In silico analysis of two novel Alpha-Galactosidase-A variants in patients with suspected Fabry disease

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Abstract

Fabry disease (FD) is caused by deficient activity of alpha-galactosidase-A (α-Gal-A) due to mutations in the X-chromosome GLA gene. The disorder affects multiple organ systems. Some α-Gal-A variants, leads to the systemic accumulation of glycolipids, mainly globotriaoslyceramide (Gb3) and globotriaosylphingosine (lyso-Gb3) in the plasma and cellular lysosomes of vessels, nerves, tissues and different organs. Lacking structural and experimental data for mutant proteins induced by different types of variants in the enzyme α-Gal-A its involvement in metabolism is unknown. In the present study, two new mutant structural models (3D) p.Phe211Ser and p.Gly360Ala were constructed for the α-Gal-A enzyme by means of a coordinated reconstruction approximation model using as a template the 1R46 protein (Protein Data Bank) previously described for the protein alpha-galactosidase-A in humans. The structural analysis of the computational models showed that changes reported here seem to significantly affect the functionality of the protein, however it is necessary to support the findings with clinical evidence.

Key Words: Lysosomal storage disease, Fabry disease, Alpha -galactosidase A, Variant, Models molecular, Computational biology

1- Introduction

Fabry disease (FD) is an X-linked genetic disease caused by the deficient enzyme activity of α-galactosidase A [α-gal A; Online Mendelian Inheritance in Man of the galactosidase (GLA) gene, 301500; Enzyme Commission number of α-gal A, 3.2.1.22], which is encoded by the GLA gene (Xq22) (Germain, 2010). This enzymatic defect leads to the systemic accumulation of glycolipids, mainly globotriaoslyceramide (Gb3) and globotriaosylphingosine (lyso-Gb3) in the plasma and cellular lysosomes of vessels, nerves, tissues and different organs (Nance et al., 2006). There are two phenotypes associated with fabry disease in men: the classic phenotype of early onset is characterized by a residual enzyme activity <1% and clinical manifestations include pain in the extremities, angiokeratomas, corneal opacity and alterations in the kidneys, heart and neurological. On the other hand, in the mild or late phenotype, there is a residual enzymatic activity between 1-10% compared to the levels in healthy individuals, and the clinical manifestations are late onset and less severe (Pisani et al., 2012). Disease manifestations in female heterozygotes have been reported, but are considered to be rare and usually mild (Beer, Reinecke, Gabbert, Hort, & Kuhn, 2002). Corneal alterations (cornea verticillata and posterior lenticular cataract) are present in about 70% of women and are useful for the detection of heterozygotes. About 30% of women have minimal angiokeratomas and <10% have infrequent attacks of neuropathic pain (MacDermot, Holmes, & Miners, 2001). However, heterozygous women with severe and early cerebrovascular disease, stroke and renal failure have been documented, but it was estimated that these severe manifestations only occurred 1%. The great variability in the severity of the phenotype has been attributed mainly to the random inactivation of the X chromosomes in each of their cells, that is, by the skewed X inactivation (Lyon hypothesis)(Perretta, Antongiovanni, & Jaurretche, 2018).

The human GAL-A gene (GLA), is organized in seven exons encompassing over 12 Kb (Kornreich, Desnick, & Bishop, 1989). The X-ray structure reveals a-GAL as a homodimeric glycoprotein of about 46 kDa (398 amino acids), with each monomer composed of two

domains, a $(\alpha/\beta)_8$ barrel domain containing the active site (encompassing amino acids 32-330) and a C-terminal domain containing eight antiparallel b strands on two sheets in a b sándwich (Garman & Garboczi, 2004).

Currently, more than 1000 mutations are reported for the GLA gene and these include missense and nonsense mutations and splice defects (Saito, Ohno, & Sakuraba, 2011; Stenson et al., 2009) and most of them are unique to a family (private) and therefore genotype–phenotype correlation is limited (Laney & Fernhoff, 2008).

