Research Report

Reviewing Large LAMA2 Deletions and Duplications in Congenital Muscular Dystrophy Patients

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Abstract.
Background: Congenital muscular dystrophy (CMD) type 1A (MDC1A) is caused by recessive mutations in laminin-α2 (*LAMA2*) gene. Laminin-211, a heterotrimeric glycoprotein that contains the α2 chain, is crucial for muscle stability establishing a bond between the sarcolemma and the extracellular matrix. More than 215 mutations are listed in the *locus* specific database (LSDB) for *LAMA2* gene (May 2014).
Objective: A limited number of large deletions/duplications have been reported in *LAMA2*. Our main objective was the identification of additional large rearrangements in *LAMA2* found in CMD patients and a systematic review of cases in the literature and LSDB.
Methods: In four of the fifty-two patients studied over the last 10 years, only one heterozygous mutation was identified, after sequencing and screening for a frequent *LAMA2* deletion. Initial screening of large mutations was performed by multiplex ligation-dependent probe application (MLPA). Further characterization implied several techniques: long-range PCR, cDNA and Southern-blot analysis.
Results: Three novel large deletions in *LAMA2* and the first pathogenic large duplication were successfully identified, allowing a definitive molecular diagnosis, carrier screening and prenatal diagnosis. A total of fifteen deletions and two duplications previously reported were also reviewed. Two possible mutational "hotspots" for deletions may exist, the first encompassing exons 3 and 4 and second in the 3' region (exons 56 to 65) of *LAMA2*.

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Conclusions: Our findings show that this type of mutation is fairly frequent (18.4% of mutated alleles) and is underestimated in the literature. It is important to include the screening of large deletions/duplications as part of the genetic diagnosis strategy.

Keywords: LAMA2, congenital muscular dystrophy, large deletion, large duplication, review

INTRODUCTION

LAMA2-related dystrophy (LAMA2-RD) collectively gathers two distinct clinical entities: the classical phenotype with congenital onset known as MDC1A, and a milder limb-girdle type muscular dystrophy with onset during childhood (late-onset or "ambulant" LAMA2-RD) [reviewed by 1, 2]. As foreseeable by this designation, these entities are caused by recessive mutations in *LAMA2* gene located on chromosome 6q22-23 and spanning 65 exons [3, 4]. This gene codes for the α2 chain of laminin-211, an extracellular glycoprotein expressed in the basal membrane of striated muscles, peripheral nerves, brain and trophoblast [5–7]. The interaction of laminin-211 with cell-surface receptors such as α-dystroglycan and integrin (mainly α7β1 in adult skeletal muscle) explains its major relevance in the overall extracellular architecture, integrity and cell adhesion [reviewed by 8].

MDC1A represents the most frequent form of CMD in western countries, accounting for 30 to 50% of cases [9]. Typical clinical features includes severe hypotonia associated with muscle weakness manifesting at birth or during early infancy, proximal joint contractures, elevated creatine kinase (CK) levels, cerebral white matter abnormalities and delayed motor milestones with affected children usually not achieving independent ambulation [10–12]. Feeding problems and respiratory insufficiency are commonly reported complications often requiring gastrostomy and/or artificial ventilator support [13, 14].

Other features such as cardiac involvement, a sensory and motor demyelinating neuropathy, epilepsy and mental retardation have been also documented in some forms of LAMA2-RD [15–19].

An important diagnostic aspect is that skeletal muscle biopsies from these patients have changes in laminin-α2 expression detected by immunohistochemical (IHC) analysis [10]. However, there is a recent report of a muscular dystrophy patient with apparently normal laminin-α2 IHC expression and having mutations in *LAMA2* gene [20].

Milder LAMA2-RD cases have been reported in the past few years expanding the phenotypic spectrum of the disease [21]. These patients have slower disease progression and acquire independent locomotion, and are usually associated with a partial expression of laminin-α2 [22–26].

