Clinica Chimica Acta 452 (2016) 82–86

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Pitfalls in the detection of gross gene rearrangements using MLPA in Fabry disease

Lorenzo Ferri [a,b], Catia Cavicchi [b], Agata Fiumara [c], Rossella Parini [d], Renzo Guerrini [a,b], Amelia Morrone [a,b,*]

[a] Department of Neurosciences, Psychology, Pharmacology and Child Health, University of Florence, viale Pieraccini 24, 50139 Firenze, Italy
[b] Paediatric Neurology Unit and Laboratories, Neuroscience Department, Meyer Children's Hospital, viale Pieraccini 24, 50139 Firenze, Italy
[c] Center for Inborn Errors of Metabolism, Department of Pediatrics, University of Catania, via S. Sofia 78, 95123 Catania, Italy
[d] Rare Metabolic Diseases Unit, San Gerardo Hospital, via Pergolesi 33, 20900 Monza, Italy

ARTICLE INFO

Article history:
Received 11 September 2015
Received in revised form 26 October 2015
Accepted 26 October 2015
Available online 1 November 2015

Keywords:
MLPA
QFM-PCR
Gene rearrangement
Allele dosage
De novo mutation
Deletion breakpoint

ABSTRACT

MLPA (Multiplex Ligation-dependent Probe Amplification) is a semiquantitative molecular technique developed to uncover gross gene rearrangements in several monogenic diseases, including the X-linked Fabry disease (FD) caused by mutations in the *GLA* gene. Heterozygosity of the X chromosome in females makes it important to combine routine sequencing analysis with at least one allelic dosage assay (i.e. MLPA).
We identified two new gross *GLA* gene rearrangements, which were not properly detected by MLPA in male patients with FD. In Patient 1, routine sequencing did not amplify *GLA* exon 7. MLPA failed to confirm such deletion. An alternative allele dosage, based on the Quantitative Fluorescent Multiplex-PCR (QFM-PCR), confirmed the mutation in the proband and excluded it in the mother, revealing that the mutation was de novo in the proband. Patient 2 harboured a gross *GLA* gene deletion encompassing almost the entire exon 5, the entire intron 5 and part of exon 6. MLPA confirmed the deletion of exon 5 but missed the partial deletion of exon 6. We characterised the breakpoint (c.652_886del452) and carried out screening for possible heterozygosity among at risk female family members.
MLPA can miss some gross *GLA* gene rearrangements making the combination with other allele dosage assays mandatory to confirm or exclude FD at the molecular level.

1. Introduction

Fabry disease (FD; OMIM 301500) is an X-linked lysosomal storage disorder characterised by the accumulation of glycosphingolipids containing terminal α-galactose residues within the lysosomes of many tissues, including kidney, arteries and heart. Fabry disease is caused by total or partial deficiency of the lysosomal α-galactosidase A (α-Gal A; EC 3.2.1.22) enzyme which catalyses the hydrolysis of α-galactosidic linkages of glycosphingolipids, glycoproteins and polysaccharides [1]. α-Galactosidase A is encoded by the *GLA* gene (OMIM 300644, RefSeq X14448), located at position Xq22.1 [2].

The phenotypic expression of FD is highly variable [3–6] and covers a wide clinical spectrum [1,5]. Several forms have been recognized that range from a less severe condition with only cardiac and/or renal abnormalities, to the more classic FD phenotype characterised by chronic pain, vascular degeneration, angiokeratoma, cardiac abnormalities, kidney manifestations leading to renal failure, and other symptoms [1,5]. Demonstration of deficient α-Gal A activity in leukocytes or fibroblasts is commonly used for diagnosis in males and the milder forms of FD usually associates with residual enzyme activity.

Heterozygous females may also develop mild to severe clinical manifestations [1,7]. However, the enzymatic assay is unreliable in females due to random X-chromosome inactivation. Hence, in order to identify affected females it is crucial to combine biochemical assays with molecular analyses of the *GLA* gene.