Mutation identification in Fabry disease is important for many reasons including heterozygote detection by enzyme assay of carriers for this X-linked recessive disease is unreliable (Brown & Brown, 1993) , treatment for Fabry disease using replacement therapy is available worldwide (Eng et al., 2001) , it is important to identify affected males and carriers for medical monitoring and early treatment (Desnick et al., 2003) and the use of specific therapies such as pharmacological chaperones dependent on the mutation type of the affected patient (Germain et al., 2016) , however, while the pathogenicity of some GLA mutations is well described, many subjects often have an unknown gene variant / GLA mutation meaning (VUS) (Smid et al., 2015) , therefore, it is necessary to elucidate the structural and functional affectation of the two novels variants described in this study on the α-galactosidase A protein, in order to provide more information and support at the time of the classification of the variants as well as to provide scientific evidence that facilitates the choice of the most appropriate treatment according to the damage to the protein level that is being generated.

Here we describe the functional analysis of two novel GLA variants, p.Phe211Ser (c.632T>C) and p.Gly360Ala (c.1079G>C) identified in two female individuals with suspected Fabry disease in Colombia by screening the population at risk for this disease.

2- Materials and methods

2.1 Patients

We report the clinical and molecular studies of two unrelated Colombian female patients with suspected Fabry disease. Patients were identified from the screening program for high

risk population for Fabry disease, among those that include individuals with alterations in renal, cardiac and nervous system level.

2.2 Patient specimens

Peripheral blood was collected, using EDTA as an anticoagulant from patients with clinical manifestations related to Fabry disease symptomatology and signed informed consent was obtained from all participants.

2.3 Analysis of genomic DNA

Genomic DNAs from patients and their relatives were isolated from blood cells from the total blood sample in EDTA tube, then the exons and the exon - intron limits of the GLA gene were amplified. The exons and the exon - intron limits of the GLA gene were amplified. The amplified product was sequenced by Sanger technology ("gold standard" technique for sequencing). The result was compared with the reference sequence: NG_007119.1.

2.4 Mapping variants onto structure of α-gal A

In Silico Variants Analysis

To predict the structural effect of the novel missense variants on resulting α-Gal-A enzymes we modeled the variants onto the three-dimensional structure of α-Gal-A previously determined by X-ray crystallography (Garman & Garboczi, 2004). Amino acid substitutions corresponding to the two mutant proteins were introduced into the wild type structure in the molecular visualization program Pymol. RMSD was determined for each model using PyMOL. The solvent-accessible surface area (ASA) value of each variant in the wild-type and the mutated model of α-Gal-A and electrostatic surface potential were calculated using PyMOL. The physicochemical characteristics of the substitutions of a single amino acid were determined with the help of the National Center for Biotechnology Information (NCBI) amino acid explorer(Chen, Huang, & Wu, 2017) and detection of hydrophobic regions in α-Gal-A by ProtScale (Wilkins et al., 1999).

The evolutionary conservation of the sequences was studied with T-Coffee (Notredame, Higgins, & Heringa, 2000) and Clustal Omega (Sievers et al., 2011) . Conservation indices were calculated with ConSurf (Ashkenazy, Erez, Martz, Pupko, & Ben-Tal, 2010). Structural disorders generated by the variants in the protein were studied using IUPred (Dosztányi, Csizmok, Tompa, & Simon, 2005) .The consequences of variants on protein stability were predicted by I-Mutant2.0 (Capriotti, Fariselli, & Casadio, 2005) , CUPSAT (Parthiban, Gromiha, & Schomburg, 2006) and MUpro (Cheng, Randall, & Baldi, 2006). The pathogenic effects of point variants were analyzed using several methods: SIFT (Kumar, Henikoff, & Ng, 2009) , PolyPhen–2 (Adzhubei, Jordan, & Sunyaev, 2013) , Provean (Choi & Chan, 2015), Mutation taster (Schwarz, Cooper, Schuelke, & Seelow, 2014) , PhD-SNP (Capriotti & Fariselli, 2017) , SNP&Go (Calabrese, Capriotti, Fariselli, Martelli, & Casadio, 2009), Mutation Assessor (Reva, Antipin, & Sander, 2007), FATHMM (Shihab et al., 2013), M-CAP (Jagadeesh et al., 2016), MetaLR & MetaSVM (Ioannidis et al., 2016), SAAP (Al-Numair & Martin, 2013).