More than 375 distinct sequence variants (215 of them with known clinical relevance) have been reported in the LSBD for *LAMA2* gene (http://www.dmd.nl/LAMA2, data accessed May 2014). Pathogenic changes include small deletions/insertions (34.9%), nonsense mutations (25.1%), changes affecting splicing (25.6%) and also missense substitutions (12.1%). In spite of the relevant amount of mutational data available there is still a limited number (2.3%) of large deletions and duplications reported in this gene. The initial suspicion of a large *LAMA2* deletion (which was predicted to include exons 23 to 56) was identified by protein truncation test in the work of Pegoraro and collaborators [27]. The first fully characterized large deletion in *LAMA2*, corresponds to an out-of-frame deletion of exon 56 (c.7750-1713_7899-2154del), which has been proven to be one of the most frequent pathogenic variants detected in Portuguese MDC1A patients [28].

One of the main objectives of this work was to describe additional novel pathogenic deletions and duplications associated with the *LAMA2* gene, identified in our cohort of CMD patients. Moreover, a systematic review of all cases with large deletions/duplications reported in the literature and mutation databases is presented. Our findings showed that this type of mutation is fairly frequent and is underestimated in the literature, reinforcing the importance to screen large deletions/duplications in *LAMA2* gene as part of the genetic diagnosis strategy.

MATERIAL AND METHODS

Patients

Over the last 10 years (2004-2014) our group performed genetic studies in 94 CMD patients. Mutations in genes related with CMD were identified in 68% (n = 64) of these patients. The majority of these patients have *LAMA2* mutations (n = 52) and were referred for molecular studies due to changes in muscle laminin-α2 detected by IHC analysis and/or compatible white matter anomalies detected by magnetic resonance imaging (MRI). In four patients of this cohort only

Table 1
Clinical data of CMD patients with novel large deletions and duplications in *LAMA2*

Patient	Sex	Age*	Age of onset	Clinical presentation	Highest CK (IU/l)	Pattern/ progression of weakness	Best motor achievement	Contractures	Central nervous system involvement/ seizures	Magnetic resonance imaging	Laminin-α2 in muscle
P1 (P19 in [28])	M	20 yr	At birth	Generalized hypotonia and areflexia	3264	Muscular weakness with axial and proximal predominance and scoliosis	Independent ambulation	Elbows and ankles	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	nd
P2 (P24 in [28])	M	3 yr	At birth	Hypotonia and feeding problems	1770	Muscular weakness with proximal predominance and hip congenital luxation	Cephalic control and assisted trunk control	Knees	No cognitive delay and no seizures	White matter changes and no gyral abnormalities	Total absence
P3	M	2 yr	4 mo	Hypotonia and muscular weakness	7644	Muscular weakness with axial and proximal UL predominance	Cephalic control and assisted trunk control	Knees, ankles and rigid spine	No cognitive delay and no seizures	White matter changes	nd
P4	M	8 mo	At birth	Generalized hypotonia	4400	Muscular weakness with proximal UL predominance	Cephalic and trunk control	Discrete equinus	No cognitive delay and no seizures	nd	Partial deficiency

CK-creatine phosphokinase; M- male; mo- months; nd- not determined; P- patient; UL- upper limbs; yr- years; *- age at last clinical follow-up.

one heterozygous mutation was detected upon *LAMA2* genomic sequencing (described in Table 1). In these patients we conducted screening of large deletions and duplications in the *LAMA2* gene. This research was approved by the ethics committee from Hospital Centre of Porto (CHP).

LAMA2 gene analysis

Genomic DNA (gDNA) was obtained from peripheral blood using the salting-out method [29]. *LAMA*2 gene sequencing was done according to [28], which comprised all coding and adjacent intronic sequences of *LAMA*2. Variants were described according to the Human Genome Variation Society (HGVS) guidelines for mutation nomenclature (version 2.0) [30] and using the cDNA reference sequence with accession number NM_000426.3.

MLPA analysis

Screening for deletions and duplications in *LAMA2* gene was performed by multiplex ligation-dependent probe application (MLPA) technique using two sets of probe mixes (P391-A1 and P392-A1) from MRC-Holland (Amsterdam, the Netherlands). These probe mixes contain one probe for each exon of the gene with the exception of exons 18, 44 and 48. Two probes are present for exon 1, 2, 4 and 65 and three probes for exon 56. Also, probemix P391 contains 9 reference probes and P392 contains 8 reference probes detecting different genomic regions. For the MLPA procedure, 150 ng gDNA was used for each patient and normal control samples. Amplification products were subsequently separated by capillary electrophoresis on an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, CA). Data analysis was conducted using GeneMarker Software V1.5 (SoftGenetics LLC, State College, PA). Population normalization method was selected and data was plotted using probe ratio.

