) had a known polymorphism in the 5′ non-coding region (c.-10C>T) in addition to c.196G>C.

Clinical data of the patient with Fabry disease identified through screening of stroke patients.

The patient was a 65-year-old Japanese woman (II-2, Figure 2A). Her father (I-1, Figure 2A) died of sepsis at age 31 and her mother (I-2, Figure 2A) died of myocardial infarction at age 60. Her 63-year-old brother (II-3, Figure 2A), 41-year-old daughter (III-1, Figure 2A), and 38-year-old son (III-2, Figure 2A) had no obvious relevant medical history. There was no family history of acroparesthesia, hypohidrosis, angiokeratoma, renal failure, cardiomyopathy, or stroke.

The patient (II-2, Figure 2A) had been well until 59 years old, when she developed cardiac failure and hypertension, which was treated with amlodipine, enalapril maleate, and betaxolol hydrochloride. At age 65, she developed acute onset of left-sided weakness and was admitted to our hospital. Brain magnetic resonance (MR) imaging and MR angiography

showed an acute infarction in the right temporal lobe due to right middle cerebral artery occlusion (Figure 2B, C). Chest X-ray revealed enlarged cardiac shadow (Figure 2D) and electrocardiography showed atrial fibrillation, left ventricular hypertrophy, and ST-T abnormality (Figure 2E). Echocardiography showed mild symmetrical thickening of the ventricular wall (diastolic thickness of the left ventricular posterior wall, 12 mm; diastolic thickness of the interventricular septum, 12 mm) and diastolic dysfunction (ratio of early mitral inflow velocity to mitral annular early diastolic velocity [E/e' ratio], 17.3), although cardiac systolic function was preserved (left ventricular ejection fraction, 57.8%; fractional shortening, 30.3%). Measurement of α-Gal A in the whole blood revealed markedly reduced activity of 5.5 AgalU (Figure 1B) and direct DNA sequencing of the *GLA* gene revealed a single base sequence change (c.2T>C) causing substitution of a methionine reside with threonine at codon 1 (p.M1T), the known mutation causing classic Fabry disease [18].

Clinical characteristics of patients with *GLA* c.196G>C variant

The clinical characteristics of the seven patients with *GLA* c.196G>C variant are summarized in Table 2. Five patients were hemizygous males, and the others were heterozygous females. Patients were > 70 years old except for two males (44 and 59 years). In the male patients with c.196G>C variant, mean blood α-Gal A activity was 13.1 ± 2.6 (ranged from 10.1 to 17.8), which was significantly lower than that in male patients without the variant (25.2 ± 8.7, ranged from 10.4 to 61.6, $P = 0.0005$, Figure 1A). On the other hand, α-Gal A activity of

female patients with c.196G>C variant (31.7 ± 10.3, ranged from 21.4 to 41.9) was not significantly different from that of female patients without the variant (25.5 ± 11.0, ranged from 9.5 to 95.0, $P = 0.402$, Figure 1B).

Four male patients (patients 2, 3, 4 and 5) had multiple small-vessel occlusions, which were accompanied by mild white matter lesions (leukoaraiosis). One patient (patient 2) with small-vessel occlusions had a history of symptomatic thalamic hemorrhage and the other two patients (patients 3 and 5) had asymptomatic multiple microbleeds detected by T2*-weighted MRI. Two male patients (patients 1 and 4) had large-artery atherosclerosis, one female patient (patient 6) had cardioembolism, and one female patient (patient 7) had small-vessel cerebral infarction. No patients with *GLA* c.196G>C variant showed characteristic symptoms of Fabry disease, except one elderly male patient that had chronic kidney disease (serum creatinine level, 2.0 mg/dL).

Statistical analysis of allele frequencies of the *GLA* c.196G>C variant in Japanese stroke patients

Statistical analysis using the Fisher's exact test indicated that the allele frequencies of the *GLA* c.196G>C variant in male patients with small-vessel occlusion (2.46%, odds ratio 3.94, $P = 0.048$) and non-cardioembolism (2.54%, odds ratio 4.08, $P = 0.012$) were significantly higher than that in the general Japanese population (0.64%, Table 3). On the other hand, the allele frequencies of the male patients with all stroke, all ischemic stroke, large-artery

atherosclerosis, cardioembolism, and hemorrhagic stroke were 1.37%, 1.65%, 2.67%, 0%, and 1.37%, respectively; these differences were not statistically significant (Table 3). These results were compatible with our previous study [15]. In this study, we analyzed female stroke patients in addition to male patients. In contrast to male patients, the allele frequency of the *GLA* c.196G>C variant in female stroke patients was not increased even in those with small-vessel occlusion (0.88%, odds ratio 1.39, $P = 0.52$) and non-cardioembolism (0.53%, odds ratio 0.83, $P = 1.00$).