3- Results

In this paper, we describe two novel variants that we found in female patients no relationed with suspected fabry disease, in whom we performed the molecular identification by sequencing the GLA gene. The absence of these variants was verified in the databases of human gene mutations (http://www.hgmd.org), in the databases of Fabry disease (http://fabry-database.org), Leiden open variation database 3.0 (http://www.lovd.nl/3.0/home) and the ClinVar database of ncbi (https://www.ncbi.nlm.nih.gov/clinvar/) [Cited Feb 15, 2019]. We named the two variants according to the guidelines for mutation nomenclature recommended by the Human Genome Variation Society (www.hgvs.org/mutnomen). Where it was possible, we also extended the molecular analysis to the relatives of probands.

3.1 Index Case 1

Patient 1 (P1), is a 42 years old woman, who is referred by a nephrologist for sequencing the GLA gene. Clinical examinations showed the presence of subnephrotic proteinuria and phospholipidosis in podocytes reported as vacuolization.

The genetic analysis of the *GLA* gene in this patient allowed us to identify a new variant in the heterozygous state which produces a change of a phenylalanine residue by a serine at position 211 (c.632T>C, p.Phe211Ser) in the exon 4 of the gene.

3.2 Index case 2

Patient 2 (P2), is a 15-year-old girl, referred by a specialist in nephrology for sequencing the GLA gene. Clinical examinations showed occasional hematuria from 6 years, acroparesthesia in hands, occasional tinnitus and episodes of depression since the age of 11, hyperhidrosis from the 13 years, dyspnea and occasional fatigue accompanied by edema of lower limbs from the age of 14. Pathological antecedents of asthma and allergic rhinitis since 2 years, consumption of psychoactive substances (marijuana).

The genetic analysis of the GLA gene in this patient allowed us to identify a new variant in the heterozygous state which produces a change of a glycine residue by alanine at position 360 (c.1079G>C, p.Gly360Ala) in the exon 7 of the gene. When extending the analysis to other family members of the patient, it was found that her 63-year-old father, previously diagnosed with Fabry disease and on enzyme replacement therapy, manifested diarrhea for 4 days / week from 8 years of age, tinnitus from the age of 25 accompanied of edema of lower limbs, cardiomegaly since 43 years, two acute myocardial infarctions for 7 years and renal failure from 58 years. In addition, her 27-year-old half sister reported myopia from age 14, iron deficiency anemia from 18 years of age with a possible bleeding disorder, dysaesthesia from the age of 24, and photophobia. She has been experiencing an episode of increased sensation for 2 years. Unexplained body temperature that yielded spontaneously, occasional bruising, episodes of bleeding gums, hematuria, rectal bleeding and abundant epistaxis. A history of appendicetomy at 15 years of age complicated by heavy bleeding, cholecystectomy (cholelithiasis) at 19 years, urolithiasis at 24 years, trigeminal neuralgia 6 months ago and blood transfusion 6 months ago was reported.

3.3 Molecular and tridimensional data

We identified the putative disease-causing mutations of the patients' α-Gal-A enzyme in two novel amino acid substitutions: p.Phe211Ser (c.632T>C) and p.Gly360Ala (c.1079G>C). The effect of the novel variants on the α-Gal-A structure was predicted by three-dimensional structural analyses of the mutated enzymes (Fig. 1). The substitution site p.Phe211Ser is within a radius of less than 20A° of the active sites reported for α-Gal-A D170 and D231. While for the p.Gly360A la substitution the distances reach between 28.7A° for the active site located at position D231 and 28.7A° for the active site at position D170. These findings are relevant because variants mapping near the active site, appear to impair protein function (Filoni et al., 2010).

