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Original Article

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Broad spectrum of Fabry disease manifestation in an extended Spanish family with a new deletion in the *GLA* gene

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Abstract

Background. Fabry disease (FD) is an X-linked inherited disease based on the absence or reduction of lysosomal-galactosidase (Gla) activity. The enzymatic defect results in progressive impairment of cerebrovascular, renal and cardiac function. Normally, female heterozygote mutation carriers are less strongly affected than male hemizygotes aggravating disease diagnosis.

Method. Close examination of the patients by renal biopsy, echo- and electrocardiography and MRI. Blood work and subsequent DNA analysis were carried out utilizing approved protocols for PCR and Sequencing. MLPA analysis was done to unveil deletions within the GLA gene locus. Quantitative detection of Glycolipids in patient plasma and urine were carried out using HPLC/MS-MS and ESI-MS.

Results. In the presented case, a female index patient led to the examination of three generations of a Spanish family. She presented with severe oto-cochlear symptoms and covert renal and cardiac involvement. While conventional sequencing failed to detect a causative mutation, MLPA analysis revealed a deletion within the *GLA* gene locus, which we were able to map to a region spanning exon 2 and adjacent intronic parts. The analysis of different biomarkers revealed elevated lyso-Gb3 levels in all affected family members.

Conclusion. Our findings highlight the broad intrafamilial spectrum of symptoms of FD and emphasise the need to use MLPA screening in symptomatic females without conclusive sequencing result. Finally, plasma lyso-Gb3 proved to be a reliable biomarker for the diagnosis of FD.

Keywords: Fabry disease; lyso-Gb3; multiple sclerosis; renal involvement

Background

Fabry disease (FD) is a rare X-linked inherited condition which, due to the absence or reduction of α-galactosidase A (*GLA*) activity in lysosomes, results in an accumulation of globotriaosylceramide (Gb3) in endothelial cells, smooth muscle cells, peripheral nerves and in other tissues [1]. Patients experience pain, gastrointestinal disturbances, disorders of the skin (angiokeratoma), eyes (cornea verticillata), ears (hypacusis) and the central nervous system [2]. The first symptoms usually occur in childhood. During their fourth or fifth decade, FD patients have a high risk of lethal renal, cardiac or cerebrovascular disease [3, 4].

Despite being X-linked, heterozygous females can suffer from symptoms of similar severity to males due to X-inactivation. Females heterozygous for FD show a wide variety of clinical symptoms ranging from *GLA* levels within normal range without clinical symptoms or disease severity similar to hemizygous males [2].

Since the majority of the affected females have a normal *GLA* activity in leucocytes, sequencing of the entire *GLA* gene including exon–intron boundaries and selective intronic areas [3, 5] was thought to be the method of choice to diagnose female patients. However, deletions of one or more exons or deletion of the entire gene are not detectable by standard sequencing in heterozygous females. The multiplex ligation-dependent probe amplification (MLPA) has proven to be an efficient tool for discovering these rearrangements [6].

Furthermore, as several mutations with uncertain pathological significance have been described [7, 8], metabolites related to impaired substrate degradation such as Gb3 in plasma and urine [8, 9], urinary Gb3, in particular its long chain N-acylisoforms [10], as well as plasma globotriaosylsphingosine (lyso-Gb3) [8, 11] have been proposed as potential biomarkers.

In this study, we describe an extended Spanish family in which a novel exon 2 deletion (exon2del) in the *GLA* gene was detected using the MLPA technique. Additionally, we were able to characterize the exact breaking point of the deletion in the affected family members. Furthermore, the proposed metabolic biomarkers in urine and blood were analysed.

Present data support the extensive intrafamilial range of FD and distinguishes the need to use MLPA screening in genetic testing of females. In line with recent findings on the role of lyso-Gb3 as a reliable biomarker for the diagnosis of FD, all females with the exon2del genotype had clearly elevated blood levels of lyso-Gb3.

Case presentation

Index patient

A 48-year-old woman (2:11, see Figure 1 for family lineage) was referred to us because of mild proteinuria and microhaematuria detected in a routine analysis, but maintained a completely normal renal function. She had a past history of severe neurological symptoms such as acroparesthesia, peripheral vertigo and an attack of sudden hypacusis. Othorhinolaryngological tests revealed bilateral vestibular areflexia, while cerebral imaging showed no relevant abnormalities. Recently she had noticed reduced sweating.

