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Research paper

# Identification of mutations in Colombian patients affected with Fabry disease

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ABSTRACT

Fabry Disease (FD) is an X-linked inborn error of glycosphingolipid catabolism, caused by a deficiency of the lisosomal α-galactosidase A (AGAL). The disorder leads to a vascular disease secondary to the involvement of kidney, heart and the central nervous system. The mutation analysis is a valuable tool for diagnosis and genetic counseling. Although more than 600 mutations have been identified, most mutations are private. Our objective was to describe the analysis of nine Colombian patients with Fabry disease by automated sequencing of the seven exons of the GLA gene. Two novel mutations were identified in two patients affected with the classical subtype of FD, in addition to other 6 mutations previously reported. The present study confirms the heterogeneity of mutations in Fabry disease and the importance of molecular analysis for genetic counseling, female heterozygotes detection as well as therapeutic decisions.



## 1. Introduction

Fabry disease (FD; OMIM: 301500) is an X-linked lysosomal storage disorder caused by the deficiency of the enzyme alpha-galactosidase A (AGAL, EC 3.2.1.22) (Brady, 1967). This enzymatic defect leads to the systemic accumulation of glycosphingolipids (mainly globotriaosylceramide -GL3-) in blood vessels from the skin, kidney, heart and brain (Desnick et al., 2003).

Today two main FD subtypes have been described. The first one, or "classical phenotype", affects males who have a markedly reduced AGAL activity. In these patients the onset of disease occurs in childhood or adolescence and is characterized by acroparesthesias, angiokeratomas, corneal opacities, hypohydrosis and progressive vasculopathy of the kidney, heart, and central nervous system (Desnick and Brady, 2004). The second phenotype, or "milder", has been described in patients with a higher residual AGAL activity and a later onset characterized by cardiac and renal symptoms (Nakao et al., 2003, 1995; Terryn et al., 2013).

Due to the late appearance of symptoms in patients with milder FD, the prevalence of this phenotype seems to be higher in relation to patients with classical FD, whose incidence has been estimated to be between 1:40.000 to 1:117.000 male births, approximately (Lin et al., 2009; Meikle et al., 2006; Spada et al., 2006).

Patients with FD can be diagnosed by evaluating AGAL activity in plasma or white blood cells, however this analysis often fails to distinguish between Fabry heterozygotes with high residual AGAL activity and normal individuals. For this reason, mutation analyses are required to detect female heterozygotes, define genotype/phenotype correlations, and to make an accurate prenatal diagnosis and take therapeutic decisions (Yoshimitsu et al., 2011; Lukas et al., 2013).

The genomic sequence of the *GLA* gene is 12 kb in length and contains 7 exons (OMIM: 300644). To date, more than 600 mutations have been described including missense, nonsense and splice-site mutations, as well as gene rearrangements (Ashley et al., 2001; Schirinzi et al., 2008; Shabbeer et al., 2006). Most of the described mutations are private (with some few exceptions found in several unrelated subjects) and are usually associated with modifications in CpG dinucleotides, known hotspots for the disease (Barker et al., 1984; Cooper and Youssoufian, 1988).

In this study, we describe the first clinical and genetic analysis of nine Colombian FD patients. Direct sequencing of the complete *GLA* open reading frame revealed eight mutations, two new and six previously reported. Patients with the classical phenotype of FD were identified,

*Abbreviations:* FD, Fabry disease; AGAL, alpha-galactosidase A; *GLA*, alpha-galactosidase A gene; GL3, globotriaosylceramide; DBS, dried blood sample.

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thereby providing additional information about the genotype–phenotype correlation.

## 2. Materials and methods

### 2.1. Patients

Nine FD Colombian patients were included in this study. All procedures were followed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). All participants completed a consent form for diagnostic testing approved by the Institutional Ethics Committee of each participating institution.

### 2.2. AGAL activity assays

AGAL enzymatic activity was first determined in dried blood spot (DBS) samples (as previously described by Chamoles et al., 2001; Civallero et al., 2006;Uribe and Giugliani, 2013) and the results were compared with the reference values reported for the Colombian population (range: 2.0–21.8 nmol/ml/h; Uribe and Giugliani, 2013). To confirm the deficiency, AGAL activity was also measured in leukocytes by a fluorometric assay following the procedure described by Shapira et al. (1989).

### 2.3. GLA mutation analysis

The mutation analyses were performed as described by Pasqualim et al. (2014). Briefly, genomic DNA was extracted from FTA Classic cards (Whatmann, USA) according to the manufacturer's instructions. In all patients the *GLA* open reading frame (exons 1–7 and their flanking regions) was amplified by PCR, as previously described by Shabbeer et al. (2005), with minor modifications. Each amplicon was purified with EXO-SAP (GE Healthcare Lifesciences, USA) and quantified with Low Mass Reader (Invitrogen, USA).

