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High-risk screening for Anderson–Fabry disease in patients with cardiac, renal, or neurological manifestations

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Abstract

Anderson–Fabry disease (AFD) is an X-linked lysosomal storage disorder caused by abnormalities in the α -galactosidase (*Gal*) A gene (*GLA*; MIM:300644). The reduced activity of the lysosomal enzyme, α -galactosidase A (α -Gal A) leads to classic early manifestations and vascular disease of the heart, kidneys, and brain. As a high-risk screening for symptomatic AFD using an enzymatic assay on dried blood spot samples, we enrolled 2325 individuals (803 females and 1522 males; median age: 66 years) with cardiac, renal, or neurological manifestations that met at least one of the following criteria: (a) family history of early-onset cardiovascular diseases; (b) typical classic manifestations, such as acroparesthesias, clustered angiokeratoma, cornea verticillata, and hypo-anhidrosis; (c) proteinuria; (d) receiving dialysis; (e) left ventricular hypertrophy on electrocardiography or echocardiography; or (f) history of stroke. Ninety-two patients displayed low α -Gal A activity. Four males and two females had different pathogenic *GLA* mutations (0.26%) including a novel mutation c.908-928del21. Four males (0.17%) harbored the *GLA* c.196G>C (p.E66Q) variant. This simple screening protocol using dried blood spot samples is useful for early diagnosis of AFD in high-risk and underdiagnosed patients suffering from various cardiac, renal, or neurological manifestations.

Introduction

Anderson–Fabry disease (AFD) is an X-linked lysosomal storage disorder caused by abnormalities in the α -galactosidase (*Gal*) A gene (*GLA*, MIM:300644). The reduced activity of the lysosomal enzyme, α -galactosidase A (α -Gal A) [1] leads to classic early manifestations that include acroparesthesias, clustered angiokeratoma, cornea verticillata, and hypo-anhidrosis, as well as vascular disease of the heart, kidneys, and brain [1].

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The worldwide incidence of AFD is reportedly ranges from 1 in 40,000–117,000 [2]. This may be a significant underestimate given the under-recognition of symptoms and delayed or missed diagnosis [1].

Screening of high-risk populations displaying various cardiac, renal, or neurological manifestations could increase the diagnostic rate of AFD and facilitate therapeutic interventions that could prevent serious complications. Currently, screening for AFD involves measuring α -Gal A activity in peripheral blood leukocytes. However, the evaluation of dried blood spot (DBS) samples can permit easier and more efficient screening. We herein report our high-risk screening program of AFD and successful detection of six patients with AFD among 2325 patients with various cardiac, renal, or neurological manifestations.

Materials and methods

Experimental design

Forty-five hospitals in Hokkaido prefecture, Japan, participated in this prospective study. From July 2012 to Table 1Demographiccharacteristics of the enrolledpatients

	Total	Female	Male
Age (interquartile range)	66 (56–74)	66 (57–73)	66 (55–74)
α-Gal A activity (standard deviation)	24.4 (11.2)	24.2 (12.4)	24.5 (10.5)
Inclusion criteria			
Family history of early-onset cardiovascular diseases	304 (13.1%)	131 (16.3%)	173 (11.4%)
Typical classic manifestations ^a	417 (17.9%)	155 (19.3%)	262 (17.2%)
Proteinuria	374 (16.1%)	129 (16.1%)	245 (16.1%)
Dialysis	1476 (63.5%)	503 (62.6%)	973 (63.9%)
Left ventricular hypertrophy	601 (25.8%)	205 (25.5%)	396 (26.0%)
Prior stroke	259 (11.1%)	89 (11.1%)	170 (11.2%)

^aTypical classic manifestations include acroparesthesias, clustered angiokeratoma, cornea verticillata, hypoanhidrosis

December 2017, we analyzed DBS samples obtained from 2325 patients with various cardiac, renal, or neurological manifestations. Patients were included if they met at least one of the following criteria: (a) family history of early-onset cardiovascular diseases (to identify patients with early and asymptomatic, especially for asymptomatic hetero-zygote); (b) typical classic manifestations, such as acro-paresthesias, clustered angiokeratoma, cornea verticillata, and hypo-anhidrosis; (c) proteinuria; (d) receiving dialysis; (e) left ventricular hypertrophy (LVH) on electrocardiography or echocardiography; or (f) a history of stroke. Patients who did not agree to undergo measurement of α -Gal A activity and genetic analysis were excluded. The standard nomenclature for representing pedigrees was used [3].