Fig 1. α-Gal-A three-dimensional structural map of the two novel amino acid substitutions together with the two active sites (spheres) in the A and B chains of the enzyme. Pymol

3.4 Physicochemical characterization of altered amino acids

The p.Phe211Ser variant that generates the substitution of a phenylalanine residue for a serine residue in position 211 reveals the following characteristics in terms of the physicochemical changes altered at the protein level. Phenylalanine is the simplest of the aromatic amino acids, its side chain is highly hydrophobic and is found more frequently in the interior of folded proteins, it is a non-polar amino acid and has a moderate flexibility in its side chain, it interacts with forces of van der Waals and its hydrophobicity is 0.951. On the other hand, serine is a hydroxylated amino acid whose flexibilty of its side chain is low with respect to phenylalanine, interacts through hydrogen bonds and van der Waals forces and can form up to 3 H bonds with its side chain. Its hydrophobicity is close to 0.601 making it a polar amino acid.

Regarding the variant p.Gly360Ala where the substitution of a glycine in position 360 by an alanine was generated, it is observed that the residues that involve the changes are

chemically of the simplest, their side chains are composed of a proton and a methyl group respectively, however, it is that simplicity that allows glycine to acquire unusual conformations and for accommodating dramatic changes in the direction of the polypeptide chain, for example in tight turns. The residue involved in the substitution interact through van der Waals forces and are classified as non-polar.

The Kyte-Doolittle scale confirmed that the p.Phe211 residue is highly hydrophobic and is also located in a hydrophobic region of the protein, while the p.Gly360 residue is highly hydrophilic as well as its location region within the α-Gal-A protein.

3.5 Sequence conservation

The sequence conservation analysis showed a semi-conservative residue in the p.Phe211Ser position, while highly conserved residues were evidenced in the p.Gly360Ala position. Pathogenic mutations typically involve conserved positions within a protein family, since these involve residues essential for structure or function of the protein (Suri et al., 2017) . In fact, the probability that a random mutation can cause a genetic disease has been shown to increase with an increase in the degree of site conservation (Vitkup, Sander, & Church, 2003) .

Fig. 2. Alignment of wild-type human α-Gal A amino acids with other organisms sequences. The rectangles indicates the amino acids at position 211 and 360 that we found mutated in the index cases.

3.6 Stability

The most frequent effect of missense variants is alteration of protein folding and stability decreased (Stefl, Nishi, Petukh, Panchenko, & Alexov, 2013) . 2/3 prediction tools show that the p.Phe211Ser substitution exerts a decrease in the stability of the protein, while for the substitution located in the p.Gly360Ala position a decrease in the stability of the protein was found in 3/3 predictive tools.

3.7 Surface and electrostatic potential effects

Both the variant p.Phe211Ser and p.Gly360Ala alter the electrostatic potential of the protein. These changes in the electrical potential around the enzyme could influence the rate of association for the substrate.

Fig 3. Electrostatic Surface potencial of the mutant proteins, products of the novel missense variants. The color scale ranges from -5KT/e (red) to 5 KT/e (blue). A) Wild type protein; B) p.Phe211Ser; C) p. Gly360Ala. Alterations in the potential of the electrostatic surface in the mutant proteins are observed.

3.8 Predictive algorithms used for sequence variant interpretation

Twelve pathogenic prediction tools were used to establish a consensus to determine the degree of tolerance for each amino acid substitution based on the physico-chemical properties. According to these results, for the variant p.Phe211Ser the results were not conclusive, since 7/12 programs classified it as benign (except SNP & GO, FATHMM, M-CAP, MetaLR_MetaSVM that classify it as cause of illness and probably harmful); while for the variant p.Gly360Ala seems to have a detrimental effect since 11/12 prediction tools (except PhD-SNP) classify it as harmful or causing disease.