Although angiokeratomas were absent, FD was suspected and consequently she underwent ophthalmological tests which revealed a Cornea verticillata, a hallmark of FD [5]. The enzymatic study showed 5% *GLA* activity in leucocytes. However, standard sequencing failed to detect a mutation in the *GLA* gene. MLPA

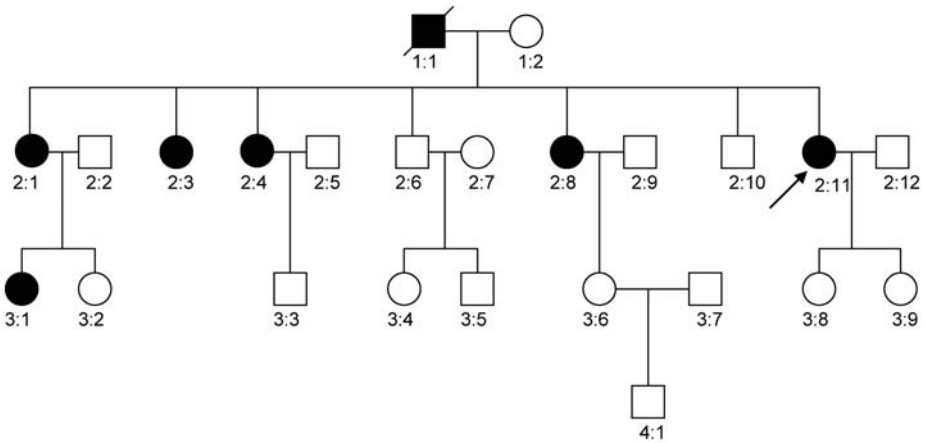

Fig. 1. Lineage of the family studied. Squares represent males and circles represent females. Black shade indicates patients suffering from FD. We observed three generations of an extended Spanish family. Patient 1:1 died at 43 years of age. Although the cause of his death remained unclear, he suffered from acroparesthesia and renal involvement with albuminuria during his lifetime. In the second generation five of his daughters carried the same exon2del (2:1, 2:3, 2:4, 2:8 and 2:11). Both 2:8 and 2:11 were index patients investigated during this analysis. Patient 2:1 presented with no symptoms except acroparesthesias, whereas 2:3 experienced acroparesthesia only during fever episodes and displayed some neurological and cardiac involvement. Likewise, 2:4 was without any symptoms except acroparesthesia. Within the third-generation, 3:1 was the only patient with a significantly reduced enzymatic activity; however, she had only mild renal involvement and no apparent symptoms.

Table 1. Phenotypic investigation of the Spanish family members studied and biomarker results of total Gb3 in plasma (µg/mL), urinary tetracosanoyl-Gb3 (ng/mg creatinine) and lyso-Gb3 in plasma (ng/mL)

No.	Enzyme activity (norm > 12%) (%)	Symptoms	Gb3 plasma (norm < 5 µg/mL)	Gb3-24 urine (norm < 52 mg/mmol creatinine)	Lyso-Gb3 plasma (norm < 1.2 ng/mL)	MLPA
1:2	>80	None	3.8	36		WT
2:1	50	Acroparesthesia	4.4	134	3.83	exon2del
2:3	10	Neurological, cardiac and renal	5.8	34	7.33	exon2del
2:4	50	Acroparesthesia, mild renal and cardiac	5.0	92	4.64	exon2del
2:6		None	4.5	28	<0.4	WT
2:8	12	Periventricular signal hyperintensities mimicking multiple sclerosis	5.5	NA	4.19	exon2del
2:10		None	4.4	40	<0.4	WT
2:11	5	Neurological, cardiac and renal	6.4	214	14.4	exon2del
3:1	37	Mild renal	4.6	83	1.89	exon2del
3:2	95	None	3.6	8	<0.4	WT
3:3		None	3.8	NA	<0.4	WT
3:6	95	None	4.9	38	0.506	WT
3:8	95	None	2.5	53	<0.4	WT
3:9	95	None	2.9	39	<0.4	WT

was required to detect a deletion of exon 2. The patient then underwent a renal biopsy, which displayed extensive lipidic deposits within glomeruli and vessels. Echo- and electrocardiography displayed a mild left ventricular hypertrophy and a moderate mitral insufficiency. In addition, the suspected biomarkers of FD were determined. All three were above the typical cut-offs, plasma Gb3 at 6.4 µg/µL, urinary Gb3 214 ng/mg creatinine and lyso-Gb at 14.4 ng/mL (Table 1). In 2006, the patient's treatment was commenced with intravenous enzyme replacement therapy (agalsidase alfa 0.2 mg/kg every 2 weeks) and renal function monitored (Supplementary Table S1).