Preparation of DBS samples

All the DBS samples were prepared from peripheral blood as previously reported [4]. Blood droplets were spotted onto filter paper (Toyo Roshi Kaisha Ltd., Tokyo, Japan), allowed to dry for ~4 h at room temperature, and sent through the mail within 1 week of preparation to Kumamoto University for analysis. The DBS samples were stored at -20 °C until use.

Measurement of $\alpha\mbox{-Gal}$ A activity and DNA analysis of GLA

We measured the α -Gal A activity of 2325 patients using a DBS test with a slight modification of a method described previously [5, 6]. α -Gal A activity <20 Agal U in females and <12 Agal U in males was identified as abnormally low. Patients with α -Gal A activity <20 Agal U in females or <12 Agal U in males had blood drawn again for the remeasurement of the α -Gal A activity on another day. Patients with low α -Gal A activity after re-measurement were assessed clinically, and they underwent a genetic analysis of *GLA* after genetic counseling by clinical

geneticist certified by the Japanese board of medical genetics and genomics, clinical genetics. For DNA analysis, total genomic DNA was extracted from the leukocytes of the patients. All seven exons and the flanking intronic sequences of *GLA* were amplified by PCR, and the amplification products were analyzed by direct sequencing. All patients were screened for the *GLA* c.196G>C (p.E66Q) variant (rs104894833) as described previously [6].

Protocol approvals and patient consent

This study was approved by the Ethical Committee of Asahikawa Medical University (no. 735) and the ethics committees of each of the participating hospitals. Written informed consent was obtained from each patient prior to enrollment.

Results

Characteristics of patients

We enrolled 2325 individuals (803 females and 1522 males; median age, 66.0 years). Table 1 summarizes their demographic characteristics. The patient group included 1476 patients (63%) undergoing hemodialysis. Of the 2325 patients, 601 (26%) had LVH, 374 (16%) had proteinuria, 259 (11%) had prior stroke, 417 (18%) had any typical classic manifestations that included acroparesthesias, clustered angiokeratoma, cornea verticillata, or hypo-anhidrosis, and 304 (13%) had a family history of early-onset cardiovascular diseases.

$\alpha\text{-}Gal\ A$ assay of DBS samples and molecular analysis of GLA

The mean (SD) blood α -Gal A activities of the females and males were 24.2 (12.4) and 24.5 (10.5) Agal U,

respectively. α -Gal A activity values <20 Agal U in females and <12 Agal U in males were observed for 410 (333 females and 77 males) of the 2325 patients (Fig. 1). Thus, we repeated the α -Gal A assay using DBS samples obtained for a second time from these 410 patients. In the second determination, α -Gal A activity values <20 Agal U in females and <12 Agal U in males were observed for 92 (80 females and 12 males) of the 410 patients. Samples from these 92 patients were analyzed for mutations in *GLA*. Four men and two women displayed a different pathogenic mutation (0.26%; Fig. 2). The four male patients had the *GLA* c.196G>C (p.E66Q) variant (0.17%), which is not associated with the full clinical manifestations of AFD.

When a subset of patients undergoing hemodialysis was analyzed separately, α -Gal A activity values <20 Agal U in females and <12 Agal U in males were observed for 52 (46 females and six males) of 1476 (503 females and 973 males) patients. Finally, two male patients (patient 4 and 6 in Table 2) had a pathogenic mutation (0.14%) among the 1476 patients undergoing hemodialysis. As for the subset of patients with LVH and stroke, five (patient 1, 2, 4, 5, and 6 in Table 2) of 601 patients with LVH (0.8%) and two (patient 1 and 3 in Table 2) of 259 patients with prior stroke (0.8%) had a pathogenic mutation.

Clinical characteristics of patients with pathogenic *GLA* mutation

The clinical characteristics of the six patients with a pathogenic *GLA* mutation (patients 1–6) and the four patients with a nonpathogenic c.196G>C (p.E66Q) variant (patients 7–10) are summarized in Table 2.

