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Low frequency of Fabry disease in patients with common heart disease

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Purpose: To test the hypothesis that undiagnosed patients with Fabry disease exist among patients affected by common heart disease.

Methods: Globotriaosylceramide in random whole urine using tandem mass spectroscopy, α-galactosidase A activity in dried blood spots, and next-generation sequencing of pooled or individual genomic DNA samples supplemented by Sanger sequencing.

Results: We tested 2,256 consecutive patients: 852 women (median age 65 years (19–95)) and 1,404 men (median age 65 years (21–92)). The primary diagnoses were coronary artery disease ($n = 994$), arrhythmia ($n = 607$), cardiomyopathy ($n = 138$), and valvular disease ($n = 568$). Urinary globotriaosylceramide was elevated in 15% of patients and 15 males had low α-galactosidase A activity. *GLA* variants found included R118C ($n = 2$), D83N, and D313Y ($n = 7$); IVS6-22 C > T, IVS4-16 A > G, IVS2+990C > A, 5′UTR-10 C > T ($n = 4$), IVS1-581 C > T, IVS1-1238 G > A, 5′UTR-30 G > A, IVS2+590C > T, IVS0-12 G > A, IVS4+68A > G, IVS0-10 C > T, IVS2-81–77delCAGCC, IVS2-77delC. Although the pathogenicity of several of these missense mutations and complex intronic haplotypes has been controversial, none of the patients screened in this study were diagnosed definitively with Fabry disease.

Conclusion: This population of patients with common heart disease did not contain a substantial number of patients with undiagnosed Fabry disease. *GLA* gene sequencing is superior to urinary globotriaosylceramide or α-galactosidase A activity in the screening for Fabry disease.

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Key Words: Fabry disease; heart disease; screening, X-linked

INTRODUCTION

Fabry disease is an X-linked genetic disorder (OMIM 301500). The incidence of the disease has been estimated to be 1 in 117,000 live male births;[1] however, recent newborn screening surveys suggest that the incidence may be 10 times higher.[2–4] The most common complication of Fabry disease is cardiac dysfunction, which may include cardiomyopathy, atrio-ventricular conduction defects, arrhythmia, valvular dysfunction, and non-atherosclerotic coronary vascular disease.[5,6] Increased stroke risk,[7] progressive renal failure,[8] and painful small-fiber neuropathy[9] occur commonly as well.[10] Fabry disease is caused by a deficiency of the lysosomal enzyme α-galactosidase A and accumulation of the glycosphingolipid globotriaosylceramide (Gb_3) in most cells and organs, as well as an increase of Gb_3 in urine.[11–13] Urinary Gb_3 is not found primarily in the filtrate, but mostly in shed renal tubular cells.[13,14] Increased blood and urine Gb_3 and globotriaosylsphingosine (lyso-Gb_3) are considered important specific biomarkers in this disease, and are often used in screening and diagnosis for this disorder.[15,16]

Since cardiac manifestations of Fabry disease are common and largely non-specific in their clinical presentation, we screened for this disorder in patients with common heart disease. We hypothesized that there are patients with undiagnosed Fabry disease, particularly with later-onset mutations, among patients with all forms of heart disease seen in the general population, although the incidence may be higher in more specific types of heart disease, such as hypertrophic cardiomyopathy.[17–19]

MATERIALS AND METHODS

Patients

We screened for Fabry disease in a population of patients with multiple forms of cardiovascular disease (ClinicalTrials.gov identifier: NCT01019629). These included coronary artery disease, conduction or rhythm abnormalities, non-ischemic cardiomyopathy, and valvular dysfunction. The patients were ambulatory, had to be over 18 years of age, and were seen at a number of institutions in Dallas: Baylor Heart and Vascular Hospital, the Heart Hospital at Baylor Plano, Soltero Cardiovascular Research Center, and cardiology outpatient clinics. More than 95% of patients who were asked to participate in the study accepted and gave written informed consent. The institutional review board of the Baylor Research Institute provided oversight for the study. We estimated that about 0.5–2% of all patients screened would be positive for

