Identification of three novel mutations in Romanian patients with Fabry disease

Identificarea a trei noi mutații la pacienții români cu boala Fabry

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Abstract

Fabry disease is an X-linked recessively inherited disease caused by the deficiency of alpha-galactosidase A, a lysosomal enzyme involved in glycosphingolipid catabolism. This disorder can be clinically suspected in patients exhibiting predominantly angiokeratoma and neuropathic pain, but also cardiac, cerebrovascular and renal symptoms. However, the diagnosis is definitively confirmed in affected males by measurement of the enzymatic activity and characterisation of the pathogenic mutations. The alpha-galactosidase A (GLA) gene, located in Xq22, was cloned and numerous mutations were described. The aim of the present study was to identify the mutations present in six Romanian Fabry disease patients, using exon-sequencing of the GLA gene. Three novel sequence variations, one deletion c.548del and two missense substitutions c.539T>G (p.Leu180Trp) and c.241T>C (p.Trp81Arg) were found in these patients, as well as two previously reported mutations (c.937G>T or p.Asp313Tyr and c.485G>A or p.Trp162Stop).

Key words: Fabry disease, mutation analysis, alpha galactosidase A gene, DNA sequencing

Rezumat

Boala Fabry este o afecțiune cu transmitere recesivă legată de cromozomul X, cauzată de deficitul alfa-galactozidazei A, enzimă lizozomală implicată în catabolismul glicosfingolipidelor. Boala poate fi suspectată la pacienții cu angiokeratoame şi dureri neuropate, dar şi la cei cu simptome cardiace, cerebro-vasculare şi renale. La pacienții de sex masculin, diagnosticul de certitudine constă în dozarea activității enzimatice şi caracterizarea mutațiilor patogene. Gena alfa-galactozidazei A (GLA), localizată în regiunea Xq22, a fost clonată şi au fost descrise numeroase mutații. Acest studiu a avut ca scop identificarea mutațiilor la 6 pacienți români cu boala Fabry, prin secvențarea exonilor genei GLA. Au fost identificate 3 noi variații genetice: o deleție - c.548del - şi două substituții punctiforme - c.539T>G (p.Leu180Trp) şi c.241T>C (p.Trp81Arg). Au fost de asemenea evidențiate două substituții descrise în prelabil: c.937G>T (p.Asp313Tyr) şi c.485G>A (p.Trp162Stop).

***Cuvinte cheie**: boala Fabry, analiza mutatiilor, gena alfa-galactozidazei A, secventarea ADN*

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Introduction

Fabry disease is an X-linked recessive disorder of the glycosphingolipid metabolism, caused by molecular lesions in the *GLA* gene, resulting in a deficiency of the lysosomal enzyme alpha-galactosidase A (EC 3.2.1.22). The enzymatic defect causes the multivisceral accumulation of non-degraded substrate, mainly neutral glycosphingolipids, such as globotriaosylceramide, identified in numerous tissues and cell types [1].

In males affected by the severe classic form of the disease, clinical signs appear since early childhood. At this stage, the picture is dominated by neuropathic pain and dermatological signs (angiokeratoma). Afterwards, a multivisceral disease develops and the main clinical manifestations are cardiac symptoms (left ventricular hypertrophy), cerebrovascular signs (stroke, transient ischaemic attacks), sensorineural deafness and renal complications (proteinuria, renal failure) [2]. Attenuated variants with either predominant cardiac, or renal involvement have recently been described. Heterozygous females who are carriers of the disease are often symptomatic because of the random inactivation of one X chromosome (lyonisation). However, their clinical presentation is generally less severe and a high degree of clinical variability has been reported [1].

Biological diagnosis of Fabry disease is simple and straightforward in males. It is based on the measurement of alpha-galactosidase A enzymatic activity in leukocytes, by comparison with normal controls. The situation is different in females, who can exhibit a completely normal alpha-galactosidase A activity, if they inactivate their mutant X chromosome. Identification of the pathogenic mutations is therefore the reference method for determining their status [3]. To date, more than 470 mutations have been reported in the *GLA* gene, located on Xq22 [4].

