Published in final edited form as:
Biochim Biophys Acta. 2010 February ; 1802(2): 247–252. doi:10.1016/j.bbadis.2009.11.003.

FUNCTIONAL STUDIES OF NEW GLA GENE MUTATIONS LEADING TO CONFORMATIONAL FABRY DISEASE

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Abstract

Fabry Disease (FD) is an X-linked multisystemic lysosomal disorder caused by mutations of α-galactosidase (*GLA*) gene. Only a few of the 450 genetic lesions identified so far have been characterised by in vitro expression studies. Thus the significance of newly identified *GLA* nucleotide variants sin FD patients which lead to α-galactosidase (GAL-A) amino acid substitutions or intronic changes can be uncertain.

We identified three *GLA* mutations: c155G>A (p.C52Y), c548G>C (p.G183A), c647A>G (p.Y216C) in as many individuals (two male; one female), and performed in vitro expression studies and Western Blot analysis, in order to clarify their functional effects.

Reduced GAL-A activity and normal or partially reduced mutant proteins were present in all overexpressed mutant systems in which, three-dimensional structural analysis showed that the active site was not directly involved. We hypothesize that the three new mutations affect the GAL-A protein, leading to conformational FD. When, mutant proteins overexpressed in COS-1 cells and in patients' lymphocytes were tested in the presence of the 1-deoxygalactonojirimicin (DGJ) chaperone, the p.G183A and p.Y216C systems showed increased GAL-A enzyme activities and protein stabilisation, while p.C52Y was not responsive.

We underline that genetic, biochemical and functional studies are helpful in clarifying the consequences of the missense genetic lesions detected in FD. ERT is the elective therapy for Fabry patients but it is not always possible to issue the enzyme's active form in all involved organs. Our study endorses the hypothesis that an active-site-specific chemical chaperone, which could be administered orally, might be effective in treating GAL-A conformational defects.

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Keywords

α-galactosidase; 1-deoxygalactonojirimicin; chaperones; Fabry disease

INTRODUCTION

Fabry disease (FD) (MIM 301500) is an X-linked recessive lysosomal storage disorder of glycosphingolipid catabolism, caused by deficient α-Galactosidase A (GAL-A, E.C. 3.2.1.22) activity with a progressive accumulation primarily of globotriaosylceramide and related glycosphingolipids in the plasma and in the lysosomes of cells throughout the body [1].

Angiokeratoma, acroparesthesias, hypohidrosis, renal, cardiac and cerebrovascular involvement, and corneal opacities are the major clinical manifestations in patients with the classic phenotype. The onset of disease manifestations occurs in childhood or adolescence and, if untreated, can lead to death within fourth or fifth decade of life. In classically affected males the GAL-A activity can be very low or undetectable, while patients with residual GAL-A activity can have a milder FD phenotype [1,2,3].

Heterozygous female patients exhibit a spectrum of clinical manifestations and severity, from asymptomatic to severe impairment likewise the male phenotype [4].

The human GAL-A gene (*GLA*), mapped on Xq22, is organized in seven exons encompassing over 12 Kb [5]. The GLA cDNA of 1290bp encodes foe a precursor protein of ~50kDa (429 amino acids) which is proteolytically cleaved into the lysosomal mature protein [6,7]. The mature GAL-A enzyme is a homodimeric glycoprotein of about 46 kDa (398 amino acids), 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 [8].

More than 450 mutations, scattered throughout all exons have been reported, about 60% of which are missense, mostly private (Human Gene Mutation Database Web site, HYPERLINK "http://www.hgmd.cf.ac.uk/"http://www.hgmd.cf.ac.uk/) [9,10]. The structure of human GAL-A has been determined by X-ray crystallographic methods [8] and the amino acid substitutions responsible for the biochemical defect have been characterized. Expression and functional studies on GAL-A aim to define the mutations effects on the mature protein in order to determine causative mutations and evaluate therapy options. Currently enzyme replacement therapy (ERT) is the only approved treatment but its success is disease progression related i.e. limited when patients have already developed severe cardiac or renal involvement. Also, Gb3 is proved not to be efficiently removed from podocytes and blood vessel walls and, as occurs in all intravenous drug administration, ERT can give rise to immuno reactions [11,12]. There is also a new therapeutic approach using small molecules termed active-site-specific chaperones (ASSC) which help the influx of otherwise degraded misfolded proteins into the lysosomes [13,14,15].

