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Original Article

Screening of Fabry disease in patients with end-stage renal disease of unknown etiology: the first Thailand study

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Abstract

We aimed to explore the prevalence of Fabry disease in Thai patients who were diagnosed with end-stage renal disease (ESRD) of an unknown origin. Venous blood samples were collected from ESRD patients for biochemical and molecular studies. Alpha-galactosidase A (α-GAL A) screening was performed from dried-blood spots using fluorometry. Molecular confirmation was performed using DNA sequencing of the *GLA* gene. A total of 142 male and female patients were included in this study. Ten patients (7.04%) exhibited a significant decrease in α-GAL A activity. There were no definitive pathogenic mutations observed in the molecular study. However, four patients revealed a novel nucleotide variant at c.1 −10 C>T, which was identified as a benign variant following screening in the normal population. In conclusion, the α-GAL A assay utilizing dried-blood spots revealed a significant false positive rate. There was no definitive Fabry disease confirmed in Thai patients diagnosed with ESRD of unknown etiology.

Keywords: Fabry disease, end-stage renal disease (ESRD), Alpha-galactosidase A (α-GAL A)

Introduction

Fabry disease (OMIM #301500) is an X-linked lysosomal storage disorder, characterized by decreased or absent activity of lysosomal α-galactosidase A (α-GAL A) due to mutations in the *GLA* gene (NG_007119). Patients with this disorder are unable to effectively degrade membrane glycosphingolipids, which causes progressive accumulation of globotriaosylceramide (GL-3) and other glycosphingolipids in the vascular endothelium, renal epithelial cells, cardiac myocytes and neurons. Patients lacking α-Gal A activity in childhood present with the "classical" phenotype, including acroparesthesia, angiokeratoma, and corneal or lenticular opacities and subsequently develop end-stage renal disease (ESRD), cardiomyopathy and

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cerebrovascular disease[1–2]. Recently, our institute has introduced the Fabry disease screening protocol to facilitate clinical services for patients suspected of having the classical form of this disorder by utilizing dried blood spot (DBS) and fluorometry. However, in addition to the classical phenotype of Fabry disease, the renal variant of Fabry disease or Fabry nephropathy is characterized by proteinuria and progresses to chronic kidney disease or ESRD without the characteristics of classical Fabry disease[3]. This variation manifests in the fifth decade of life, and greater residual α-GAL A activity is observed in these patients[2-3]. Previous studies in various ethnic groups demonstrated 0–1.17% of chronic kidney disease patients present with Fabry disease[3–21]. Therefore, α-GAL A screening in high-risk patients, e.g., ESRD of unknown etiology, is crucial to determine if the patients are at risk for Fabry nephropathy. A definitive diagnosis can facilitate a carrier testing process in the family, leading to proper genetic counseling. In addition, early detection can result in early treatment, which can decrease the deterioration of kidney function with treatments such as angiotensin receptor blockers to minimize proteinuria. With the advent of enzyme replacement therapy (ERT) for the treatment of Fabry disease, improvements in the renal pathology have been observed[26–28]. In addition, patients with ESRD can be protected against cardiovascular and cerebrovascular morbidity and mortality by ERT[22].

This study across three medical centers in Thailand aimed to explore the prevalence of Fabry disease in Thai patients who were diagnosed with ESRD of an unknown origin.