To date, more than 750 *GLA* disease causing mutations have been identified [1,8], 6% of which are gross *GLA* gene rearrangements leading to single or multi-exon deletions. Routinely PCR-based analyses can easily detect *GLA* deletions in males, due to hemizygosity of the X chromosome, but is unreliable in females due to the wild-type copy of the *GLA* allele.

MLPA (Multiplex Ligation-dependent Probe Amplification) is a semiquantitative molecular technique developed to detect abnormal copy number variations escaping detection by routine sequencing of exon-based PCR amplifications. Although not certified for diagnostic purposes, MLPA has been set up to confirm or exclude gross gene rearrangements in several monogenic diseases, including FD.

* Corresponding author at: Molecular and Cell Biology Laboratory, Paediatric Neurology Unit and Laboratories, Meyer Children's Hospital, viale Pieraccini n. 24, 50139 Firenze, Italy.
E-mail address: a.morrone@meyer.it (A. Morrone).

http://dx.doi.org/10.1016/j.cca.2015.10.027
0009-8981/

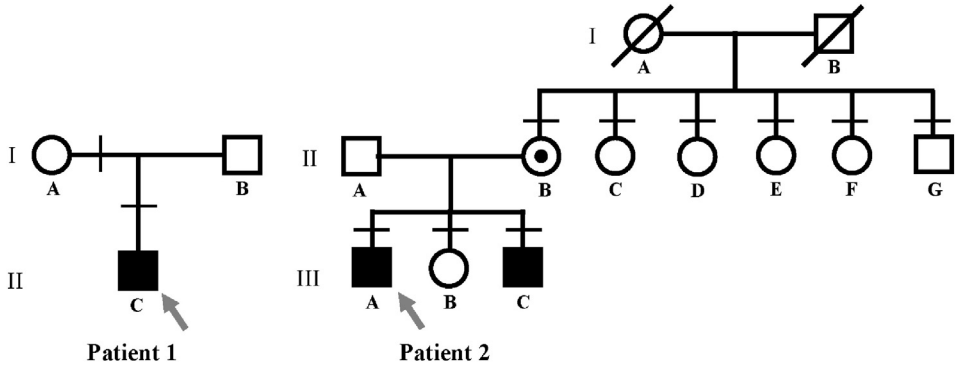

Fig. 1. Pedigree charts of Fabry patients. The arrow indicates the probands. Dashes indicate individuals subjected to molecular analysis.

Heterozygosity of the X chromosome in females makes it important to combine routine sequencing analysis with allelic dosage assays, such as MLPA, to more reliably exclude or confirm FD [9–14].

Two additional allelic dosage assays have been developed for the *GLA* gene, over MLPA. One is an adaptation of the Quantitative Fluorescent Multiplex PCR (QFM-PCR) strategy [13] which is based on the use of a mixture of 6-FAM-labelled primers designed to amplify each *GLA* exon. QFM-PCR requires capillary electrophoresis to read the signals. A second molecular tool for *GLA* gene dosage has been developed by adapting the Real-time PCR technique [10].

Here we report two new gross *GLA* gene rearrangements resulting in exonic deletions, which were not properly detected by MLPA in symptomatic male patients with Fabry disease.

2. Materials and methods

2.1. Patients and controls

Whole blood DNA samples from patients with Fabry disease and their relatives were examined after informed consent was obtained for all individuals, in accordance with local ethical committee recommendations. The family pedigree is shown in Fig. 1; the clinical presentation of the two patients analysed is summarized as follows:

Patient 1 is a 21 year old man with classic Fabry disease who was diagnosed when 12 year old (α-Gal A on leukocytes was 0.33 nmol/mg protein/h; normal range: 2–7 nmol/mg protein/h). Since the age of 8 years he complained of burning pain of fingers and toes, mainly during fever, responding to paracetamol or ibuprofen. ERT was soon introduced, after he also started complaining of sporadic abdominal pain. After starting ERT with agalsidase beta, typical episodes of pain still persisted for several months to subsequently become less frequent. In 2011, due to the shortage of agalsidase beta, he started treatment with agalsidase alpha. He currently exhibits sporadic abdominal and hand and feet pain and tinnitus. No heart or eye involvement has appeared, but ecocholor Doppler of spermatic vases showed bilaterally renospermatic reflux during Valsalva manoeuvre. Scrotal echography demonstrated mild ectasia of venous plexus.