Forward and reverse sequences were analyzed with the same primers used for PCR amplification in an ABI3500 genetic analyzer and BigDye Terminator v3.1 (Applied Biosystems, USA). The obtained sequences were compared to the one reported on GenBank (accession number X14448.1) and sequence variation was confirmed by repeated PCR amplification and sequencing. Sequence variations were described according to the international mutation nomenclature guidelines as set forth by the Human Genome Variation Society (http://www.hgvs.org/mutnomen).

Data from the Fabry Database (http://www.fabry-database.org) and Human Genome Mutation Database (http://www.hgmd.cf.ac.uk) were used to define novel *GLA* sequence variants and their associated phenotype. Each mutation was analyzed to determine the relative conservation of the substituted amino acid by comparison with other vertebrate species. To predict the effect of newly identified mutation we used SIFT, PolyPhen2, MutPred, MutationTaster, NetGene2 and Fabry CEP software.

SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (Ng and Henikoff, 2003). In this way, if a score is smaller than 0.05, the corresponding neutral substitutions (NS) are predicted as “damaging”, while if it is greater, the NS are predicted as “tolerated”. PolyPhen2 is an automatic tool for prediction based on several features, including the sequence, phylogenetic and structural information characterizing the substitution (Adzhubei et al., 2010). The score of PolyPhen2 ranges from 0 to 1, and the corresponding prediction is “probably damaging” (coded as “D” if the score is greater than 0.85); “possibly damaging” (coded as “P” if it is between 0.85 and 0.15) and “benign” (coded as “B” if it is smaller than 0.15).

MutPred is a web application tool developed to classify an amino acid substitution as disease-associated or neutral in human. In this way, it can be used to predict the molecular cause of disease. Another application (MutationTaster) integrates information from different biomedical databases and uses established analysis tools to predict evolutionary conservation sites, splice-site changes, loss of protein features and changes that might affect the amount of mRNA within the cell (Schwarz et al., 2010). We also used the NetGene2 server to predict possible splice sites in human GAL gene (Hebsgaard et al., 1996) and, in order to determine if the mutations found were candidates for the treatment with chaperones, the program Fabry CEP.

## 3. Results

### 3.1. AGAL enzymatic activity

The nine Colombian FD patients included in this study had low or undetectable levels of AGAL activity in DBS (0.0 to 0.4 nmol/ml/h) and leukocytes (0.0 to 0.4 nmol/protein mg/h) (Table 1), showing an AGAL residual activity ranging from 0.0 to 0.6%.

### 3.2. GLA mutations

The sequence analysis of the complete coding region of *GLA* in the nine evaluated patients revealed six previously described mutations: c.1024C > T (p.Arg342*), c.804-2_-3delCA (IVS5-2_-3delCA), c.195-1G > C (IVS1-1G > C), c.1072_1074delGAG (p.Glu358del), c.334C > A (p.Arg112Ser) and c.1072G > A (p.E358K) (Table 1). Patients FD-2 and FD-8 displayed two novel variants: c.1051_1052delGT (p.Val351SerfsX23) and c.806G > T (p.Val269Gly) (Fig. 1A and B). The mutations and enzymatic activities are summarized in Fig. 2.

At the protein level, comparative *in silico* analysis of the novel c.806G > T mutation (p.Val269Gly) revealed a strict conservation of valine at position 269 among vertebrate species. Polyphen2 predicted that this missense mutation is probably damaging with a score of 0.999, and SIFT software showed probabilistic scores compatible with a potential deleterious effect (p.Val269Gly = 0,0). For the c.1051_1052delGT variant the MutationTaster software predicted a pathogenic effect with a changed splice site and a truncated protein, while NetGene2 predicted that an acceptor splice site would be lost with this mutation.

### 3.3. Clinical correlation

All the patients showed a classical FD phenotype (Table 2). In relation to the patients who showed new variants, patient FD-2 was a 50-year-old male who had experienced intermittent neuropathic pain in the lower extremities since 6 years of age, fever crisis, heat intolerance and hypohidrosis. Later, when he turned 33, he showed an increase in proteinuria that quickly progressed to an end-stage renal disease and kidney transplantation.

Patient FD-8 was a 51-years-old male who presented with angiokeratoma at age 6, and suffered from acroparesthesias, fever crisis, hypohidrosis and heat intolerance from the age of 8.

At the age of 30 he showed increases in proteinuria and developed chronic renal failure.

## 4. Discussion

This is the first study carried out in Colombia that evaluates the molecular aspects of FD. The mutation analysis of the *GLA* gene in nine FD Colombian patients allowed us to identify eight mutations, six of them previously described and two new variants, illustrating the genetic heterogeneity underlying this disease at the molecular level.