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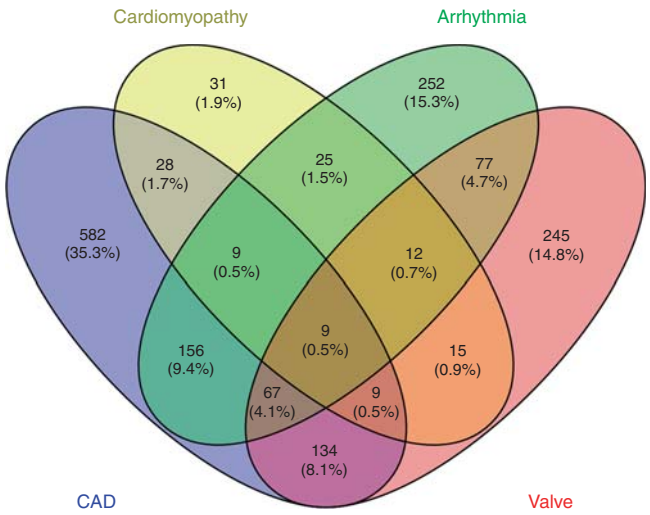

Figure 1 Venn diagram of cardiac diagnoses in this study. CAD, coronary artery disease; Valve, valvular disease.

Fabry markers and the screening of at least 1,000 patients was considered adequate.

Screening for Fabry disease was performed by measuring urinary Gb_3 in randomly collected samples of whole urine using ultra high pressure chromatography–tandem mass spectrometry, measuring α-galactosidase A activity in dried blood spots by flow injection analysis–tandem mass spectrometry, and testing for *GLA* gene mutations by parallel sequencing of the whole gene in pooled genomic DNA samples.

Urinary Gb_3 analysis by mass spectrometry

Analysis was performed as previously described.[20]

α-Galactosidase A activity evaluation by tandem mass spectrometry

Enzyme analysis was performed as previously described.[20]

GLA gene analysis

GLA gene variants were searched for in pooled DNA using massively parallel sequencing[21] or in individual genomic DNA samples using the Illumina MiSeq platform (Illumina, Inc.; San Diego, CA). Briefly, the SmartChip MyDesign TE system (WaferGen Bio-systems, Inc., Fremont, CA) was designed to enrich multiple samples for multiple resequencing targets simultaneously using a 4–polymerase chain reaction primer amplification strategy. The system consisted of SmartChip MyDesign chips that contain 5,184 nanowells. The separate, 100-nL reactions were then amplified using the Techne Prime Thermal Cycler (Techne, Staffordshire, UK), which was preprogrammed with the recommended thermal cycling program and equipped with an adapter plate for cycling 1 or 2 chips. After the polymerase chain reactions, pooled amplicons were collected from the chip(s) via centrifugation using single-use components supplied in the SmartChip TE Collection Kit. The library was then loaded on the MiSeq platform. Raw image files were processed by base-calling software with default parameters and the sequences of each individual were generated as 250 base pairs paired-end reads. Conventional Sanger sequencing was used to further analyze individual samples from selected patient DNA pools.

Table 1 Clinical and biochemical characteristics

	Mean	Median	SD	Min	Max	Range
Weight (kg)	88.76	86.05	21.88	38.7	200.66	361.96
Height (cm)	172.69	172.72	10.6	105.45	200.66	95.21
BMI	29.74	28.48	7.49	15	180.45	165.45
HDL (mg/dl)	45.94	43	17.77	11	357	346
LDL (mg/dl)	94.94	90.5	35.12	10	279	269
Urine Gb_3 (ng/ml)	133.29	101	169.51	9	4145	4136
α-Galactosidase A (μmol/L/hr)	6.06	5.51	3.58	0.59	105.62	105.03
HDL (mg/dl)	45.94	43	17.77	11	357	346
LDL (mg/dl)	94.94	90.5	35.12	10	279	269