Discussion

Stroke is one of the most common and serious complications of Fabry disease. According to the natural history data from the Fabry Registry, the prevalences of stroke in Fabry disease were 6.9% in male patients and 4.3% in female patients [19]. Importantly, stroke may occur before diagnosis of Fabry disease and in the absence of other clinical manifestations of the disease [19]. In fact, a recent large-scale screen (The Stroke in Young Fabry Patients Study) identified previously undiagnosed Fabry disease patients in 0.5% (definite Fabry disease) to 0.9% (definite + probable Fabry disease) of young stroke patients [13]. On the other hand, Fabry disease was considered to be very rare in elderly stroke patients [15, 20]. The present study identified a 65-year-old female Fabry patient that developed cardiogenic cerebral embolism. This is the first elderly patient identified through screening for Fabry disease in a stroke population, suggesting that Fabry disease should be considered in the differential diagnosis of

underlying disease of stroke even in elderly patients, particularly in patients with left ventricular hypertrophy and/or chronic kidney disease.

Another intriguing finding in the present study was the high prevalence of c.196G>C variant in male patients with small-vessel occlusions and non-cardioembolism. *GLA* c.196G>C is associated with relatively low α-GAL activity as compared to individuals without variants and had been considered as a pathogenic mutation, which induces late-onset Fabry disease associated with ventricular hypertrophy [8, 21, 22], renal failure [2, 8, 22, 23], or cerebrovascular disease [24, 25]. On the other hand, recent studies showed that c.196G>C is not a rare mutation, but is found at certain frequencies in the general Korean [26] and Japanese [27] populations, suggesting that this variant is a functional polymorphism. In addition, pathological studies of biopsied renal [28], cardiac [29], and skin tissues [27] from male subjects with c.196G>C variant showed no abnormal accumulation of GL-3. At present, there is a consensus that c.196G>C variant is not associated with the full clinical manifestation of Fabry disease, but its pathogenesis remains controversial [15, 24-29]. Recently, Nakamura et al. [15] reported that *GLA* c.196G>C variant is a genetic risk factor (allele frequency, 2.08; odds ratio = 3.34) for cerebral small-vessel occlusion in elderly Japanese men. However, the frequency of c.196G>C in stroke patients may had been underestimated in the previous study as DNA sequencing was performed only in patients with reduced α-Gal A activity [15] and individuals with this variant occasionally showed blood α-Gal A activity within the normal range. Therefore, we screened for *GLA* c.196G>C variant in all patients in this study and confirmed

that the allele frequencies of *GLA* c.196G>C in male patients with cerebral small-vessel occlusion and non-cardioembolism were significantly higher than that in the general Japanese population (Table 3). In contrast to male stroke patients, the allele frequency of c.196G>C was not high in female patients with stroke, even in cases of small-vessel occlusion or non-cardioembolism suggesting that heterozygosity of this variant is not pathogenic. This appears to be reasonable considering the relatively mild impact of c.196G>C variant on onset of stroke in hemizygous male patients and the observation that blood α-Gal A activity was not decreased at all in heterozygous female patients (Figure 1B).

In Caucasian populations, the *GLA* c.937G>T (p.D313Y) variant is found with an allele frequency of 0.45% [30] and showed relatively preserved α-Gal A activity [11] similar to c.196G>C variant in the Japanese population. In the largest screen for Fabry disease in a young stroke population performed to date [13], patients with c.937G>T variant were regarded as "probable Fabry disease", as patients harboring this variant showed significantly increased Gb3 and Gb3-C24 in addition to decreased α-Gal A activity. Similarly, Maruyama et al. reported that two of nine patients with c.196G>C showed detectable levels of lyso-Gb3, a promising biomarker for Fabry disease [31]. Taken together, these studies and the results of the present study suggest that *GLA* variants associated with relatively high residual α-Gal A activity may add to the risk of stroke, although they are not associated with full clinical presentation of Fabry disease with classical Mendelian effects.

Female Fabry patients may have been missed in the present study, as female patients could show normal α-Gal A activity. Therefore, plasma globotriaosylsphingosine (lyso-Gb3) [31] in addition to α-Gal A activity should be analyzed in screening of female patients for Fabry disease in future studies. Our study was also limited by the relatively small sample size. Further large-scale epidemiological studies, and biochemical and pathological examinations are necessary to elucidate the precise pathomechanism by which the c.196G>C variant increases the risk of stroke.

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Figure legends

Figure 1. The distribution of α-Gal A activity in all of the patients included in this study. The y-axis indicates α-Gal A activity (AgalU). The cut-off α-Gal A activity was 10 AgalU.

Figure 2. Pedigree (A), brain magnetic resonance (MR) imaging (B, diffusion-weighted image; C, MR angiography), chest X-ray (D), and electrocardiographic (E) findings of the Fabry patient with *GLA* c.2T>C (p.M1T) mutation.