Patients and methods

Patients

This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects of the Faculty of Medicine at Ramathibodi Hospital, Mahidol University, Thailand (ID 10-54-25 and 08-54-07). Thai patients affected with ESRD of unknown origin were enrolled in our study at three centers: 1) Department of Medicine, Ramathibodi Hospital, 2) Renal Disease Unit, Uttaradit Hospital, and 3) Hemodialysis Center, Banphaeo Hospital-Prommitr Branch. Informed consent was obtained and clinical data were collected. Subsequently, 10 mL of peripheral venous blood was taken from each individual and processed for biochemical and molecular studies. In patients with Fabry disease, it is common to reach ESRD within the fifth decade of life. Therefore, the inclusion criteria in this study were subjects between 20 and 60 years of age, diagnosed with ESRD with no documented cause and undergoing renal replacement therapy (RRT) (i.e. either hemodialysis or peritoneal dialysis). Patients with any of the following conditions were excluded from the study: 1) evidence of renal dysplasia, 2) polycystic kidney disease or nephronophthisis, 3) diagnosed with diabetic nephropathy or diabetes mellitus for more than 15 years, 4) obstructive uropathy, 5) history of renal malignancy, including both solid and hematologic tumors, 6) history of microscopic or macroscopic haematuria, and 7) kidney biopsy results compatible with any particular syndromes or diseases (e.g., lupus nephritis, glomerulonephritis, minimal change disease, etc.), except glomerulosclerosis and nephrotic syndrome of unknown pathology, which can be manifestations of Fabry disease. Subjects who declined to be enrolled were also excluded. Gender and age matched negative control populations were selected from volunteers with confirmed eGFR of more than 90 mL/(minute·1.73 m^2).

α-GAL A enzyme testing assay

Dried blood spots (DBS) were prepared from venous blood samples using Guthrie's paper. Each bloodspot contained 75 μL of sample and was subsequently dried while protected from light at room temperature for 4 hours. The prepared DBS were placed in long-term storage at –4°C for future use. Protein was extracted from 3-mm punches through each DBS and incubated at 37°C with 4-methylumbelliferyl-α-D-galactopyranoside (4-MUGaL; Sigma-Aldrich, MO, USA) as a substrate. N-acetyl-galactosamine (Sigma-Aldrich) was added to inhibit α-galactosidase B, which can suppress α-galactosidase A activity. The fluorescence of the 4-methylumbelliferone product of α-GAL A was quantified using a fluorescence plate reader (BioTek, VT), with excitation at 360 nm and emission at 460 nm. Each sample was processed in duplicate. Quality control (QC) in the assay consisted of using QC-DBS pools (Center for Disease Control and Prevention, GA). A positive control sample was donated by a male patient affected by classical Fabry disease with the p.E66Q mutation. Relying on several references and the Mayo Clinic Fabry Disease Testing Algorithm, α-GAL A activity less than 1.2 nmol/(mL·hour) in males and 2.8 nmol/(mL·hour) in females were considered positive (Mayo Medical Laboratories).

Molecular assay of G*LA* gene

DNA was extracted from peripheral blood leukocytes using the Flexigene DNA kit according to the manufacturer instructions (Qiagen, Hilden, Germany). DNA collected from patients who screened positive in

the enzyme assay was processed for PCR and direct DNA sequencing of the seven exons of the *GLA* gene using primers designed by a web-based tool (***Table 1***). The 25-µL PCR mixture contained 100 ng of genomic DNA, 200 µmol of each dNTP, 20 pmol of each primer, 2.5 mmol/L of $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase (GIBCO BRL, Life Technologies, NY) in 10X PCR buffer supplied by the manufacturer (GIBCO BRL). Each PCR began with initial denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The final extension was at 72°C for 15 minutes for 35 cycles. DNA sequencing was performed in both directions, forward and reverse, by a standard automated method using an ABI PRISM 3100™ genetic analyzer (Applied Biosystem).

Screening of a novel variant in the general population

A novel variant, c.1 −10C>T, was discovered in this study. To confirm the frequency of this minor allele in the general population, a PCR-RFLP-based detection strategy was developed, since this variant creates a *Hph*I restriction site. Ten microliters of PCR product amplified by E1 primers were incubated at 37°C for 16 hours with five units of *Hph*I according to the manufacturer's instructions (NE Biolabs, MA). The digested products were electrophoresed on a 3% agarose gel. Following ethidium bromide staining, the gel was visualized using a UV transilluminator. The expected fragment lengths for the C allele were 256, 113 and 52 bp, whereas the lengths for the T variant were 308 and 113 bp.