It has been reported that some missense mutations are more responsive to chaperone 1-deoxygalactonojirimycin (DGJ) than others. It appears that this variability is possibly connected to both the position of the mutations and the nucleotide substitution that leads to a different amino acid, even within one codon [15,16,17].

Here we report biochemical, genetic and cellular studies on three FD patients in whom we identified two new and one known but functionally uncharacterised GLA mutations. In order to evaluate the influence of these mutations on the structure and activity of the protein, we performed transient expression and western blot analysis on novel missense mutations. To

complete our results we tested the therapeutic effects of DGJ and galactose on these expression systems and that of DGJ alone on patients leukocytes.

METHODS

Patients

We report the clinical, biochemical and molecular studies of three unrelated Italian patients with Fabry disease (two males and one female). The male patients exhibited classic phenotypes, with renal and cardiac involvement. All patients are being treated with ERT (dosage: 1mg/kg b.w. per 1 day every 15 days for agalsidase beta, and 0,2mg/kg/b.w. every 15 days for agalsidase alpha).

Patient 1 (P1, genotype c.155G>A, p.C52Y) is a classic Fabry male patient and his clinical features have been summarised in a previous report [Patient 22 in 17]. He has presented the typical symptoms of Fabry disease since adolescence: achroparhesthesia, hypohydrosis, intolerance of cold, fever attacks and recurrent abdominal pain. From the age of 27 to 41 years he had 8 acute episodes of cerebrovascular ischemia with a diagnosis of cerebral vasculitis. The neurological clinical picture was progressive: at 29 years of age hemyplegia with dysartria and onset of dyplopia that acutely worsened at 33 years; at 36 he developed hemyanopsia on the left side and acute worsening of right hemyplegia necessitating a cane for walking; at 41 mild acute left hemyplegia occurred. Brain MRI performed at 42 years showed multiple small lesions in: bilateral pontocerebellar, left mesencephalic, posterior thalamic, lenticular nucleus, right peritrigonal and bilateral periventricular white matter. When the patient was 42 years of age, the diagnosis of FD was made. He also had proteinuria (552 mg/4h), moderate hypertrophic cardiomyopathy, and bilateral neurosensory hypoacusia. He started ERT with agalsidase alpha at the age of 41 years and 8 months. After 5 months he had a new acute ischemic vertebral-basilar episode. After that he began to need a wheel-chair and his dysartria worsened presenting a very reduced personal autonomy. When last seen, 2008, renal and heart function had not worsened. Proteinuria was stable around 500 mg/24 h and plasma creatinine was 0,8 mg/dl. Left ventricular mass had passed from 170,5 g/m2 to 138, 4 g/m2.

The female patient (P2, genotype: heterozygous for c.548G>C, p.G183A) is 54 years old (age at diagnosis 49 years) with a wide clinical presentation of FD. She had mild hypertension and renal involvement; her mother underwent dialysis for several years because of renal failure. Classic symptoms such as fever pain, acroparesthesia, and angiokeratoma were absent. Before ERT was started at 51 years of age, she presented podocytes damage with “zebra bodies”, proteinuria (250 mg/h), very mild mitralic insufficiency, and a developing diabetes mellitus type 2. ERT with agalsidase beta was initiated in order to prevent the development of the disease, and in particular to reduce renal damage, also considering diabetes mellitus and hypertension as independent risk factors of cardiovascular disease and end stage renal disease. Despite ERT, albuminuria and proteinuria remain unchanged, even if the concentration of “zebra bodies” has decreased. Also, the mild mitralic valve insufficiency disappeared after 30 months of therapy.

