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Functional and pharmacological evaluation of novel *GLA* variants in Fabry disease identifies six (two *de novo*) causative mutations and two amenable variants to the chaperone DGJ

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ABSTRACT

Background: Allelic heterogeneity is an important feature of the *GLA* gene for which almost 900 known genetic variants have been discovered so far. Pathogenetic *GLA* variants cause alpha-galactosidase A (α-Gal A) enzyme deficiency leading to the X-linked lysosomal storage disorder Fabry disease (FD). Benign *GLA* intronic and exonic variants (*e.g.* pseudodeficient p.Asp313Tyr) have also been described. Some *GLA* missense variants, previously deemed to be pathogenetic (*e.g.* p.Glu66Gln and p.Arg118Cys), they have been reclassified as benign after re-evaluation by functional and population studies. Hence, the functional role of novel *GLA* variants should be investigated to assess their clinical relevance.

Results: We identified six *GLA* variants in 4 males and 2 females who exhibited symptoms of FD: c.159C > G p.(Asn53Lys), c.400T > C p.(Tyr134His), c.680G > C (p.Arg227Pro), c.815A > T p.(Asn272Ile), c.907A > T p.(Ile303Phe) and c.1163_1165delTCC (p.Leu388del). We evaluated their impact on the α-Gal A protein by bioinformatic analysis and homology modelling, by analysis of the *GLA* mRNA, and by site-directed mutagenesis and *in vitro* expression studies. We also measured their responsiveness to the pharmacological chaperone DGJ.

Conclusions: The six detected *GLA* variants cause deficient α-Gal A activity and impairment or loss of the protein wild-type structure. We found p.Asn53Lys and p.Ile303Phe variants to be susceptible to DGJ.

1. Introduction

Allelic heterogeneity is a predominant feature of the *GLA* gene (located at position Xq22.1, OMIM 300644, RefSeq X14448) which counts 900 known variants (Human Gene Mutation Database Professional, www.biobase-international.com) in a protein of 429 amino acids: the lysosomal α-galactosidase A (α-Gal A; EC 3.2.1.22). Pathogenetic variants of the *GLA* gene cause total or partial α-Gal A enzyme deficiency and induce the progressive development of the X-linked lysosomal storage disorder, Fabry disease (FD; OMIM 301500) [1]. The α-Gal A enzyme catalyses the hydrolysis of α-galactosidic linkages of glycosphingolipids, glycoproteins and polysaccharides [1]. Deficiency of α-Gal A enzyme activity induces accumulation of glycosphingolipids containing terminal α-galactose residues within the lysosomes of many tissues, including the kidney, arteries and heart [1,2].

Laboratory confirmation of FD in males relies on the demonstration of deficient α-Gal A activity in leukocytes or fibroblasts followed by *GLA* gene sequencing. Milder forms of FD are usually associated with residual enzyme activity. Heterozygous females may develop mild to severe clinical manifestations [1,3], but enzyme assay is unreliable in females due to random X-chromosome inactivation. Hence, *GLA* gene sequencing is crucial for confirming FD in females.

Abbreviations: α-Gal A, alpha-galactosidase A; FD, Fabry disease; DGJ, 1-deoxygalactonojirimycin; VUS, variants of unknown significance

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FD is characterized by a heterogeneous spectrum of clinical manifestations [1,2] and most of the signs and symptoms resemble those of other common diseases. Several FD forms have been recognized, ranging from a milder condition with only cardiac and/or renal abnormalities, to the more classic FD phenotype characterized by chronic pain, vascular degeneration, angiokeratoma, cardiac abnormalities, kidney manifestations leading to renal failure, and other symptoms [1,2]. Establishing a correlation between *GLA* nucleotide variants and patients' phenotypes can represent a challenge. Some missense *GLA* variants have been reported in clinical case reports which lack functional assessment. Other missense *GLA* variants, which as yet lack correlation with a phenotype, have been reported in newborns through newborn screening programs.

Besides the pathogenetic variants, several intronic polymorphic variants and one missense change (*i.e.* p.Asp313Thr) which is responsible for a pseudodeficiency have also been described [4–7]. Pseudodeficiency variants cause false positives in the enzyme assay but are not disease causing. Some *GLA* missense variants (p.Pro60Leu, p.Glu66Gln, p.Arg118Cys, p.Ala143Thr, p.Ile198Thr; aliases p.P60L, p.E66Q, p.R118C, p.A143T and p.I198T) that had been reported as causative when first discovered, have resulted as benign polymorphisms in subsequent clinical, functional and population studies [4,8–11]. Such findings stress the importance of performing exhaustive assessments of the effects of new *GLA* VUS when detected in patients with suspected FD.

Such a high allelic heterogeneity makes the identification of *GLA* variants of unknown significance (VUS) in routine molecular confirmation for FD quite frequent. VUS produce uncertainty both for those responsible for giving a laboratory report, and for clinicians managing a patient. They increase the risk of misdiagnosis, especially if the patient does not exhibit obvious signs of classic FD [12]. Early diagnosis of FD in affected patients is of great importance since a specific enzyme replacement therapy (ERT) [12] is available. A clear-cut exclusion of FD in suspected patients is of equal importance to avoid distress in families and inappropriate initiation of ERT, which is invasive and extremely expensive [12].

Besides ERT, a new alternative treatment option based on the small-molecule pharmacological chaperone 1-deoxygalactonojirimycin (or DGJ) is available [13]. DGJ reversibly binds to the active site of α-Gal A and stabilises the 3D structure of mutant forms of the enzyme which are affected by conformational mutations, rescuing enzyme activity [13–18]. Responsiveness to this therapeutic option can be tested by administering DGJ to cell lines expressing mutant forms of α-Gal A and by measuring the rescue of the α-Gal A enzyme activity [5,19].