Alpha galactosidase recombinant enzymes have recently been developed and several clinical trials have proved their efficacy and tolerance. Enzyme replacement therapy is now the standard treatment for this disease in various countries around the world [5-8].

The specific diagnosis of Fabry disease by measurement of alpha-galactosidase A enzymatic activity is available in Romania since 2003. It has allowed the identification of 12 hemizygous male patients, but the characterisation of their genotype had not yet been performed. The aim of the present study was to identify the mutations responsible for Fabry disease in Romanian patients and to evaluate their phenotypic impact.

Patients and methods

This study was approved by the ethical commission of the University of Medicine and Pharmacy, Cluj-Napoca, Romania. Informed consent was obtained for all patients. Six hemizygous male patients (age between 23 and 51 years) belonging to 5 different families were included. The diagnosis was first confirmed by enzymatic assay. Measurement of alpha-galactosidase A activity was performed on leukocytes using a fluorescent substrate (4-methylumbelliferyl-alpha-D-galactopyranoside) in the presence of N-acetylgalactosamine (inhibitor of alpha-galactosidase B). The diagnosis was confirmed if the enzymatic activity was at most 10% of that measured in control males, tested in the same reaction [9].

The extraction of genomic DNA was performed for each patient using the Quick-gDNA™ MiniPrep (ZymoResearch), according to the recommendations of the manufacturer. The seven exons of the *GLA* gene (including the intronic flanking sequences) were amplified using the primers given in *Table 1*. DNA amplification was performed for each fragment in a 100 µl final volume containing 100 ng genomic DNA, 0.25 mM of each dNTP, 0.2 µM of each primer, 2.5 units of *Taq* polymerase and 10 µl of 10X buffer. The concentration of $MgCl_2$ was 2.5 mM for exons 2, 5, 6 and 3 mM for exons 1, 3, 4, 7.

Table 1. Experimental conditions used for the amplification of *GLA* exons

Exon	Direction	Sequence of primers (5'-3')	Annealing temp (°C)	Product size (bp)
1	Forward Reverse	TTGCCAGAGAAAACAATAACGTCA AACACACCCAAACACATGGAAAAG	55	370
2	Forward Reverse	CCAAGGTGCCTAATAAATGGG GCTATTTCTAAACAAGCTTC	50	330
3	Forward Reverse	CTCTCTTTCTGCTACCTCACG CTCTGGCACATGGTGAATA	55	381
4	Forward Reverse	ACTCCACCCTGGATGACAGACT CTTGGTTTCCTTTGTTGTCAAG	53	295
5	Forward Reverse	AGGGTTTAGACCTCCTTATGGAGAC TAAGTACTCTCACATAAAG	45	413
6	Forward Reverse	TATGTGAGAGTACTTAGCGC ACTGATAGTAACATCAAGAGCAAGGG	53	421
7	Forward Reverse	CATTAAGAATGAATGCCAAACTAA TGTTGTCCAGGCGGGTCTAAAG	58	530

temp: temperature, bp: base pairs

The thermocycling conditions were: 5 min at 94°C, followed by 32 cycles of amplification consisting of 45 seconds at 72°C, 45 seconds at 94°C and 45 seconds at a specific annealing temperature (*Table 1*), and a final elongation of 7 min at 72°C, in a 2720 Thermal Cycler (Applied Biosystems). PCR products (size given in Table 1) were then purified with Multiscreen HTS filter plates (Millipore) and sequenced in the forward and reverse directions. Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit, following the instructions of the manufacturer and the sequences were analyzed on the ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The nomenclature used for the description of the mutations is based on the recommendations of the HGVS (Human Genome Variation Society).

Results

Exon-sequencing of the *GLA* gene in the six Romanian patients allowed the identification of their complete genotype. The sequencing diagrams are presented in *Figure 1* and the mutations are described in detail in *Table 2*. Three novel mutations were identified: c.548del, c.539T>G and c.241T>C.