Patient 3 (P3, genotype c.647A>G, p.Y216C) is a 56 year classic Fabry male patient, diagnosed at 20 years of age. He presented with diffuse angiokeratoma, acroparesthesia, pain and limb edema. Before starting ERT, at 54 years of age, he also presented cornea verticillata, tinnitus, vertigo and mild left ventricular hypertrophy. After starting ERT with agalsidase alpha, acroparesthesia and fever pain crises disappeared, but proteinuria severely worsened from 300mg/h to 2,5g/h. He is currently treated with Triatec 2,5 mg/day and proteinuria decreased to <500mg/h. Heart involvement remains unchanged.

Analysis of genomic DNA

Genomic DNAs from patients and their relatives were isolated from leukocytes. 200ng of genomic DNA were amplified with *GLA* specific oligonucleotide primers. The sequences of genomic primers and the amplifying conditions used to amplify Exons 1–7 of *GLA* gene had been previously reported [18].

PCR products were checked on a 2% agarose gel, excised and purified using Nucleospin Extraction kit (Macherey-Nagel). Approximately 100 ng of purified fragments were used in sequencing reactions. The sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Ready Reaction Kit reagents (Applied Biosystems). The reactions were run on an ABI 310 sequencer (PE Biosystems) and were analyzed using Sequencing Analysis Software, version 3.3.

Restriction-Site Analysis

The known p.C52Y (c.155G>A) and the new p.G183A (c.548G>C) and p.Y216C (c. 647A>G) amino acid substitutions were confirmed in patients and in their relatives. A possible polymorphism effect was excluded by testing by restriction analysis 200 control alleles for each mutation. The restriction enzymes used were the following: Bsp1407I for p.C52Y (c.155G>A), AluI for p.G183A (c.548G>C) and Bsp1407I for p.Y216C (c. 647A>G). All mutated fragments showed a natural restriction site. 6 µl of PCR product were incubated for 1h at the temperature recommended by the manufacturer's instructions, with a reaction mixture containing 1.5 µl of 10X reaction buffer (BM) and 5 U of specific restriction enzyme. The total volume was brought up to 15 µl.

Mapping mutations onto structure of GAL-A

To predict the structural effect of the novel missense mutations on resulting GAL-A enzymes we modeled the mutations onto the three-dimensional structure of GAL-A determined by x-ray crystallography. Amino acid substitutions corresponding to the three mutant proteins were introduced into the wild type structure in the molecular graphics program O. Amino acid side chain rotamers for the substitutions were chosen to minimize steric clashes. The resulting three dimensional model of the mutant protein were energy minimised in the program CNS. Molecular figures were prepared with the program Molscript.

WT GLA vector preparation

The GLA cDNA obtained was used as the PCR template. The forward and the reverse primers were designed to introduce a restriction site for specific endonuclease (XbaI and EcoRI). The amplification reactions were performed in a total volume of 50 µl containing 2.5U of Expand High fidelity Taq polymerase (Roche), 25 mM of dNTPs, 200 ng of forward (5'CTCTAGAATGCAGCTGAGGAA3') and reverse (5'CCGAATTCTTAAAGTAAGTCTTTTAA3') primers and 1X PCR reaction buffer. Cycle conditions were: 94°C for 2 min, 28 cycles 94°C 15 sec, 55°C 30 sec and 68°C 2min, with a 10 min 68°C final extension cycle.

Site directed mutagenesis

Fragment replacement was used to introduce the full length GLA cDNA in transient expression vectors, derived from pCD-X vector kindly provided by Dr. D'azzo, St. Jude, Memphis.

The pCD-X expression vector and the full length *GLA* cDNA were cut with endonucleases XbaI and EcoRI that recognised respectively a unique restriction site. The digested

fragments were linked by a ligase (TaKaRa Bio Inc., Japan). In the final construct the integrity of the DNA and the presence of only the expected *GLA* cDNA insert were verified by sequencing. This expression vector was used to transform the E. coli strain Solo pack gold cells (Stratagene, Amsterdam Zuidoost, The Netherlands), obtaining a great quantity of vector. Plasmid DNAs were purified by Perfectprep Plasmid Maxi kit (Eppendorf, Hamburg, Germany).