Table 1. Demographic characteristics of the stroke patients

	All stroke	All ischemic stroke	Subtypes of ischemic stroke					Cerebral hemorrhage
			Large-artery atherosclerosis	Cardio-embolism	Small-vessel occlusion	Stroke of other determined etiology	Stroke of undetermined etiology	
Number of male patients (age, mean ± SD)	363 (71.9 ± 12.2)	303 (72.7 ± 12.2)	75 (74.5 ± 9.9)	47 (78.8 ± 10.0)	122 (70.2 ± 12.6)	6 (59.8 ± 8.1)	53 (72.5 ± 13.0)	73 (68.2 ± 11.0)
Number of Female patients (age, mean ± SD)	225 (77.8 ± 12.2)	173 (77.8 ± 11.9)	38 (77.5 ± 10.9)	41 (82.9 ± 8.1)	57 (75.3 ± 12.8)	2 (63.0 ± 5.0)	35 (80.2 ± 12.8)	53 (75.8 ± 12.9)

Table 2. Clinical characteristics of patients with *GLA* c.196G>C variant

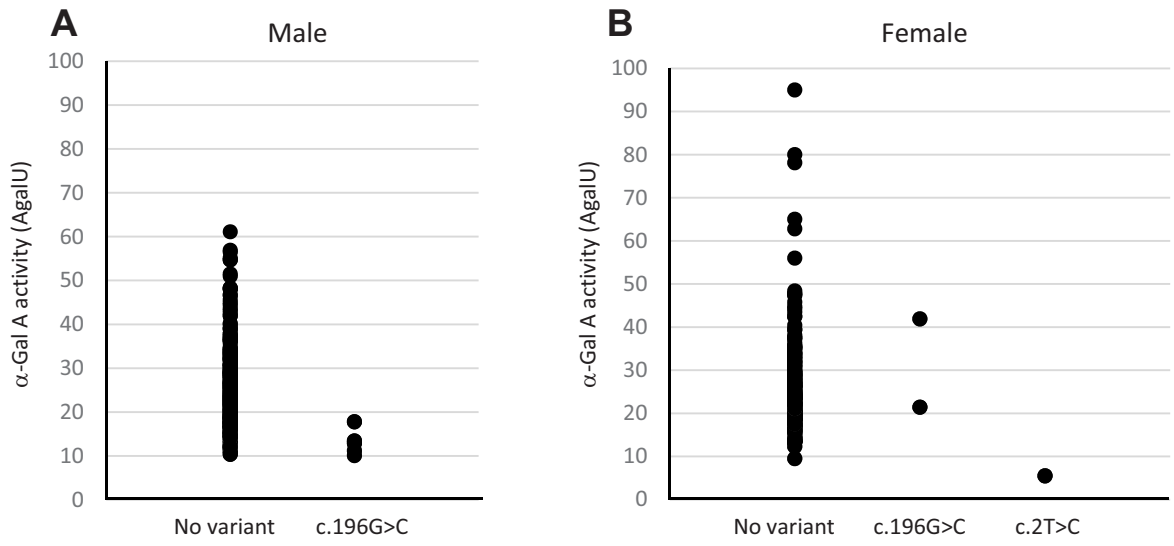
Patient	Age	Sex	Blood αGalA activity (AGalU)	Type of stroke	Risk factor for stroke	Other complications
1	44	M	13.4	*IS (large-artery)	Hypertension / Hyperlipidemia	–
2	59	M	17.8	*IS (small-vessel, multiple) **HS (thalamic)	Hypertension / Diabetes mellitus	–
3	80	M	12.9	*IS (small-vessel, multiple) Asymptomatic microbleeds	–	–
4	81	M	11.2	*IS (large artery, multiple)	Hyperlipidemia	–
5	87	M	10.1	*IS (small-vessel, multiple) Asymptomatic microbleeds	Hypertension	Chronic kidney disease
6	77	F	41.9	*IS (cardioembolism, multiple)	Hypertension	Left atrial enlargement
7	90	F	21.4	*IS (small-vessel)	Hypertension	-

*IS, ischemic stroke; **HS, hemorrhagic stroke

Table 3. Allele frequencies of the *GLA* c.196G>C variant in Japanese stroke patients