Patient 1 was a 42-year-old female with aseptic meningitis, ischemic stroke, LVH, and schizoaffective psychosis, as reported previously [7]. α -GAL activity determined using DBS samples was low (11.9 Agal U) and genetic analysis revealed the presence of a heterozygous mutation in intron 4 of *GLA* c.IVS4-1G>A, which has been reported previously [8]. Genetic analyses of the patient's parents and daughter did not detect this mutation. Therefore, the patient was diagnosed with de novo AFD (Fig. 2a).

Patient 2 was a 36-year-old male with LVH detected by a health check-up (Fig. 2b, d and e). Cardiovascular magnetic resonance imaging revealed concentric LVH with late gadolinium enhancement in the basal posterolateral wall (Fig. 2f). He also suffered from coronary spastic angina, acroparesthesias, clustered angiokeratoma, hypo-anhidrosis, and gastrointestinal disorders. α -GAL activity was markedly low (2.8 Agal U) and genetic analysis revealed the presence of a hemizygous nonsense mutation in exon 5 of *GLA* c.707G>A (p.W236X), which has been reported previously [9]. His mother had a heterozygous mutation and LVH with coronary spastic angina (Fig. 2b). Patient 3 was a 37-year-old female with nephrotic range proteinuria (4.0 g/gCr) and juvenile minor ischemic stroke. Her renal function and serum albumin were preserved with a serum creatinine of 0.70 mg/dL and serum albumin of 4.0 g/dL. She had a kidney biopsy, which demonstrated podocyte vacuolization on light microscopy sections with podocyte inclusions on semi-thin sections (Fig. 2g). α -GAL activity was low (9.0 Agal U) and genetic analysis revealed the presence of a hemizygous deletion mutation in exon 7 of *GLA* c.1072_1074del (p.358delE), which have been reported previously [10]. Genetic analyses of the patient's parents did not detect this mutation. However, she had two boys with acroparesthesias and this hemizygous *GLA* mutation (Fig. 2c).

Patient 4 was a 64-year-old male who underwent hemodialysis for 20 years after being diagnosed with glomerulonephritis without kidney biopsy. He also had hypoanhidrosis and LVH. α -GAL activity was markedly low (1.9 Agal U) and genetic analysis revealed the presence of a hemizygous missense mutation in exon 4 of *GLA* c.559A>G (p.M187V), which has been reported previously [11]. His mother died due to heart failure at 83 years of age and his maternal aunt underwent hemodialysis and died at 82 years of age, although they had undiagnosed AFD (Fig. 2h).

Patient 5 was a 56-year-old male with nephrotic range proteinuria (3.8 g/g Cr), renal dysfunction with a serum creatinine of 2.0 mg/dL and mild LVH. α -GAL activity was markedly low (2.8 Agal U) and genetic analysis revealed the presence of a hemizygous missense mutation in exon 7 of *GLA* c.1124G>A (p.G375E), which has been reported previously [12]. His mother had undiagnosed AFD, underwent hemodialysis, and died due to colon cancer at 76 years of age (Fig. 2i). He had two daughters who in theory must be heterozygous (Fig. 2i). Therefore, they will be analyzed for mutations in *GLA*.

Patient 6 was a 55-year-old male patient who underwent hemodialysis for 4 years after being diagnosed with glomerulonephritis without kidney biopsy. He had also acroparesthesias, hypo-anhidrosis, and LVH. α -GAL activity was markedly low (3.7 Agal U) and genetic analysis revealed the presence of a hemizygous deletion mutation in exon 7 of *GLA* c.908-928del21, which is a novel mutation not described before in the literature. His mother had undiagnosed AFD, but died due to heart failure at 82 years of age (Fig. 2j).

Among these patients with AFD, patients 2, 3, and 4 had resting angina and were diagnosed with coronary spastic angina by acetylcholine-loaded coronary spasm provocation tests as reported most recently [13]. All six patients with AFD had been regularly treated using enzyme replacement therapy (ERT), whereas the four patients with the *GLA* c.196G>C (p.E66Q) variant had not begun ERT.