Gene sequencing and MLPA

It is noteworthy that standard sequencing of the exons including the exon–intron boundaries did not show any exon–intron or splice mutations in the examined individuals. Only after performing standard MLPA analysis, a heterozygous deletion of exon 2 was revealed (Figure 1; Supplementary Figure S1).

Analysis of break point demonstrating the exon 2 deletion

PCR with the patients' DNA with exon2del gave rise to two differently sized products. All affected individuals displayed an identical pattern. We were able to determine the exact break point by sequencing the different fragments. We demonstrated a deletion of a 553 bp fragment [NM_000169.2:c.195-91_369+287del; DNA level genomic: Chr X (NCBI 36):g.100545168_100545720del] including the entire 175 bp of exon 2 and adjacent intronic regions (start: IVS1 -91; end: IVS2 +287).

Second patient (2:8)

The second patient was a 53-year-old woman (2:8, Figure 1) who was diagnosed with multiple sclerosis in 1995. Magnetic resonance imaging revealed significant white-matter lesions, especially in the frontal and parietal subcortical area. She was suffering from unspecific symptoms such as headache and pruritus, especially in her hands, as well as polyarthralgia and Raynaud syndrome. She complained of several relapses of the suspected multiple sclerosis and accordingly received various treatments including corticosteroids. However, the neurological symptoms progressed and she was confined to a wheelchair due to paraplegia and became doubly incontinent.

After the diagnosis of FD in her sister, she underwent analysis of *GLA* activity in leucocytes which detected a moderate reduction in her enzyme activity to 12%. No other organ involvement could be detected aside from the neurological manifestation. Since it remained unclear whether the patient was suffering from isolated neurological FD symptoms or a chronic inflammatory process in the brain due to other reasons, no enzyme replacement therapy was administered. In 2008, Invernizzi *et al*. [12] described the case of a 36-year-old woman presumably affected by both FD and multiple sclerosis, which we cannot dismiss for the patient described here. Regardless, due to the exon2del within the examined family members and the low *GLA* activity of this patient, we concluded FD as the most consistent cause of the patients' neurological symptoms.

Of the six living affected family members, three presented evidence of a covert renal involvement, while all displayed neurological symptoms on various degrees (see the Supplementary Lineage and Clinical Characterization of the Family section, available as Supplementary data online).

Glycolipids in plasma and urine

Aside from the gene sequencing and MLPA analysis (Figure 2), possible biomarkers of FD were investigated (Table 1).

Gb3 in plasma

Plasma Gb3 was determined by an HPLC/MS-MS method as described in detail recently [13]. Only moderate changes in plasma Gb3 concentrations (max 2.6-fold) were found in the family members. Eleven of 14 subjects in this family displayed Gb3 concentrations above the threshold of 95th percentile of the normal range (>3.6 mg/L, interassay coefficient of variation ~10%) including all carriers of the exon2del allele as well as five subjects with the wild type (WT) allele (Table 1).

Urinary tetracosanoyl-Gb3

Four of the five subjects (2:1, 2:4, 2:11 and 3:1) carrying the exon2del allele excreted markedly elevated amounts of Gb3-24 (2.4 to 6.1-fold above cut-off) while in patient 2:3 only borderline amounts were found (34 ng Gb3-24/mg creatinine; 96% of calculated cut-off), similar to subjects carrying the WT allele. Isoforms of urinary Gb3 were determined by electrospray ionization mass spectrometry (ESI-MS) using stable-isotope-dilution/internal standardization [14]. According to recent results [10], tetracosanoyl-Gb3 (Gb3-24) provides the best diagnostic performance among N-acyl isoforms of Gb3. For a calculated cut-off of 35 ng tetracosanoyl-Gb3/mg creatinine, a sensitivity of 100% and a specificity of 87% can be expected.

Lyso-Gb3 in plasma

All six female Fabry patients with the exon2del (2:1, 2:3, 2:4, 2:8, 2:11 and 3:1) showed clearly elevated concentrations of lyso-Gb3 in plasma. All WT family members analysed revealed plasma lyso-Gb3 levels below the detection limit, the cut-off being 0.51 ng/mL. Lyso-Gb3 in plasma was analysed by HPLC-MS/MS and protein precipitation of plasma and serum samples with ethanol by a new method designed to detect plasma lyso-Gb3 together with other mono-, di- and triglycosylated ceramides and corresponding lyso-derivatives.