To introduce the genetic lesions detected in the patients in the transient expression vector, the pCD-GLA was amplified by PCR with specific mutated oligonucleotides primers (Table 1) and the mutated constructs were verified by sequencing. These mutated transient expression vectors were used to transform the E. coli strain Solo pack gold cells (Stratagene, Milano, Italy).

Transfection into COS-1 cells

Normal and mutant vectors were transiently overexpressed into African green monkey kidney cells (COS-1). Transfection of COS-1 cells was mediated by DEAE- dextran [19]. The amount of vector used for each transfection was about 4 μg/ml. The efficiency of transfection was increased by treatment with cloroquine 100μM (SIGMA, Milano, Italy).

Cell cultures and biochemical enzymatic assay

COS-1 cells were cultured in Dulbecco's modified Eagles-Hams F10 medium (1:1 vol/vol) with fetal bovine serum (10%) and antibiotics. Primary cultures of T- lymphocytes were cultured as previously reported [12]. GAL-A enzyme assays were performed in triplicate by fluorogenic method previously reported [20] with the following modifications: 1mg/ml cell lysates (diluted in 10μl of heat inactivated 0,2% BSA + 0,02% Na Azide) were added to 20μl substrate (4-MU-a-D-galactopyranoside 5 mM in 0,1M Na-acetate buffer, pH 4,5 + 150mM N-acetyl-D-galactosamine and 0,02% Na Azide); stop solution: 200μL of 0,5M Na-HCO_3-Na_2Co_3 buffer, pH 10,7 + 0,025% Triton X-100. The Micro BCA protein Assay kit (Pierce Rockford, USA) was used to set up the starting proteins used in each enzyme assay performed in black 96-well microplates. Fluorescence was read on a Spectra Max M2 microplate Reader (Molecular Devices, Toronto, Canada).

Western blot analysis

Transfected COS-1 cells were harvested by trypsin and lysed by sonication (10 sec). 10 μg of total COS-1 cells were used in the blots. For analytical purposes, western blots were prepared from 12.5% polyacrylamide gels and probed as previously described [21]. Following electrophoresis, proteins were transferred onto nitrocellulose (Bio-Rad, Hercules, CA) and the filters incubated with the anti GAL-A antibody provided by Genzyme Corporation. The results of the blots were visualised by reaction with the secondary antibody anti-rabbit IgG (whole molecule) Alkaline phosphatase conjugate (SIGMA, Milano, Italy), revealed by AP Conjugate Substrate kit (Bio-Rad, Hercules, CA).

In vitro pharmacological administration

The effects of DGJ were tested on both COS-1 cells transfected with p.C52Y, p.G183A, p.Y216C mutated vectors and on patients' lymphocytes. COS-1 cells were incubated in supplemented medium with and without DGJ 20μM [12,16] and with and without galactose 200mM as previously reported [22]. DGJ 20μM was administrated in patients' lymphocyte cultures for 72 hours. For the p.C52Y expression system, three different concentrations of DGJ were used (1μM, 20μM and 100μM) and incubation was followed by a washout period as previously reported [20]. After 48 hours the cells were harvested by trypsin and were tested in triplicate by GAL-A enzyme assay.

Statistical analysis

In each experiment the value of GLA activity in non transfected COS-1 cells was used to set up the GAL-A intrinsic activity of the expression systems.

Standard deviation determination was obtained using Microsoft Excel 97 SR-2 (HYPERLINK "http://www.microsoft.com/"www.microsoft.com)

RESULTS

Clinical and biochemical features

In the three patients reported herein the diagnosis of FD was confirmed by demonstrating the reduction or absence of GAL- A enzyme activity in leukocytes and by molecular analysis. The highest GAL-A activity (46% of mean control values) was detected in the female patient (P2), and lymphocytes from P1 and P3 showed respectively 4 and 2,5% of control activity. After starting ERT, no improvement was observed in P1's condition. However, he has had no more ischemic attacks and kidney and heart functions have not worsened and ERT may have played a role in this stabilization. The concentration of "zebra bodies" in P2's podocytes and her very mild mitralic valve insufficiency disappeared after 30 months of ERT.