The mutation c.548del found in patient 1 is a 1 bp deletion in exon 4. It is a frameshift mutation, responsible for a Gly>Val substitution at codon 183 and the occurrence of a premature stop codon (UGA), 8 positions downstream Gly183 (p.Gly183ValfsX9). Patient 2 carries the novel missense substitution (c.539T>G) in exon 3, that replaces a leucine residue with tryptophan at codon 180 (p.Leu180Trp). The third novel sequence variation, found in patient 4, is a T>C transition (c.241T>C) in exon 2, responsible for the substitution of a tryptophan by an arginine at codon 81 (p.Trp81Arg). Patient 5, who is the brother of patient 4, carries the same substitution, as expected.

Two other previously reported mutations were also identified in our study. Patient 3 carries the mutation c.937G>T, located in exon 6 and resulting in an Asp>Tyr substitution at codon 313 (p. Asp313Tyr) [10]. The other known substitution c.485G>A in exon 3 was found in patient 6. It is a non-sense mutation (p.Trp162Stop), resulting in a premature termination of polypeptide synthesis 161 amino acids downstream codon 162 [11].

Table 2. Enzymatic activity and mutations identified in the 6 Romanian patients with Fabry disease

Patients	Enzymatic activity** (% of controls)	Nucleotide change	Location (exon)	Structural effect
1	20.00 (10%)	**c.548del**	4	p.Gly183ValfsX9
2	18.60 (10%)	**c. 539T>G**	3	p.Leu180Trp
3	1.68 (2.5%)	c.937G>T	6	p.Asp313Tyr
4*	5.23 (3.3%)	**c.241T>C**	2	p.Trp81Arg
5*	11.85 (7.2%)	**c.241T>C**	2	p.Trp81Arg
6	6.95 (1.6%)	c.485G>A	3	p.Trp162Stop

The novel mutations are in bold.
** These patients are brothers, **in nmol/h/mg protein, reference range 160-320 nmol/h/mg*
c.: cDNA, p.: protein, fs: frameshift, X: stop codon.

Discussion

This study allowed the complete genotype characterisation of 6 Romanian Fabry disease patients. Their clinical presentation was suggestive for this pathology (angiokeratoma, severe renal impairment, neuropathic pain) [12], and prompted their referral to our laboratory for biochemical confirmation of the diagnosis (*Table 2*). This study comprising only a limited number of patients is preliminary, but it offers the first glimpse into the molecular pathology of Fabry disease in Romanian patients.

The deletion c.548delG found in patient 1 is a novel abnormality on the GLA gene and is likely deleterious, as it is a frameshift mutation, responsible for the occurrence of a premature stop codon. Therefore, the mRNA produced might be targeted for nonsense-mediated decay (NMD). Another novel mutation, present in patient 2, is the missense substitution c.539T>G. This sequence variation is probably deleterious, as it occurs on an amino acid highly conserved among different species (p.Leu180Trp). The third alteration c.241T>C (p.Trp81Arg), found in two brothers (patients 4 and 5), is responsible for the replacement of a hydrophobic amino acid (tryptophan) by a highly polar arginine residue. It has not been previously described, but other anomalies - p.Trp81Stop [13] and p.Trp81Ser [14] - have been reported at the same codon, that is probably prone to mutate.

The deleterious potential of these newly described missense substitutions can be inferred from their gene location and non-conservative status, but molecular modelling and expression studies are further required to accurately establish whether they represent clearly pathogenic mutations or polymorphisms. In addition, the allele frequency of these variants should be determined in a larger population sample, as in previously reported studies [15-17].

The missense substitution c.937G>T (p.Asp313Tyr) identified in patient 3 has already been described in the literature as responsible for Fabry disease [10]. However, its deleterious potential is controversial [18, 19]. Patient 3 exhibits typical signs of Fabry disease and displays very low alpha-galactosidase A activity. The mutation c.937G>T is likely responsible for his phenotype, as no other mutation was found after a complete DNA sequencing on both strands. However, it is not possible to exclude the presence of another mutation, not detectable by the classical sequencing strategy. The other known mutation, c.485G>A (p.Trp162Stop), found in patient 6, is clearly deleterious, as it is responsible for the occurrence of a premature stop codon in exon 3 and consequently for an early arrest of the translation.