Gb_3: 200 ng/ml 99th percentile; α-galactosidase A activity: normal > 2 μmol/L/hr.
BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

RESULTS

A total of 2,256 consecutive patients were screened for Fabry disease; 1,404 were male (62.2%) and 852 were female (37.8%). The median age of women was 65 years (19–95) for women and men was 65 years (21–92) for men. Eighty-four patients were Hispanic, 124 were African Americans, and 2,048 were non-Hispanic Caucasians. All types of complications related to heart disease were represented in this study. Overall, the primary diagnosis was coronary artery disease in 994 patients, arrhythmia in 607, cardiomyopathy in 138, and valvular disease in 568. Patients often had more than one type of clinical cardiac abnormality (**Figure 1**). **Table 1** describes physiologic and biochemical characteristics of the screened patient population with heart disease, including urine Gb_3 and whole-blood α-galactosidase A activity. **Table 2** describes the *GLA* gene variants that were identified. α-Galactosidase A was abnormally low in 1% (15/1,404) of men and 0.8% (7/852) of women (**Figure 2**). Urine Gb_3 was abnormally elevated in 9.8% (138/1,404) of men and 8.1% (69/852) of women. Of the 22 patients with abnormally low α-galactosidase A levels, 6 had abnormally high Gb_3, but none had *GLA* gene variants. We found that none of the 19 patients with variants had abnormally low α-galactosidase A, while 9 of them had abnormal urine Gb_3 levels (**Figure 2**). Interestingly, several of the missense mutations and complex intronic haplotypes that were identified have been reported in the medical literature to cause Fabry disease or Fabry-related symptomatology in specific cases.[22–25] However, the male patients with these variants identified in this population did not have sufficiently low α-galactosidase A enzyme activity to result in clinical Fabry disease. About half of the patients identified were female, and α-galactosidase A

Table 2 *GLA* gene variants identified and subject clinical characteristics

Alpha-galactosidase A activity	Urine Gb_3	Mutation	Gender	Age at consent	Ethnicity	Arrhythmia	CAD	MI	Valve	ICM	HCM	CHF	CVA	CKD/ ESRD	Hypothyroid	DM	COPD	HTN	HLP	Septal hypertrophy	LVH
4.03	564	5′UTR-10C > T, IVS1-518T > C, IVS2-77delC, IVS2 +990C > A, IVS4-16A > G, IVS6-22C > T,	M	82	W	X	X	X										X	X		
6.97	805	IVS1-1238G > A, IVS4-16A > G	M	63	W	X	X	X				X				X		X	X		
3.55	2208/ 4	5′UTR-30G > A	M	56	W		X	X									X	X	X		
7.48	1261	5′UTR-10C > T, IVS1-518T > C, IVS2-77delC, IVS2 +990C > A, IVS4-16A > G, IVS6-22C > T	M	57	W		X	X											X		
6.86	289	IVS2+590T > C	M	59	W	X															
5.96	274	IVS1-1238G > A	M	62	W	X	X	X		X								X	X		
3.34	119	Asp83Asn/ c.247G > A, Gat/Aat	F	71	W	X			X	X					X				X		
10.02	96/ 23	Asp313Tyr/ c.937G > T, Gat/Tat (het)	F	49	W		X									X		X	X		
3.34	368/ 192	IVS0-12G > A IVS4 +68A > G IVS6-22C > T (hemi)	M	59	W		X	X		X			X	X		X		X	X		
3.47	83	Arg118Cys/ c.352C > T, Cgc/Tgc	F	35	W	X															
8.44	67	Asp313Tyr/ c.937G > T, Gat/Tat (het)	F	71	W	X						X			X	X		X			
2.39	449/ 146	IVS0-10C > T, IVS2-81–77delCAGCC, IVS4-16A > G, IVS6-22C > T (het)	F	60	B		X	X			X	X	X	X	X			X	X		
3.27	216	IVS0-10C > T, IVS2-81–77delCAGCC, IVS4-16A > G, IVS6-22C > T (hemi)	M	58	H		X		X									X			