Table 1. Predictions of the effect of novel variants on α-Gal-A protein

4- Discussion

Fabry disease has been regarded as a rare lysosomal storage disease with an incidence rate of 1 in 40,000 to 1 in 117,000 live births (Meikle, Hopwood, Clague, & Carey, 1999). However, newborn screening results have shown a relatively high incidence, ranging from

1 in 1250 to 1 in 4600, with 6–7 times more late-onset patients than those with classical Fabry disease (Hwu et al., 2009). Fabry disease is progressive, whereas the clinical phenotype, both at age of onset and throughout the course of the disease, is very variable, even within the same family (Schaefer, Mehta, & Gal, 2005). Because mutations in most cases are currently private, more than 1000 mutations are reported in the GLA gene. Although, thanks to scientific and clinical evidence, many variants reported in patients with suspected Fabry disease have been classified as causing many other diseases due to the lack of clinical criteria and experimental trials are classified as of uncertain significance since it is unknown how the mutations affect the protein and its function generating an uncertainty both in the affected patient and in the treating doctor regarding their therapeutic management.

Here we report for the first time the variant p.Phe211Ser (c.632T> C), in a 42-year-old female patient with suspected Fabry disease due to renal alterations. The variant is within a radius of less than 20A ° of the active sites reported for the α-Gal-A protein, possibly affecting the function of the protein due to alterations that can be generated in the interaction with the substrate. This substitution is in the domain melibiasa_2 reported for the α-Gal-A protein as a domain belonging to the glycoside hydrolase family 27 (PF16499) (Finn et al., 2014) whose function is to break the glycosidic link between two or more carbohydrates, in this case two galactoses of the globotriaosylceramide (Gb3) compound allowing its conversion to lactosylceramide avoiding the progressive accumulation of Gb3 (Garman & Garboczi, 2004). The p.Phe211Ser substitution seems to alter the electrostatic potential and the conformation of the protein since it shows the change of a non-polar residue to a polar one, as well as the stability of the protein justifying a possible detrimental effect on the protein's function. It was determined that the conservation of the residue affected by the variant is of a semi-conservative type since it was present in 5 of the 6 organisms evaluated (figure 2). On the other hand, 7/12 algorithms used for the interpretation of sequence variants classified the variant as benign, however, SNP&GO, FATHMM, M-CAP, MetaLR, MetaSVM that classify it as a cause of illness and probably

harmful (Table 1). To date, other changes in residues for position 211 in the protein have not been reported in the literature.

In relation to the p.Gly360Ala (c.1079G>C) variant reported for the first time in a 15-year-old female patient who reported acroparesthesias, hyperhidrosis, fatigue, occasional tinnitus and episodes of depression since childhood, it was found that it is a variant that, despite being far from the active sites of the α-Gal-A protein with more than 20A ° distance and that the residues involved in the substitution seem to have no relevant physicochemical differences, 3/3 programs predict that the variant the stability of the protein decreases, likewise, the ancestral residue involved in the substitution is a conserved residue in all the evaluated species (figure 2.) supporting a possible alteration on the function of the protein. The folding process of soluble proteins decreases the surface in contact with the solvent. This is related to the secondary structures of proteins (Lins, Thomas, & Brasseur, 2003). Further, because active sites of proteins are often located at the surface of the protein, greater insight into residue accessibility would be important in understanding and predicting structure/function relationships. Finally, 11/12 prediction tools for the degree of tolerance for amino acid substitution p.Gly360Ala determined by consensus that this variant is probably harmful and causing disease (Table 1). In the literature, a variant in an Italian man in the same position described here was reported p.Gly360Asp (c.1079G> A) by (Mignani et al., 2008) which was associated with a residual enzyme activity <1%, as well as a loss of protein function that correlated positively with renal involvement of the patient who was treated early with enzyme replacement therapy thus preventing the progression of the disease.

5- Conclusion

We present a complete bioinformatic analysis of 2 new variants in the GLA gene found in individuals with suspected Fabry disease. The results of the prediction of the biological effect of these variants in the protein suggest changes that would compromise its function, with greater evidence for the change p. Gly360Ala than for p.Phe211Ser. It is necessary to

have more clinical and paraclinical information (biochemical and pathological studies) to clearly determine the clinical significance of these variants.

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