Discussion

In our study, we present a Fabry family with several affected heterozygous females, two of them presented with severe neurological manifestations at the time of their first presentations. Routine evaluation of organ involvement revealed that one of them showed typical

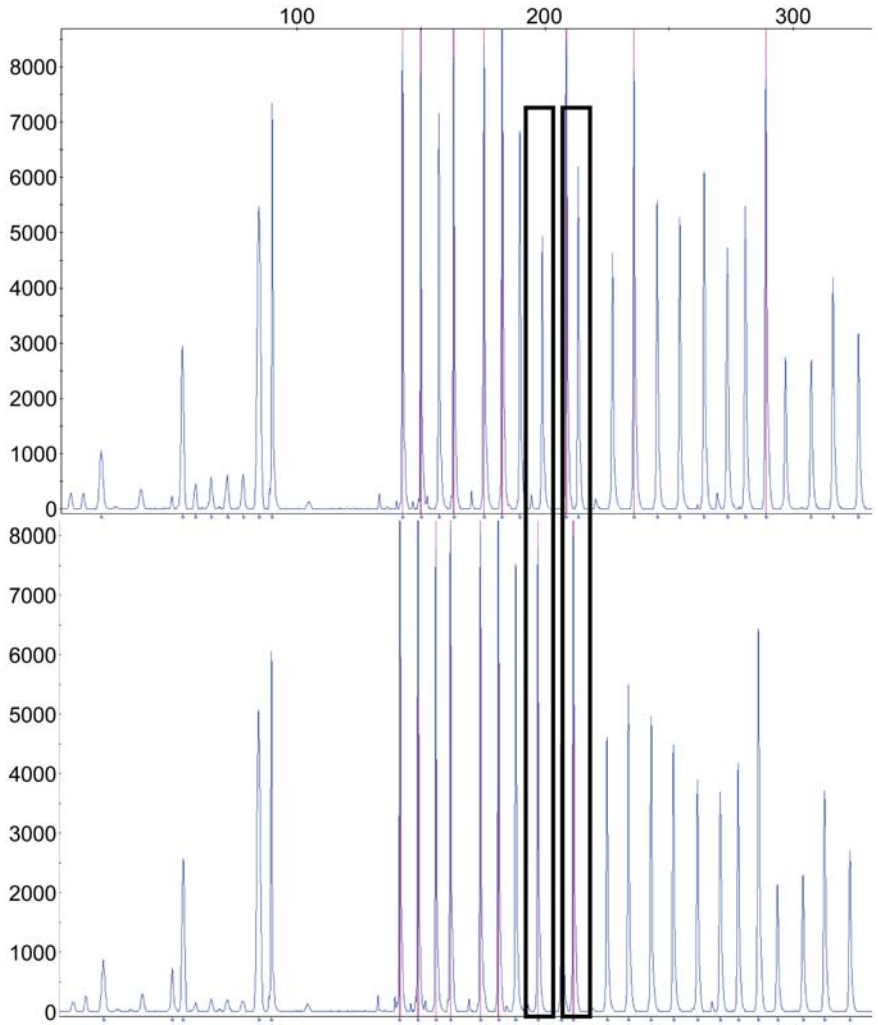

Fig. 2. Example of a typical MLPA result. Female patient of the Spanish family studied with a deletion of Exon 2 (at the top). The MLPA probe-set contains two probes for exon 2 (black boxes). The height of the peaks is reduced to ~50% compared with the genomic control DNA sample (at the bottom) indicating a loss of one exon 2 copy in the genome.

cardiac involvement and concealed renal manifestation; the other had no other organ involvement and had been diagnosed with and treated for multiple sclerosis.

The female patients from the second generation of our investigated family showed typical clinical manifestations of FD as well as a reduction in *GLA* activity in line with the clinical diagnosis of the disease.

Neurological features of FD can mimic a variety of neurological diseases such as multiple sclerosis, stroke or neuropathies which can cause diagnostic difficulties [3, 4, 8, 12]. In the second patient described here, the diverse and unspecific neurological symptoms along with the family history and low enzyme activity indicated that she has been suffering from FD. Co-existence of FD and multiple sclerosis has been reported previously [12].