Statistical analysis

The results were analyzed using descriptive statistics. α-GAL A activity was reported in units of nmol/mL/hr and reported as mean±standard deviation (S.D.). Data processing and statistical analysis were performed using GraphPad Prism 6.0 (Graphpad Software Inc., CA).

Results

Validation of α-GAL A in negative controls

To demonstrate the distribution of α-GAL A activity in the normal population, the biochemical assay was initially characterized using normal controls. Mean α-GAL A for male negative controls was 7.88±5.01 nmol/(mL·hour) (range 2.78–27.62; n = 81). For female controls, mean α-GAL A activity was 6.98±3.18 nmol/(mL·hour) (range 2.83–16.88; n = 37). These data confirmed that none of the negative control samples presented with α-GAL A activity less than the set cut-off value for positivity in the screening.

α-GAL A screening and *GLA* genotyping

A total of 142 patients of both genders (81 males and 61 females) were recruited for the α-GAL A screening assay and subsequent molecular confirmation in positive cases (***Fig. 1***). Ten patients (7.04%; 1 male, 9 females) exhibited a significant decrease in α-GAL A activity (***Fig. 2***). There were no patients that presented with an accepted pathogenic nucleotide variant (0%), as observed using DNA sequencing. Four cases (1 male, 3 female) revealed a novel single nucleotide variant (SNV) at position c.1 −10 C>T (***Fig. 3***).

Screening a novel SNV in the general population

To confirm whether this novel c.1 −10 C>T allele indicated susceptibility for Fabry disease in Thai patients; genotyping of this allele was performed in 267 X chromosomes of 202 unrelated individuals. This minor T allele was found in up to 0.1 of the general population (***Fig. 4***). This result indicated that this novel SNV is a benign variant in the Thai population.

Discussion

In this study, we did not observe cases of Fabry disease in Thai patients affected by ESRD of unknown

Table 1 **The nucleotide sequences of the primers used for DNA sequencing of *GLA* in this study**

Primer names	Forward (5′ → 3′)	Reverse (5′ → 3′)
E1	GGTTAGCGGAACGTCTTACG	ACCCAAACACATGGAAAAGC
E2	CCACACTATTACTGGGTTGGAA	GTTGGGATTACAGGCGTGAG
E3	CCCCCAATACCTGGTGAAGT	CCCCCAATACCTGGTGAAGT
E4	CAGACTGAACCCCATCTCAAA	TGGGAGAGATGGTAGGATGA
E5	TGGCCTACTTCTGAAGCAAA	AACCACTTTCCACAGCATCC
E6	GGATGCTGTGGAAAGTGGTT	GGGCCATCTGAGTTACTTGC
E7	CCAAACTAACAGGGCCACTT	CTCCCAAAGTGCTGGGATTA