However, Mainz Severity Score Index (MSSI) for all the three patients was not significantly changed after treatment. In addition, P3 has developed severe renal involvement as a new symptom.

Molecular and tridimensional data

We identified the putative disease-causing mutations of the patients' GAL-A enzyme in three novel amino acid substitutions: p.C52Y (c.155G>A), p.G183A (c.548G>C), p.Y216C (c.647A>G). A polymorphic effect of such mutations was excluded by restriction enzyme analysis of 200 control alleles. Molecular analysis extended to other family members led to the identification of two additional heterozygous females, who subsequently underwent ERT.

The effect of the novel mutations on the GAL-A structure was predicted by three-dimensional structural analyses of the mutated enzymes (Figure 1). All the analyzed mutations, mapping near the active site, appear to impair protein function.

The amino acid substitutions were inserted into the pCD-GAL-A vector by site directed mutagenesis and transiently expressed in COS-1 cells to evaluate their in vitro activity with respect to the wild type expression vector. Immunoblot analysis was performed to evaluate the GAL-A stability obtained in the expression systems (Figure 2). Reduced (p.G183A and p.Y216C) or quite normal (p.C52Y) amounts of GAL-A proteins were detected in all transfected COS-1 cells. These results show that the p.C52Y mutated enzyme is normally expressed, while p.G183A and p.Y216C mutations partially affect enzyme stability and protein folding. The addition of DGJ (1μM, 20μM and 100μM) to p.C52Y system resulted in no difference from the untreated corresponding line. Interestingly, in both p.G183A and p.Y216C systems, the GAL-A enzyme amount clearly increased, in particular for the p.G183A system (Figure 2).

In vitro supplementation with DGJ and galactose

Taking into account molecular and tridimensional data, expression studies and western blots analysis, the deleterious effects of the analysed mutations appear to affect enzyme activity or folding. Assuming a potential role of the mutations as conformational defects, we tested the

effects of the pharmacological chaperone DGJ at subinhibitory concentrations (20μM) [12] on in vitro expression systems. To provide a positive control, we also tested the effects of galactose at 200 mM [22].

In p.G183A and p.Y216C in vitro systems, DGJ administration increased residual GAL-A activity. p.Y216C was more responsive to DGJ than to galactose, and p.G183A was not influenced by galactose. No significant variation on residual GAL-A activity in the presence of either pharmacological chaperones was detected in the p.C52Y system (Figure 3), despite three different concentrations of DGJ being used. DGJ treatment tested on lymphocytes of patients and controls confirmed the data obtained from the experiment on p.C52Y, p.G183A and p.Y216C expression systems (Figure 3).

DISCUSSION

The clinical onset and disease course of Fabry patients are difficult to trace. Clinical signs and symptoms are multisystemic and nonspecific, and they may mimic other disorders such as rheumatoid diseases or fevers. Diagnosis in male patients is made by an enzymatic assay measuring GAL-A activity. The detection of residual GAL-A enzyme activity could complicate a diagnosis, both females and in males. It has been reported that higher residual enzyme activities can lead to milder phenotype [9].

To better define genotype-phenotype correlations, we carried out molecular and biochemical analyses. These procedures can also be used to identify the ‘at risk’ family members. Out of the 450 genetic lesions until now reported, 60% are missense and could affect either the active site or the protein structure. Orally administrable drugs such as small molecules acting as chaperones (ASSC) seem to be useful in helping misfolded enzymes to reach the lysosomes [13,14,15].

Data in this study contribute to Fabry mutation database two new missense mutations p.G183A (c.548G>C), p.Y216C (c.647A>G) confirming *GLA* molecular heterogeneity in Fabry disease. All three missense mutations were further analysed in western blots, expression studies, three-dimensional structure, and ASSC administration.