Table 2 Continued

Alpha-galactosidase A activity	Urine Gb_3	Mutation	Gender	Age at consent	Ethnicity	Arrhythmia	CAD	MI	Valve	ICM	HCM	CHF	CVA	CKD/ESRD	Hypothyroid	DM	COPD	HTN	HLP	Septal hypertrophy	LVH
4.1	70/ 41	Asp313Tyr/ c.937G>T, Gat/Tat	F	72	W				X									X		X	
2.98	95	Arg118Cys/ c.352C>T, Cgc/Tgc	M	69	W	X	X	X	X			X						X	X		
5.94	NA	Asp313Tyr/ c.937G>T, Gat/Tat	F	82	W	X			X					X	X			X	X		X
3.46	29	Asp313Tyr/ c.937G>T, Gat/Tat	F	61	W		X											X	X		
7.93	13	Asp313Tyr/ c.937G>T, Gat/Tat	F	63	W		X		X												
2.15	19	Asp313Tyr/ c.937G>T,Gat/Tat	F	52	W	X	X		X				X		X			X			X

B, black; CAD, coronary artery disease; CHF, congestive heart failure; CKD/ESRD, chronic kidney disease/end-stage renal disease; COPD, chronic obstructive pulmonary disease; CVA, cerebrovascular accident; DM, diabetes mellitus; F, female; H, Hispanic; HCM, hypertrophic cardiomyopathy; HLP, hyperlipidemia; HTN, arterial hypertension; ICM, insertable cardiac monitor; LVH, left ventricular hypertrophy; M, male; MI, myocardial infarction; Valve, valvular disease; W, white.
Urine Gb_3: 200 ng/ml 99th percentile. α-galactosidase A activity: normal > 2 μmol/Hr.

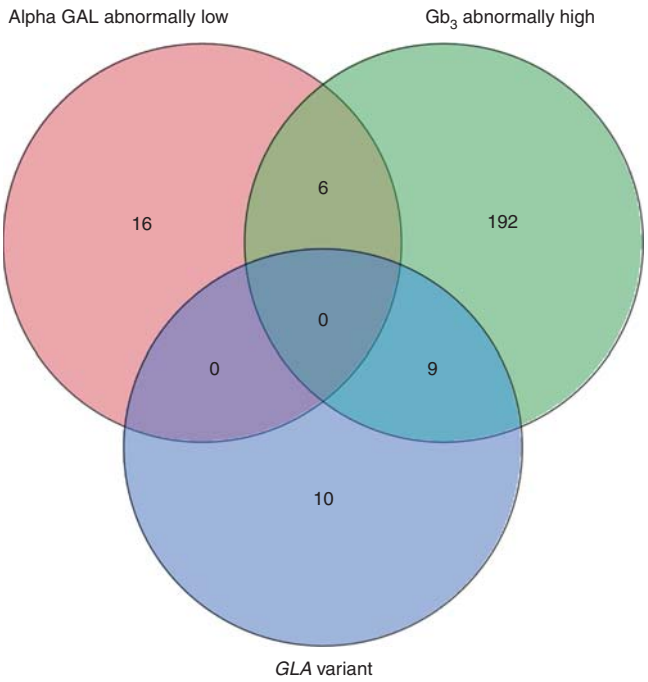

Figure 2 Venn diagram of cardiac patients with *GLA* gene variant abnormality (*GLA* variant), abnormal α-galactosidase A activity (alpha GAL abnormally low), and elevated urine Gb_3 (Gb_3 abnormally high).

activity was normal in this group, as is common even in females with Fabry disease due to the presence of the wild-type *GLA* allele. The frequencies of the exonic and intronic variants we identified in our patient population were no higher than their respective frequencies in the general population based on the 1000 Genomes (http://www.internationalgenome.org/data) or ExAc (http://exac.broadinstitute.org) databases (data not shown). The incidence of specific combinations of haplotypes in the general population is unknown.