Fig. 1 a Flow chart for screening patients with AFD. Two female and four male patients had a different pathogenic mutation. Further, four male patients had the GLA c.196G>C (p.E66Q) variant (0.17%), which is not associated with the full clinical manifestations of AFD. **b**, **c** Distribution of α-galactosidase A activity determined using DBS samples in female a and male b patients in the study. The mean (SD) α -Gal A activities of the female and male study populations were 24.2 (12.4) and 24.5 (10.5) Agal U, respectively. α-Gal A activity values <20 Agal U in females and <12 Agal U in males were observed for 410 (333 females and 77 males) out of 2325 patients



Discussion

In this study, we confirmed the diagnosis of AFD in four men and two women who had different pathogenic *GLA* mutations (0.26%) including a novel mutation c.908-928del21, and four men who had the *GLA* c.196G>C (p. E66Q) variant (0.17%) among 2325 patients who suffered from various cardiac, renal, or neurological manifestations. This simple screening protocol using DBS samples is useful for the early diagnosis of AFD in high-risk and underdiagnosed patients suffering from these manifestations.

The prevalence in selected patient cohorts was previously reported to be between 0.25% and 3.5% in male patients undergoing hemodialysis [14–17], 0.9–3.9% in male patients with hypertrophic cardiomyopathy [17–20], and 0.13–5% in patients with cryptogenic stroke [17, 21]. In the present study, the prevalence of AFD was 0.26% for the 2325 patients who suffered from various cardiac, renal, or neurological manifestations; 0.14% for the 1476 hemodialysis patients; and 0.8% for the 601 patients with LVH and 259 patients with prior stroke. These rates were similar to previously reported prevalence rates even though our screening included females. In general, many females who are heterozygous for pathogenic *GLA* mutations have normal plasma α -Gal A activity. Thus, screening studies for AFD have predominantly focused on male patients. We presently confirmed the diagnosis of AFD in two women with stroke and LVH or proteinuria, suggesting that the inclusion of female patients with multiple cardiac, renal, or neurological manifestations might be taken into consideration in screening studies for AFD.

In the present study, roughly 40% of the enrolled female patients required re-measurement of α -GAL A activity because of measured values <20 Agal U. The rate of low α -GAL A activity was reduced to 10% upon repeat testing. This discrepancy in the measured enzyme activity might have been caused by inappropriate preparation and storage of DBS. Exposure of DBS to elevated heat and humidity can rapidly degrade enzyme functions [22]. Therefore, proper and consistent DBS preparation and storage are very important when screening for deficiencies of lysosomal enzymes.



Fig. 2 Family pedigrees and representative findings of the patients with AFD in this study. **a**, **b**, **c**, **h**, **i**, **j** Family pedigrees of patient 1 (**a**), patient 2 (**b**), patient 3 (**c**), patient 4 (**h**), patient 5 (**i**) and patient 6 (**j**). Males are denoted by squares, females by circles. Filled symbols indicate those with AFD. Open circles with vertical black lines indicate asymptomatic hemizygote patients with AFD. Open symbols indicate normal patients. Symbols with a question mark indicate family members who may carry the pathogenic mutation of AFD. The

dashes indicate deceased patients. d-f Chest X-ray (d), electrocardiography (e) and cardiovascular magnetic resonance (f) of patient 2 showed LVH with complete right bundle branch block (e) and with late gadolinium enhancement in the basal posterolateral wall (f). g Pathologic findings from light microscopy of patient 3; all glomeruli show marked swelling of podocyte with expanded foamy or vacuolar cytoplasmic change (periodic acid-methenamine-silver stain, ×400). GT genetic testing

Cardiac symptoms including LVH, arrhythmia, angina, and dyspnea have been reported in ~40–60% of patients with AFD [23, 24]. Furthermore, most recently, we reported that a high prevalence of coronary spastic angina was complicated with AFD in eight of the nine patients with AFD including three cases in the present study [13]. Coronary spasms and multivessel coronary spasms have been reported to be more prevalent in Japanese people than in Westerners [25], suggesting that AFD might be an underlying cause of coronary spastic angina in Japanese patients.