		Number of subjects	Number of alleles	Number of c.196G>C alleles	c.196G>C allele frequency (%)	Odds ratio (vs. control)	*P*-value (vs. control)
All stroke	Both	588	813	7	0.86	1.36	0.482
	Male	363	363	5	1.37	2.19	0.098
	Female	225	450	2	0.44	0.70	1
All ischemic stroke	Both	476	646	7	1.08	1.73	0.199
	Male	303	303	5	1.65	2.65	0.054
	Female	173	346	2	0.58	0.92	1
Large-artery atherosclerosis	Both	113	151	2	1.32	2.11	0.259
	male	75	75	2	2.67	4.30	0.088
	Female	38	76	0	0	0	1
Cardioembolism	Both	88	129	1	0.78	1.23	0.566
	Male	47	47	0	0	0	1
	Female	41	82	1	1.22	1.94	0.413
Small-vessel occlusion	Both	179	236	4	1.69	2.70	0.075
	Male	122	122	3	2.46	3.95	0.048
	Female	57	114	1	0.88	1.39	0.522
*Non-cardioembolism	Both	292	387	6	1.55	2.48	0.050
	Male	197	197	5	2.54	4.08	0.012
	Female	95	190	1	0.53	0.83	1
Cerebral hemorrhage	Both	179	179	1	0.56	0.88	1
	Male	73	73	1	1.37	2.18	0.378
	Female	53	106	0	0	0	1
Control (newborn screening)	Male	5051	5051	32	0.64		

*Non-cardioembolism = large-artery atherosclerosis + small-vessel occlusion



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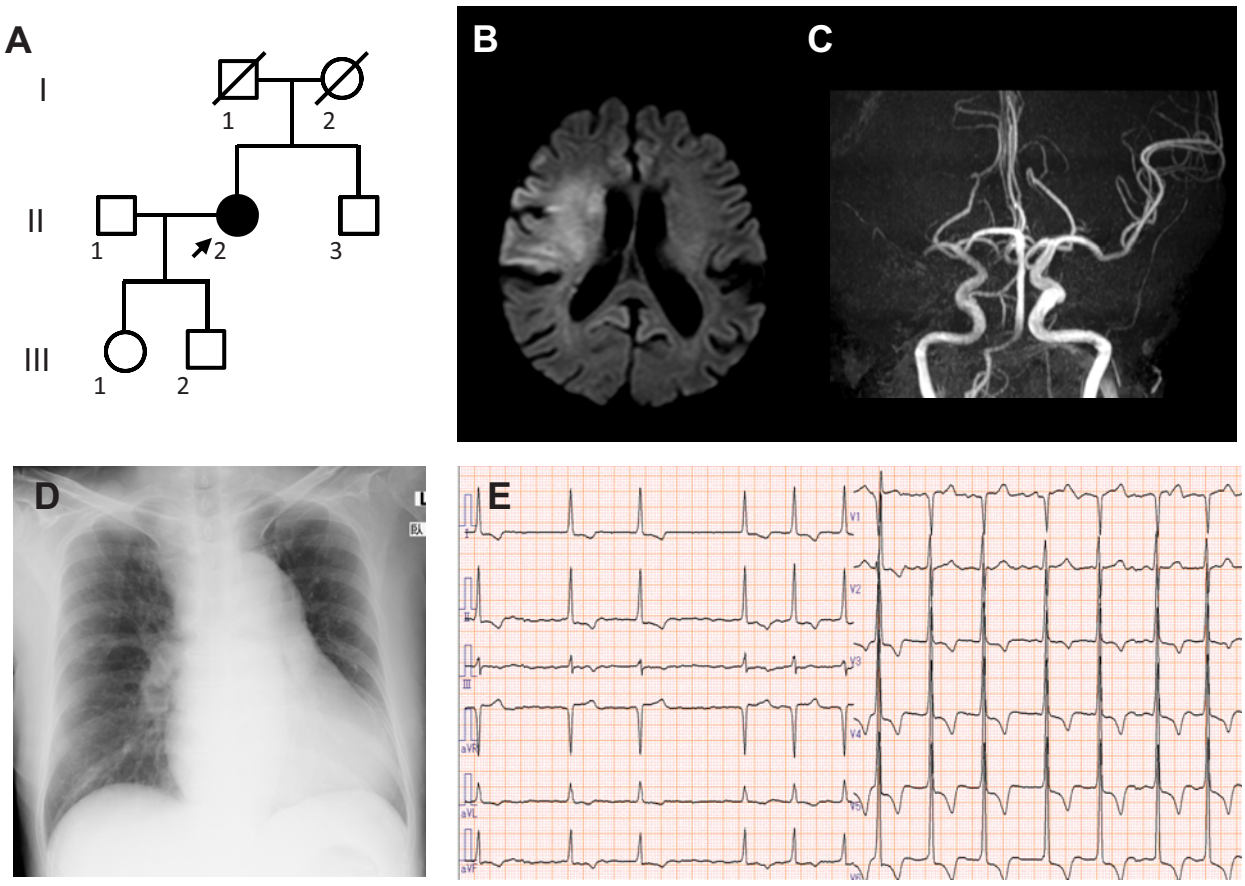

Nagamatsu et al. Figure 2