Each mutation influenced the protein structure in different ways, from changes in polarity to side chain volume. It is therefore important to assess nucleotide substitutions individually [16]. All three mutations here reported map in the $(\beta/\alpha)_8$ barrel domain containing the active site but do not directly affect the amino acids involved in the catalytic site.

The p.C52Y missense mutation, identified in P1, abolishes a required disulfide bound near the active site. The disulfide bond is found near p.W47, an active site residue critical for recognition of the terminal galactose moiety on the substrate and product. Transient expression studies of the p.C52Y showed very low GAL-A activity in vitro, consistent with the activity detected in patients' leukocytes. The severe nature of this mutation was confirmed by in vitro chaperone treatment: neither DGJ (20μM) nor galactose improved residual GAL-A activity (Figure 3). GAL-A enzyme activity measured in p.C52Y system supplemented with DGJ (1mM, and 100mM), also remains unchanged compared to untreated COS-1 cells transfected with the p.C52Y mutation (data not shown).

The p.Y216C amino acid substitution was detected in a male patient (P3) referred from a cardiac unit. Using molecular modeling of the mutant protein, the substitution of the p.Y216 amino acid with a Cys makes contacts to a critical loop at the active site, the b5-a5 loop. Perturbation of this loop by the Y216C substitution may then partially alter the geometry of the active site. GAL-A enzyme assay on lymphocytes revealed 2.5% activity. Chaperone administration on the p.Y216C expression system showed a significant increase in GAL-A

activity; in particular the effect of DGJ was greater than that of galactose. These data were confirmed on patient's lymphocytes (Figure 3).

For p.G183A, detected in the female patient P2, a long side chain (Ala) replaced a shorter chain (Gly183), likely causing a conformational GAL-A change affecting enzyme folding. According to three-dimensional modelling of the mutant, there is little room in the wild type structure to accommodate the larger side chain in the p.G183A substitution. This mutation proved to respond well to DGJ treatment in vitro, while it did not respond to galactose administration. Data from lymphocytes showed that DGJ administration moves the GAL-A residual activity's percentage from 40% to 92%, almost completely restoring normal enzyme activity (Figure 3).

Although none of the three mutations showed direct effects on the fifteen residues that make up the active site of the enzyme, but they were all found in the second shell of residues around the active site. Thus, local changes in the structure of the protein due to the mutations, leads to a less stable enzyme and a loss of enzyme activity. These types of mutations are likely to be good candidates for ASSC. We believe the p.C52Y mutant lacks a critical disulfide bond very close to the active site, and the active site of the enzyme is not correctly formed, thus it is less efficiently rescued by ASSC using DGJ.

In physiological conditions, proteins with altered tertiary structures are held by molecular chaperones at an endoplasmic reticulum (ER) level and degraded in this cellular compartment [23].

Our results on the p.C52Y mutation are in keeping with recent studies on DGJ, in which the molecule has been proved to be unable to reverse the enzyme defects of all missense mutations [14,16,24,25,26]. It therefore likely that the p.C52Y mutation influences the GAL-A active site whereas p.G183A and p.Y216C are related to protein folding alterations leading to conformational FD. Such an hypothesis was supported by western blot analyses that showed quite normal amount of GAL-A in p.C52Y system, while GAL-A amounts detectable in p.G183A and p.Y216C systems were reduced. Addition of DGJ (20μM) to p.G183A and p.Y216C expression systems significantly stabilised the protein amounts. Indeed, mutant p.C52Y GAL-A did not show any increase in protein stabilisation despite three different concentrations of DGJ being used (Figure 2).

Since 2001 the only approved therapy for FD has been ERT [11] but it has also been demonstrated that the cellular distribution of the replacement enzyme is heterogeneous [27] and is unable to remove the substrate (Gb3) from affected tissues [11,28]. In addition, oral administration is an easily manageable therapy against intravenous administration as ERT needs.