Patient 2 is a 21 year man with classic Fabry disease in whom diagnosis was made at the age of 16 because of angiokeratoma (α-Gal A on leukocytes was 0 nmol/mg protein/h; normal range: 20–65 nmol/mg protein/h). During infancy he complained of occasional acroparesthesia mainly in warm and cold environments and had many episodes of fever sine causa. At the time of diagnosis he had normal kidney and heart functions with the exception of bradycardia (50/min). Brain MRI was normal. ERT was started with agalsidase alpha immediately after diagnosis. After about 5 years of ERT he developed high titre IgG antibodies against alpha-galactosidase, vital organs functions was unchanged and pain was reduced compared to 5 years earlier.

2.2. Analysis of genomic DNA

Genomic DNA was isolated using the EZ1 DNA Blood 350 μl Kit (Qiagen, Hilden, Germany). The entire coding region with intron–exon boundaries and a region of the intron IV which includes the c.936 + 861C>T [15] and c.936 + 919G>A [16] deep intronic mutations were amplified and sequenced using the oligonucleotides listed in Table 1. PCR mixtures were prepared in a 25 μl reaction mixture containing 1 U of AmpliTaq Gold polymerase (Roche, Basel, Switzerland), 250 μM each of dNTPs, 30 pmol of each forward and reverse primer, 1× PCR buffer and 1.5 mM of $MgCl_2$. After a primary denaturation for 2 min at 95 °C, amplification was carried out for 30 cycles consisting of 15 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, with a final extension of 7 min at 72 °C. PCR products were assessed by 2% agarose gel electrophoresis and purified by mixing 5 μl of the PCR product with 2 μl of ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and incubating for 30 min at 37 °C, followed by additional 15 min at 80 °C.

Sequencing PCR reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and purifications were carried out using Sephadex G-50 Fine (GE Healthcare, Little Chalfont, UK). Capillary electrophoresis was performed using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems) as recommended by the manufacturer. GenBank: X14448 was used as reference sequence of the human *GLA* gene nucleotide sequence [17].

2.3. Multiplex PCR analysis

Multiplex PCR analyses were performed using the primer pairs listed in Table 1. Different combinations of PCR fragments were tested in order to confirm multi-exon deletions in the *GLA* genes of Pt1 and Pt2. PCR fragment combinations GLAex4 + GLAex5 + GLAex7 and GLAex4 + GLAex6 + GLAex7 were successfully assayed. PCR mixture was prepared as described above in Section 2.2. After a primary denaturation for 5 min at 95 °C, amplification was carried out for 30 cycles consisting of 30 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C, with a final extension of 7 min at 72 °C. Multiplexed PCR products were checked by agarose gel electrophoresis using 2% Agarose Low Melting (Eurobio, Les Ulis, France) + 1% Standard Agarose (AB Analitica, Padova, Italy).

2.4. Analyses of the breakpoint junctions of gross GLA rearrangements

For the analysis of the breakpoint junctions in Pt1 we performed the following experiments. We designed the reverse primer GLAEx7-R (5′-ACTGATAGTAACATCAAGAGC-3′), which anneals in the same annealing site of the MLPA probe specific for *GLA* exon 7. We coupled this primer with primer GLA7F (Table 1) in a PCR reaction to confirm that part of exon 7 was present in the Pt1's DNA. We designed a second primer