Hence, performing functional and pharmacological studies of novel *GLA* variants can be twofold helpful in assessing their clinical significance and deciding which therapeutic options are likely to be effective. Such studies can be performed *in vitro* on cell lines transfected with mutated vectors or directly on patients' derived cell lines, when available [5].

Here we report the clinical, functional and pharmacological characterization of six new *GLA* gene variants found in patients with symptoms on the FD clinical spectrum. We performed *in vitro* expression studies and studies on patients' derived cells to evaluate impact on the α-Gal A activity and on the α-Gal A protein and to estimate responsiveness to DGJ.

2. Materials and methods

2.1. Patients

Patients' clinical data and α-Gal A activities are reported in Table 1. Whole blood DNA samples from patients and their relatives were examined after informed consent was obtained for all individuals, in accordance with local ethical committee recommendations. The family pedigrees are shown in Fig. 1.

2.2. Analysis of genomic DNA

Genomic DNA was isolated using the QIAsymphony DSP DNA Midi Kit and the QIAsymphony robot (Qiagen, Hilden, Germany).

The entire coding region with intron-exon boundaries and a region of the intron IV which includes the NM_000169.2:c.639 + 861C > T [20] and NM_000169.2:c.639 + 919G > A [21] deep intronic mutations were amplified and sequenced using previously published oligonucleotides and reaction conditions [22].

Two allelic dosage assays, MLPA (Multiplex Ligation-dependent Probe Amplification) and Quantitative Fluorescent Multiplex PCR (QFM-PCR) [22], were both applied on DNA samples from the two female FD patients (Pt1 and Pt4) in order to exclude gross *GLA* gene rearrangements [22].

2.3. Bioinformatics analysis

The identified *GLA* variants were analysed in the HGMD professional database (http://www.biobase-international.com/product/hgmd), dbSNP database (http://www.ncbi.nlm.nih.gov/snp), ExAC browser (http://exac.broadinstitute.org) and 1000 genomes project browser (http://browser.1000genomes.org/index.html).

We used Alamut Visual (http://www.interactive-biosoftware.com) to predict the possible pathogenicity of the identified *GLA* variants. The Alamut software integrates 4 different prediction tools for missense changes: Mutation Taster (http://www.mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.bii.a-star.edu.sg) and Align GVGD (http://agvgd.iarc.fr). Alamut also provides phylogenetic conservation data of interrogated protein amino acid residues.

Possible alterations in *GLA* mRNA splicing processing were also checked by Alamut Visual which integrates 5 different specific algorithms (SpliceSiteFinder, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing Finder) predicting if a given variant impairs mRNA splicing.

2.4. Site-directed mutagenesis

We generated mutant plasmid to express the p.Asn53Lys, p.Tyr134His, p.Arg227Pro, p.Asn272Ile, p.Ile303Phe variants using PCR-based site-directed mutagenesis of *GLA* cDNA, cloned into the expression vector pCD-X, as previously described [23]. Forward primers: 5′-TTCATGTGCAAGCTTGACT GCCAG-3′ (p.Asn53Lys), 5′-CTAGGGATTCATGCAGATGT-3′ (p.Tyr134His), 5′-AATCACTGGCCAAATTTTGCTG-3′ (p.Arg227Pro), 5′-GTGATTGGCATCT TTGGCCTC-3′ (p.Asn272Ile) and 5′-CCTCCGACACTTCAGCCCTCAA-3′ (p.Ile303Phe) and reverse primers: 5′-CTGGCAGTCAAGCTTGCACATGAA-3′ (p.Asn53Lys), 5′-ACATCTGCATGAATCCCTAG-3′ (p.Tyr134His), 5′-CAGCAA AATTTGGCCAGTGATT-3′ (p.Arg227Pro), 5′-GAGGCCAAAGATGCCAAT CAC-3′ (p.Asn272Ile) and 5′-TTGAGGGCTGAAGTGTCGGAGG-3′ (p.Ile303Phe) were used for the mutagenesis reactions according to the standard protocols. For the construction of the plasmid carrying the p.Leu388del variant, we amplified a DNA fragment containing this variant directly from the Pt6's DNA using forward primer 5′-GGAGACAACTTTGAA GTGTG-3′ and reverse primer 5′-CCGAATTCTTAAAGTAAGTCTTTTAA-3′, both annealing to *GLA* exon 7. All the PCR amplifications were performed with FastStart High Fidelity PCR System (Hoffmann-La Roche, Basilea, Switzerland). Ligations were carried out using Solution I of the DNA Ligation Kit Ver.2.1 (TaKaRa Bio Inc., Kusatsu, Japan). *Escherichia coli* strain Solo pack gold cells (Stratagene, Milan, Italy) were used for cloning. We confirmed the mutated plasmid constructs obtained by sequencing the full-length *GLA* cDNA. We prepared large-scale plasmid preparations using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany).

2.5. COS-1 cell transfection and effect of DGJ on α-Gal A activity

We transiently overexpressed normal and mutant plasmids pCD-Asn53Lys, pCD-Tyr134His, pCD-Arg227Pro, pCD-Asn272Ile, pCD-Ile303Phe and pCD-Leu388del into African green monkey kidney cells (COS-1). COS-1 cells were grown at 37 °C, 5% CO_2 in DMEM (Dulbecco's modified Eagle medium) (1:1 vol/vol) with fetal bovine