Renal involvement is reported in ~50% of patients suffering from FD, proteinuria being the most frequent symptom [15]. Biopsy studies in FD have shown that glomerular and vascular changes are present before progression to overt proteinuria [16, 17]. Renal biopsy may therefore be a useful tool for the early detection of renal disease in FD facilitating an early enzyme replacement therapy to block Gb3 accumulation in renal tissue and thus prolonging the development of a chronic progressive kidney disease.

Based on the enzymatic analysis of *GLA* in the female patients of the family, only patients 2:3 and 2:11 would have been diagnosed correctly; patient 2:8 demonstrated a normal value in the lower range (12%). The fact that at least half of the six female patients showed almost normal enzyme activity emphasizes the need for an adequate testing strategy especially in females. Therefore, we additionally evaluated possible biomarkers for FD.

As demonstrated earlier [9, 18], our data suggest that urinary long-chain N-isoforms of Lyso-Gb3 are an insufficient biochemical marker for disease severity and therapy control [19].

A deacylated metabolite of Gb3 in plasma, lyso-Gb3, has been shown to be clearly elevated [10, 20] and it was suggested to be more directly involved in disease pathology [20]. Quite recently, minor amounts of lyso-Gb3 were also detected in urine [21]. Rombach *et al.* [22] in particular showed that males and females with classic symptoms, but not symptom-free individuals carrying specific genotypes (p.R112H and p.P60L), exhibit highly elevated lyso-Gb3 levels. However, their correlation to

disease severity may rather depend on the duration and extent of exposure to plasma lyso-Gb3 than the mutation type.

Our results of glycolipid analysis are highly consistent with the previous data. Although total plasma Gb3 showed only minor variations in plasma Gb3 among all tested family members and were without a clear correlation to the presence of the exon2del allele, their concentration range for urinary tetracosanoyl Gb3 was much larger (26.5-fold) and four of the five female heterozygotes clearly exceeded the calculated cut-off for Fabry patients. In contrast, plasma lyso-Gb3, measured using a newly developed MS/MS technique with improved sensitivity, was clearly correlated to the presence of the exon2del allele in all tested cases. Interestingly, even though all carriers of the exon2del genotype display pathological lyso-Gb3 values, they vary by a magnitude of factor 10 (1.89 ng/mL in patient 3:1 versus 14.4 ng/mL in patient 2:11).

In summary, it can be stated that a combination of exon sequencing, MLPA and assessment of biomarkers like *N*-tetracosanoyl-Gb3 and plasma lyso-Gb3 provides sufficient evidence for the presence of FD in females, independent of *GLA* activity measurements and the presence of a classic clinical phenotype. MLPA is of particular importance in symptomatic females where standard sequencing of the entire coding region may be insufficient, because in ∼2–3% of all Fabry patients large deletions can be seen in the *GLA* gene [6, 23–25]. Therefore, standard Fabry guidelines should consider MLPA as a mandatory analysis when only female heterozygotes can be tested.

Patients with other genotypes have to be studied to determine whether urinary Gb3 isoforms and lyso-Gb3 can be used for a prognosis of disease severity of novel *GLA* mutations. The newly developed, highly sensitive mass spectrometry techniques applied here can provide an important contribution to achieve an early diagnosis of FD in female patients.

Consent

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review from the Editor-in-Chief of this journal.

Supplementary data

Supplementary data are available online at http://ckj.oxfordjournals.org.