Clinically, all patients evaluated in this study had the classical phenotype and all had some degree of renal impairment. None of the three novel mutations described in this study

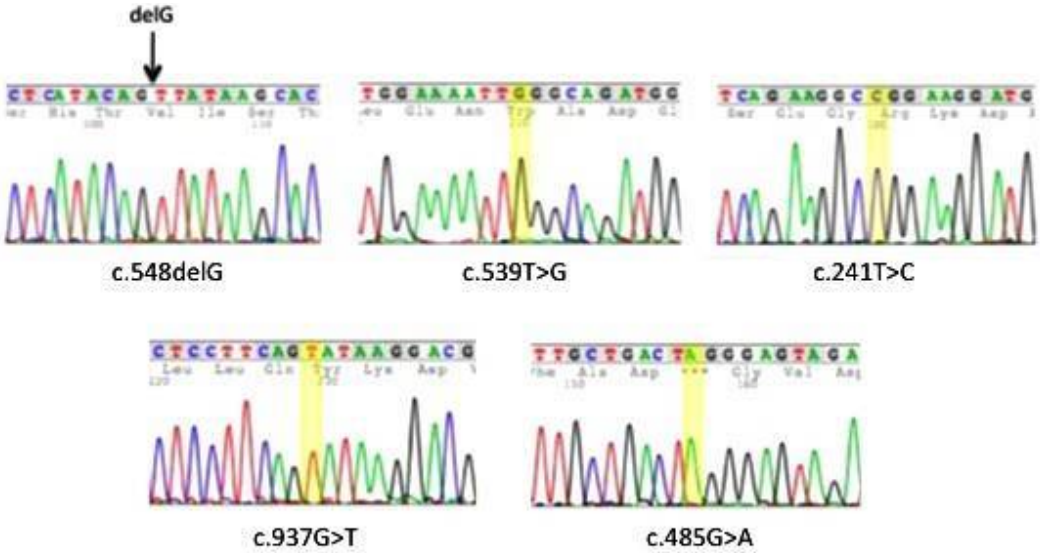

Figure 1. Sequencing diagrams showing the mutations identified in the 6 Romanian Fabry disease patients: novel mutations (first line), previously reported mutations (second line).

seem to cause an atypical clinical course, but are, as most mutations, responsible for the classical phenotype of Fabry disease.

The clinical phenotype of Fabry disease is highly heterogeneous. Therefore, the genotype-phenotype correlations are mainly imperfect. Their limited predictive value is further restricted by the age-related variability of the clinical picture. These phenotypic variations are mainly attributed to the complexity of the *GLA* gene and to environmental factors [20]. Nevertheless, the genetic heterogeneity of Fabry disease plays a primordial role in its phenotypic expression [21]. Three phenotype predictive models have been proposed in Fabry disease, taking into account the structural modifications induced by amino acid substitutions: the classic model (caused by major protein conformational changes), the attenuated phenotype (caused by minor conformational changes) and the intermediate phenotype, situated halfway between the previous presentations. These phenotype predictive models are useful for the early establishment of optimal treatment regimens [22].

The majority of reported mutations are "private", namely proper to a certain family. Although some mutations have been recurrently reported in non-related families (N215S, R227Q, R227X, R301X, R301Q) [23], the molecular heterogeneity of Fabry disease remains an important feature of this disorder and argues in favour of the investigation of larger patient groups, in order to improve the genotype-phenotype correlations, which are still a challenge [24].

Our preliminary results, established on a limited number of patients, confirm this molecular heterogeneity and emphasize the importance of *GLA* sequencing in the elucidation of the molecular pathogenesis of this disease. This study further defines the heterogeneity of mutations causing the classical Fabry disease phenotype, and allows precise heterozygote detection and prenatal diagnosis in the affected families. Further research on the structural and functional effects of the newly reported missense substitutions is however required, in order to optimally characterise the molecular profile of our patients.

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