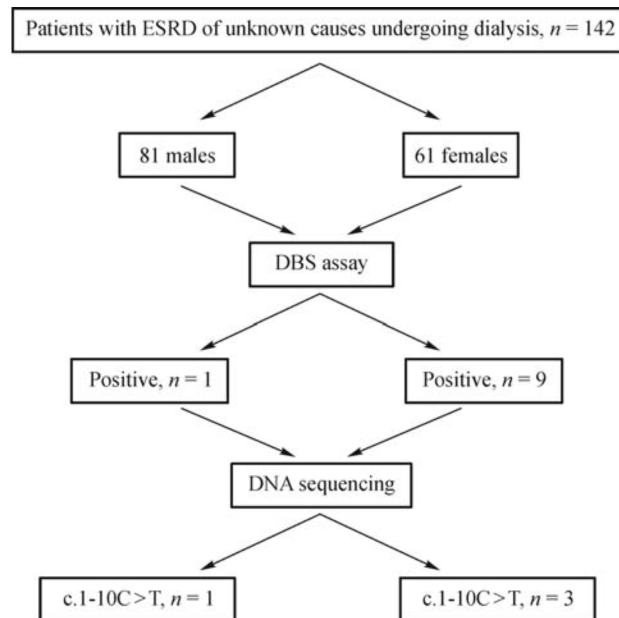

Fig. 1 **Summary of Fabry disease screening results of the study**

origin. Various studies in multiple countries have reported the prevalence of newly identified Fabry disease to be approximately 0–1.17% in chronic kidney disease patients (***Table 2***). Our data suggest that it is necessary that larger centers participate in the study to increase its statistical power. Alternatively, we have presented the first data from the Southeast Asian population, since all prior screening data in Asians was performed in the Japanese population[3-4,8,10,15,18]. Although enzyme screening using DBS is linked to a high false positive rate, this technique remains popular due to its technical feasibility and acceptable cost-benefit. DBS are not only easy to transport and process in the laboratory, but also inexpensive and can be preserved in long-term storage[23]. Theoretically, an enzyme assay done on packed leukocytes and plasma is more accurate; however, the specimen collection protocol requires fresh preparation that samples be kept frozen in the interim, which is not convenient for transportation from hospitals at remote sites. Nevertheless, molecular confirmation is highly recommended in all cases that are positive in the biochemical screening, especially in females, in which α-GAL A activity may be unreliable[24].

Our study proposed that c.1 −10C>T of *GLA* (rs2071225) is benign and not the susceptible marker for Fabry nephropathy in the Thai population. This was demonstrated by 10% of 202 healthy unrelated individuals with normal α-GAL A activity exhibiting this genotype. Our data confirm that this SNV is common in Thai ethnicity. However, several studies revealed that this genotype may be related to small fiber neuropathy and stroke of unknown etiology[25–26]. Recently, this genotype has been coincidentally found with a pathological mutation, p.E66Q, in a Fabry disease screening program in Japanese newborns[27]. Hence, the clinical impact of c.1 −10C>T SNV is less likely a functional mutation.

This is the first Thailand study to explore the prevalence of Fabry disease in patients affected by ESRD of unknown etiology. We aim to establish a National Screening Program to select under-diagnosed patients who can benefit from ERT as standard treatment. ERT is capable of delaying glycosphingolipid accumulation in the various tissues and improving the long-term clinical outcome in Fabry disease. Therefore, identification of the patients at risk is crucial to minimise renal and cardiovascular complications using

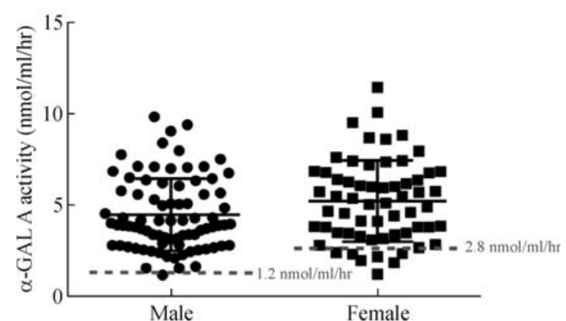

Fig. 2 **Distribution of α-GAL A activity.** One male and nine females had activities below the cut-off values, identified as "positive" in the screening.