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References

1. Stratta P, Quaglia M, Messina M *et al*. The challenges of diagnosing Fabry disease. *Am J Kidney Dis* 2008; 51: 860–864
2. Mehta A, Beck M, Eyskens F *et al*. Fabry disease: a review of current management strategies. *QJM* 2010; 103: 641–659
3. Rolfs A, Bottcher T, Zschiesche M *et al*. Prevalence of Fabry disease in patients with cryptogenic stroke: a prospective study. *Lancet* 2005; 366: 1794–1796
4. Sims K, Politei J, Banikazemi M *et al*. Stroke in Fabry disease frequently occurs before diagnosis and in the absence of other clinical events: natural history data from the Fabry Registry. *Stroke* 2009; 40: 788–794
5. Falke K, Buttner A, Schittkowski M *et al*. The microstructure of cornea verticillata in Fabry disease and amiodarone-induced keratopathy: a confocal laser-scanning microscopy study. *Graefes Arch Clin Exp Ophthalmol* 2009; 247: 523–534
6. Schirinzi A, Centra M, Prattichizzo C *et al*. Identification of *GLA* gene deletions in Fabry patients by Multiplex Ligation-dependent Probe Amplification (MLPA). *Mol Genet Metab* 2008; 94: 382–385
7. Linthorst GE, Bouwman MG, Wijburg FA *et al*. Screening for Fabry disease in high-risk populations: a systematic review. *J Med Genet* 2010; 47: 217–222
8. Tanislav C, Kaps M, Rolfs A *et al*. Frequency of Fabry disease in patients with small-fibre neuropathy of unknown aetiology: a pilot study. *Eur J Neurol* 2011; 18: 631–636
9. Mills K, Morris P, Lee P *et al*. Measurement of urinary CDH and CTH by tandem mass spectrometry in patients hemizygous and heterozygous for Fabry disease. *J Inherit Metab Dis* 2005; 28: 35–48
10. Paschke E, Fauler G, Winkler H *et al*. Urinary total globotriaosylceramide and isoforms to identify women with Fabry disease: a diagnostic test study. *Am J Kidney Dis* 2011; 57: 673–681
11. Aerts JM, Groener JE, Kuiper S *et al*. Elevated globotriaosylsphingosine is a hallmark of Fabry disease. *Proc Natl Acad Sci USA* 2008; 105: 2812–2817
12. Invernizzi P, Bonometti MA, Turri E *et al*. A case of Fabry disease with central nervous system (CNS) demyelinating lesions: a double trouble? *Mult Scler* 2008; 14: 1003–1006
13. Krüger R, Bruns K, Grunhage S *et al*. Determination of globotriaosylceramide in plasma and urine by mass spectrometry. *Clin Chem Lab Med* 2010; 48: 189–198
14. Fauler G, Rechberger GN, Devrnja D *et al*. Rapid determination of urinary globotriaosylceramide isoform profiles by electrospray ionization mass spectrometry using stearoyl-d35-globotriaosylceramide as internal standard. *Rapid Commun Mass Spectrom* 2005; 19: 1499–1506
15. Branton M, Schiffmann R, Kopp JB. Natural history and treatment of renal involvement in Fabry disease. *J Am Soc Nephrol* 2002; 2: S139–S143
16. Ramaswami U, Najafian B, Schieppati A *et al*. Assessment of renal pathology and dysfunction in children with Fabry disease. *Clin J Am Soc Nephrol* 2010; 5: 365–370
17. Ortiz A, Oliveira JP, Waldek S *et al*. Nephropathy in males and females with Fabry disease: cross-sectional description of patients before treatment with enzyme replacement therapy. *Nephrol Dial Transplant* 2008; 0: 1–8
18. Young E, Mills K, Morris P *et al*. Is globotriaosylceramide a useful biomarker in Fabry disease? *Acta Paediatr Suppl* 2005; 94: 51–54

19. Schiffmann R, Waldek S, Benigni A *et al*. Biomarkers of Fabry disease nephropathy. *Clin J Am Soc Nephrol* 2010; 5: 360–364
20. Togawa T, Kodama T, Suzuki T *et al*. Plasma globotriaosylsphingosine as a biomarker of Fabry disease. *Mol Genet Metab* 2010; 100: 257–261
21. Auray-Blais C, Ntwari A, Clarke JTR *et al*. How well does urinary lyso-Gb3 function as a biomarker in Fabry disease? *Clin Chim Acta* 2010; 411: 1906–1914
22. Rombach SM, Dekker N, Bouwman MG *et al*. Plasma globotriaosylsphingosine: diagnostic value and relation to clinical manifestations of Fabry disease. *Biochim Biophys Acta* 2010; 1802: 741–748
23. Fukuhara Y, Sakuraba H, Oshima A *et al*. Partial deletion of human alpha-galactosidase A gene in Fabry disease: direct repeat sequences as a possible cause of slipped mispairing. *Biochem Biophys Res Commun* 1990; 170: 296–300
24. Dobrovolny R, Dvorakova L, Ledvinova J *et al*. Relationship between X-inactivation and clinical involvement in Fabry heterozygotes. Eleven novel mutations in the alpha-galactosidase A gene in the Czech and Slovak population. *J Mol Med* 2005; 83: 647–654
25. Shabbeer J, Yasuda M, Benson SD *et al*. Fabry disease: identification of 50 novel alpha-galactosidase A mutations causing the classic phenotype and three-dimensional structural analysis of 29 missense mutations. *Hum Genomics* 2006; 2: 297–309

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