DISCUSSION

Using three different screening methods, we found *GLA* sequence variants that have been previously reported to cause Fabry disease in certain patients. However, in this group of 2,256 patients with common heart disease, no previously undiagnosed patients with definitive Fabry disease were identified. Therefore, the cardiovascular common heart disease population, estimated to be approximately 85 million in the United States,[26] is not likely to contain a sizable percentage of undiagnosed patients with Fabry disease; however, we could be expected to find some patients with undiagnosed Fabry disease in this population. It is likely that screening much larger cohorts or those selected high-risk populations with specific abnormalities such as idiopathic cardiomyopathy or patients on dialysis will provide a better diagnostic yield.[17,27,28] Also, measurement of additional biomarkers, such as lyso-Gb_3 and related analogues, may

improve the sensitivity of methods for identifying Fabry disease.[29]

In this study, we used a general screening approach that is novel and may serve as a template for future screening studies of at-risk populations (e.g., idiopathic stroke, hypertrophic cardiomyopathy). We looked for Fabry disease at the level of the gene, the enzyme activity in blood, and the substrate in the urine in each patient, thus providing a unique opportunity to compare the different methods. We found that screening for Fabry disease using the sequencing of the whole *GLA* gene is the most useful method. This approach identifies pathogenic and benign variants of the *GLA* gene and provides a discriminating method to identify patients truly affected by Fabry disease.[30,31] On the other hand, our study demonstrates that analysis of enzyme activity or the amount of substrate in the urine alone lacks the required specificity for large-scale screening. Urinary Gb_3 was elevated in about 15% of patients with heart disease who otherwise did not have *GLA* mutations,[20] and it is known to be normal in patients with mild mutations and in females,[29] or is falsely elevated for other reasons.[32] α-Galactosidase A activity was not a useful measurement either, because females often have normal enzyme levels[33] and a substantial number (15) of males screened had enzyme activity near or below 2 μmol/L/hr (data not shown), a threshold found to contain patients with Fabry disease.[34]

The pathogenicity of the exonic variants that we found, R118C and D313Y, has been controversial. The R118C and D313Y variants were described in clinically diagnosed patients,[35] in newborn screenings,[2] in stroke in young patients, and in patients on renal dialysis in the Portuguese and Spanish populations, as well as in Brazilian patients. However, more comprehensive and critical investigations showed that these variants do not definitively lead to Fabry disease–related complications.[31,36] D83N is a novel variant, but the patient had normal urine Gb_3 levels and no other characteristics of Fabry disease. This variant was found to have high residual α-galactosidase A activity in the HEK-293 in vitro assay.[37] However, assaying the enzyme activity in peripheral white blood cells of males is required to confirm non-pathogenicity. The intronic variants we found were described in some patients and may lower enzyme activity to a certain extent, but not enough to typically cause Fabry disease on their own, and elevated Gb_3 was not present in skin biopsies (data not shown).[38] A female with a Fabry-compatible phenotype who was heterozygous for a complex intronic haplotype has been reported, but the causality of this *GLA* gene abnormality was not conclusive.[23]

The combination of gene sequencing, assaying protein function, and substrate levels is an approach that may be applied to screening for other genetic/metabolic disorders. Such a strategy will allow not only determination of the most reliable initial screening method, but will serve to evaluate the pathogenicity of a particular novel genetic variation by assessing whether the genetic variant is associated with a significant reduction of protein function and an elevation of the related harmful metabolite. In our opinion, an enzymopathy like Fabry disease can be considered in a patient only when an abnormality is present in the entire biologic pathway, at least at the organ/tissue level.[30]

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DISCLOSURE

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