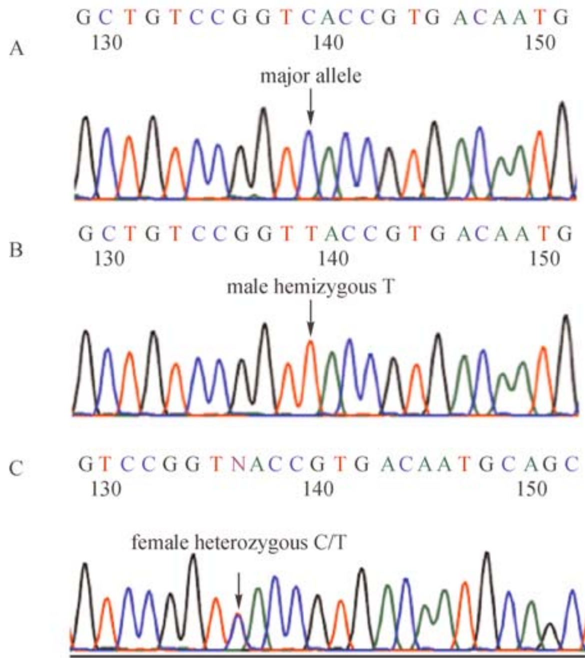

Fig. 3 **The novel nucleotide variant c.1 –10 C>T found in this study.** A: a major C allele similar to one in the NCBI database, B: a male hemizygous T allele from a subject with a positive α-GAL A screening, and C: a female heterozygous C/T allele found in three subjects who screened positive in the biochemical assay.

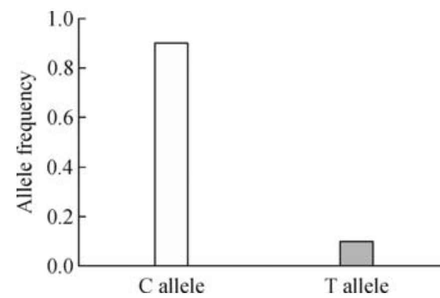

Fig. 4 **The frequency of C and T alleles.** The T allele at c.1 –10 position is identified as a minor variant with an allele frequency of 0.1.

this therapy[2]. To date, the cost of ERT is high and patients in low-income countries, such as Thailand, cannot afford the treatment. The only viable option for patients affected by such a rare disease to receive the therapy is to add ERT onto the National Health Policy for reimbursement. However, the Ministry of Public Health of Thailand has limited data regarding this disease. Therefore, our research group has attempted to identify new cases among patients presenting with common manifestations, i.e., ESRD, hypertrophic cardiomyopathy and cryptogenic stroke. These studies will be crucial in order for Thailand's Health Economics working group to consider including ERT in the National Reimbursement for special patients group. Although ERT is not available at the present time, early disease detection affords the patient an opportunity to prevent clinical deterioration using several medications, such as an angiotensin receptor blocker to prevent proteinuria[28]. In situations requiring kidney transplantation (KT), definitive diagnosis of affected, asymptomatically affected and unaffected family members is also essential for the selection of an appropriate living-relative donor[29].

In addition to ERT, few therapeutic strategies for Fabry disease have been discovered. Therefore, patients with definitive biochemical and molecular diagnosis are routinely recommended for clinical trials. An example of this clinical trial is a pharmacological chaperone as an additional therapy on ERT. Migalastat (1-deoxygalactonojirimycin HCl; GR181413A) acts as a pharmacological chaperone by facilitating the proper folding of the mutant enzyme by binding to the active sites. Therefore, this action improves its stability and trafficking to the lysosomes. The oral administration of this drug to transgenic mice expressing human mutant α-GAL A resulted in an increase of α-GAL A in various tissues. In addition, data showed that accumulation of the pathological substrate, GL-3, was reduced[30]. Oral migalastat in Fabry patients was proven to reduce plasma and urinary GL-3 and has been approved as a long-term treatment in adults and adolescents with a confirmed diagnosis of Fabry disease[31]. Another pharmacological chaperone currently being researched is 1-aminodeoxy-DMDP (ADMDP) stereoisomers. ADMDP acts as an inhibitor against α-GAL A and is able to impart thermal stabilization of this enzyme. Recent data indicated that ADMDP was capable of rescuing α-GAL A activity in the lymphoblast of N215S Fabry patient-derived cell line. Hence, this mediator is promising for Fabry disease treatment in the near future[32].