Two classes of *GLA* mutations are recognized according to their pathogenicity [26]. Class 1 mutations, also termed pathogenic mutations, have a high probability of causing disease. Class 2 mutations are nonpathogenic polymorphisms and include five missense changes (p.E66Q, p.S102L, p.S126G, p.D313Y, p.F396Y) [27]. In the nonpathogenic polymorphisms, the c.196G>C (p.E66Q) variant has been frequently observed in previous screening studies of Japanese hemodialysis [14, 16, 28, 29] and stroke patietnt [6, 30]. Therefore, the four patients (two patients with prior stroke, one with LVH, and one undergoing hemodialysis) (Table 2) in this study with the *GLA* c.196G>C (p.E66Q) variant had not begun ERT. However, recent reports demonstrated that the *GLA* c.196G>C (p.E66Q) variant is a genetic risk factor for cerebral smallvessel occlusion in elderly Japanese males [30] and noncardioembolism in Japanese males, but not in females [6]. Thus, further epidemiological studies and biochemical and

Table	2 Clinical ch	aracteristics of th	he six patients with pathogenic GL	A mutation and of the four patient	s with non-pathe	genic c.196G>C	(p.E66Q) variant	
Patient	Age (years)/ sex	α-Gal A activity (AgalU)	<i>GLA</i> mutation Nucleotide change (amino acid change)	Typical classic symptoms	Proteinuria	Dialysis	LVH Ischemic Stroke	Others
1^{a}	45/F	11.9	c.IVS4–1G>A (unkonwn)	I	I	I	++	Aseptic meningitis, psychiatric symptoms
2^{b}	36/M	2.8	c.707G>A (p.W236X)	Acroparesthesias, clustered angiokeratoma, hypo-anhidrosis	I	I	ı +	CSA, gastrointestinal disorders
3^{b}	37/F	9.0	c.1072_1074del (p.358delE)	Acroparesthesias, hypo-anhidrosis	+, 4.0 g/g Cr	I	+	CSA
4 ^b	64/M	1.9	c.559A>G (p.M187V)	1	N/A	+, For 20 years	- +	CSA
5	56/M	2.8	c.1124G>A (p.G375E)	I	+, 3.8 g/g Cr	I	- +	1
9	55/M	3.7	c.908-928del21 (unknown)	Acroparesthesias, hypo-anhidrosis	N/A	+, For 4 years	- +	I
Ζ	63/M	10.8	c.196G>C (p.E66Q)	I	I	I	++	1
8	63/M	9.6	c.196G>C (p.E66Q)	1	I	I	+	1
6	42/M	9.0	c.196G>C (p.E66Q)	1	I	I	- +	1
10	86/M	7.9	c.196G>C (p.E66Q)	I	N/A	+, For 4 years	I	Ι
a-Gal .	A α-galactosi	dase A, GLA α-C	3al A gene, LVH left ventricular h	13 13 13 13 13 13 13 13 13 13 13 13 13 1	angina, N/A not	applicable		
^a The d	etailed clinics	al profile of patie	ant 1 is described in ref. [7]					

The detailed clinical profile of patients 7, 8, and 9 is described in ref. [13]

SPRINGER NATURE

N. Nakagawa et al.

pathological examinations are necessary to elucidate the precise pathological mechanism by which the *GLA* c.196G>C (p.E66Q) variant increases the risk of stroke.

Currently, ERT is available for the treatment that is necessary to prevent the irreversible organ damage that occurs in AFD, as well as in other lysosomal storage disorders, which include Gaucher disease, Pompe disease, and mucopolysaccharidosis. Furthermore, most recently an oral pharmacological chaperone migalastat for the treatment of AFD has become available for specific mutant (amenable) forms of *GLA* [31]. Therefore, it is important to identify patients with AFD and to perform pedigree analysis as soon as possible so that ERT or pharmacological chaperone therapy can be initiated in patients with AFD before irreversible organ damage and complications occur.

This study has several limitations. First, female patients with AFD might have been missed, as female patients could have normal α -Gal A activity. Therefore, plasma globotriaosylsphingosine (lyso-Gb3) [16] in addition to α -Gal A activity should be analyzed when screening female patients for AFD in future studies. Second, some mutation such as intronic mutation and large deletion might have been missed by sequencing only exon and exon/intron boundaries of GLA gene. Therefore, we are now carefully following patients with low enzyme activity, without mutation in exon and exon/intron boundaries. Third, there was a relatively small sample size. Further large-scale epidemiological studies are necessary to elucidate the prevalence of AFD among patients with various cardiac, renal, or neurological manifestations.

In conclusion, the high-risk screening system for AFD using DBS samples appears to be an effective method for identifying the specific disorder in individuals with cardiac, renal, or neurological manifestations caused by unidentified reasons. This simple diagnostic test is useful for specialists and general physicians to screen patients with various cardiac, renal, or neurological symptoms to identify patients with AFD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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