DGJ administration on P2's lymphocytes (the female proband with the p.G183A mutation) shows a complete reversion of the enzyme defect in vitro. It would thus appear that this patient is a good candidate for in vivo treatment.

We believe that ASSC could become a useful therapeutic alternative for several forms of FD caused by GAL-A conformational defects, a combined therapy using ERT and DGJ administration could also be tested.

It is becoming clear that different nucleotide substitutions have different impacts on GAL-A protein conformation. We therefore support the creation of a catalogue listing changes responding and not responding to DGJ [16].

Expression studies and structural analyses which clarify the nature of the amino acid lesions involved in FD are essential to improve therapeutic approaches available for the disease. As response to DGJ administration is variable, each patient should be tested for new mutations before ASCC therapy is introduced.

A phase 2 trial, using DGJ molecules called AT1001 migalastat hydrochloride (Amigal^{tm}) distributed by Amicus Therapeutics, has been completed for some Fabry patients carrying responder conformational mutations.

Acknowledgments

This work was partially supported by grants from the Genzyme Corporation, Ateneo (MURST ex 60% and Prin) and Shire Italia. Dr. D'Azzo (Department of Genetics, St. Jude Children's Research Hospital, Memphis, TN, USA) is kindly acknowledged for providing pCD-X vector. We also thank Dr. Otto Van Diggelen (Erasmus University, Rotterdam) for his precious assistance in performing the enzyme assays. GAL-A antibody was kindly provided by Genzyme Corporation. Monza's Metabolic Unit thanks Pierfranco e Luisa Mariani Foundation, Milano, for their continuous financial support to their clinical activity. S.C. Garman gratefully acknowledges support from the NIH (DK76877).

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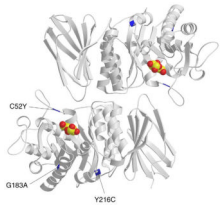

Figure 1.
GAL-A three-dimensional structural map of the three novel amino acid substitutions.

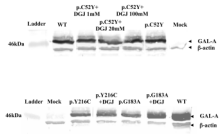

Figure 2. Western blot analyses of lysates from COS-1 cells transfected with wild type and mutant constructs

10 μg of total proteins were used in the blots. Western blot were carried out with the anti GAL-A antibody kindly provided by Genzyme Corporation. **Mock**. Negative controls. B-Actin is reported as a control protein. Where not indicated, DGJ concentration is 20μM.

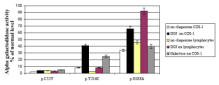

FIGURE 3. GAL-A activity resulting from DGJ and galactose administration in transfected COS-1 cells and from DGJ administration in lymphocytes

Three independent experiments performed in triplicate both for DGJ (20μM) and galactose (200mM) administration in COS-1 transfections and for DGJ (20μM) administration in lymphocytes, were performed. In the expression systems the value of GAL-A activity of non-transfected COS-1 cells was used to set up the GAL-A intrinsic activity in each experiment. Standard deviation was obtained using Microsoft Excel 97 SR-2. Values are expressed as the percentage of wild type lymphocytes for the DGJ administration in patients' lymphocytes and as the percentage of COS-1 cells transfected with wild type GAL-A for the expression systems. T- lymphocytes arise from primary cultures and they were not immortalized.

Table 1

Mutated oligonucleotides for site direct mutagenesis

Patient	Nucleotide change	Forward Primer	Reverse primer
P1	c.155G>A	5’ GCGCTTCATGT**A** CAACCTTGAC 3’	5’ GCTAAGGTTG**T**ACA TGAAGCGC 3’
P2	c.548G>C	5’TGGCAGATG**C** TTATAAGCACA TG3’	5’ CATGTGCTTATA A**G**CATCTGCCA A 3’
P3	c.647A>G	5’CAAAAGCCCA ATT**G**TACAGAA ATC 3’	5’ GATTTCTGTA**C**A ATTGGGCTTTTG 3’

Underlined base correspond to the mispairings with the normal GLA sequence used to introduce the patients’ mutations in the pCD-GLA vectors.