Apart from therapeutic purposes, the definitive diagnosis of Fabry disease is helpful to organize the patient's support group and a systematic rare disease database can be subsequently established. Since a large number of clinical research regarding Fabry disease is ongoing worldwide, a strong national database will be beneficial for regional researchers when they require a contribution from the patients. Recent discovery in this field that requires further validation include long-term clinical outcome evaluations, development of biomarkers, generation of disease-specific induced pluripotent stem cells and development of new pharmacological therapy.

Screening patients that present with other clinical manifestations is intriguing. Similar to our study, which

Table 2 **Summary of Fabry disease screening studies in chronic kidney disease patients in various countries**

Study countries	Gender	Types of assay	No. of patients with Fabry disease	Prevalence (%)	References
Japan	M and F	plasma	2/722	0.28	Utsumi et al., 2000
Japan	M	plasma, WBC, genetics	6/514	1.17	Nakao et al., 2003
Holland	M	plasma	1/508	0.20	Linthorst et al., 2003
Austria	M and F	DBS, WBS, genetics	4/2480	0.16	Kotanko et al., 2004
France	M and F	WBC, genetics	1/106	0.94	Bekri et al., 2005
Japan	M	plasma, genetics	1/450	0.22	Ichinose et al., 2005
Japan	M and F	plasma, WBC, genetics	5/696	0.72	Tanaka et al., 2005
Czech R.	M and F	DBS, WBC	5/3370	0.15	Merta et al., 2007
Lithuania	M	DBS	0/536	0	Maslauskiene et al., 2007
Canada	M	plasma, WBC	0/499	0	Andrade et al., 2008
Brazil	M	DBS, plasma	2/558	0.36	Porsch et al., 2008
Holland	M and F	plasma, genetics	3/922	0.33	Terryn et al., 2008
Spain	M and F	DBS, genetics	5/911	0.55	Gaspar et al., 2010
UK	M	DBS, plasma, WBC	0/155	0	Wallin et al., 2011
Japan	M and F	DBS, genetics	3/933	0.32	Nishino et al., 2012
Japan	M	serum, genetics	2/1080	0.19	Doi et al., 2012
Turkey	M	plasma, genetics	2/808	0.25	Kalkan Ucar et al., 2012
Japan	M	plasma, genetics	3/1453	0.21	Maruyama et al., 2013
Turkey	M and F	DBS, genetics	2/1136	0.18	Okur et al., 2013
Lebanon	M	plasma	0/275	0	Kabalan et al., 2013
Spain	M and F	DBS, genetics	11/3650	0.30	Hererra and Miranda, 2014
Thailand	M and F	DBS, genetics	0/142	0	Trachoo et al., 2017 (this study)

M: male; F: female; DBS: dried blood spots; WBC: white blood cells.

focused on the renal system, the definitive diagnosis of Fabry disease in other disease states, (i.e., cardiovascular, neurological and rheumatological systems), will acquire the benefits on clinical and basic science researches. In regards to cardiovascular involvement, Fabry disease is found in patients carrying hypertrophic cardiomyopathy (HCM). Approximately 4.6% of patients met at least one in four clinical criteria: 1) atypical HCM, 2) history or presence of documented arrhythmia, 3) short PR interval < 120 ms on electrocardiogram, and 4) symptoms of autonomic dysfunction. However, the detection rate is increased to 18.8% if at least 3 criteria are met[33]. Concerning the neurological presentation, Fabry disease was found in approximately 1%-5% of patients with cryotogenic stroke[34–35]. Another difficult neurological situation is that Fabry disease may mimic multiple sclerosis due to glycosphingolipid deposition in multiple small vessels of the central nervous system and peripheral nerves causing small fiber neuropathy and paresthesia[36–37]. Additionally, the clinical phenotype of Fabry disease may mimic Familial Mediterranean Fever (FMF) due to overlapping rheumatological manifestations, such as fever, abdominal pain, extremity pain and proteinuria. The prevalence of Fabry disease among these patients is approximately 0.5-7%[38–40].

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