Southern blot

gDNA samples from patient P3, respective parents and normal controls were digested with *BglI* (NewEngland Biolabs, Beverly, MA), resolved on a 0.7% agarose gel and vacuum transferred to a GeneScreen Plus membrane (Perkin Elmer, Waltham, MA) using a saline method. A cDNA probe recognizing exons 2–4 was prepared using digoxigenin (DIG) DNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA) and incubated overnight using the Easy Hyb Buffer (Roche Applied Science). The membrane was washed

at 60°C in 1xSSC (Saline-Sodium Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) (Sigma-Aldrich, St. Louis, MO) and twice in 0.5xSSC/0.1% SDS for 15 min each. Subsequently, the membrane was prepared with DIG Wash and Block Buffer Set (Roche Applied Science), incubated with Anti-DIG-AP conjugate (Roche Applied Science), and the DIG-labeled probe detected with ready-to-use CDP-Star (Roche Applied Science).

LAMA2 cDNA analysis

cDNA studies were carried out in patient P2. Total RNA was extracted from patient and control muscle biopsy samples using the PerfectPure RNA Fibrous Tissue kit (5 PRIME, Germany) and converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). *LAMA2* transcripts were subjected to PCR amplification using specific primers for the region corresponding to exons 27–32 (27F- 5′AAATTTCATGCGACAAAGCAGG; 32R-5′GCTTGCAGCCGTCACACTTC). Resulting PCR products were purified and sequenced as described before.

Long-range PCR

The deletion breakpoints encompassing exon 3 (patient P3) and also exon 17 (patient P1) were determined by amplification of gDNA using the BIO-X-ACT Long DNA Polymerase kit (Bioline, Taunton, MA). Specific primers were designed for each case; complementary to intron 2 and exon 4, for the deletion of exon 3 (2i-F:5′ACAAAGCCTGATGGAGG GAAAC; 4-R:5′AAAGCGTTAGGCACTCCGTGTC) and complementary to regions in exons 16 and 18, for the deletion of exon 17 (16F:5′-TTGGTCATG CGGAGTCCTG; 18R:5′ TGGCACGTTGGGC-TAAAGC). Resolved PCR fragments were purified using the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA), and subsequently sequenced.

RESULTS

Novel large deletions and duplications

We previously reported a large deletion encompassing exon 56 of the *LAMA2* gene which is relatively frequent in our laboratory patient cohort (present in 23% of our cases) [28]. This initial study suggested that it is clinically relevant to screen this type of mutations in CMD patients, and indirectly drove the development of a MLPA commercial kit for *LAMA2* gene (P391-A1

and P392-A1 from MRC-Holland). As part of this work we initially assessed the effectiveness of the MLPA kit using previously genotyped DNA samples from our patient cohort. Patients presenting homozygous and heterozygous deletions encompassing *LAMA2* exon 56 were tested. MLPA technique successfully detected this mutation in homo- and heterozygous states (Supplementary data I).

Four patients are presented in detail in this work; P1 and P2 were previously described in the literature as P19 and P24, respectively in [28] while two additional patients (P3 and P4) were more recently referred for *LAMA2* gene analysis. All of these patients had incomplete molecular characterization: only one heterozygous mutation was detected by genomic sequencing and thus the molecular defect in the other *LAMA2* allele remained unknown. MLPA technique was performed in DNA samples from these patients to tentatively identify the second pathogenic mutation in *LAMA2*.

Patient P1 presents a congenital muscular dystrophy (neonatal onset) and remained ambulatory until the age of 17 years. In this patient a heterozygous codon deletion (c.1798_1800del, p.Gly600del) was initially detected in *LAMA2*. MLPA analysis for this patient revealed reduced hybridization of one probe corresponding to exon 17 (Fig. 1A), compatible with the presence of a heterozygous deletion involving this exon. By reviewing genomic sequencing data we excluded a potential sequence change which could compromise the affinity of the MLPA probe. A long-range PCR experiment was performed using primers designed to bind regions presumably not involved in the deletion (exons 16 and 18). Upon amplification, two PCR products were detected in the patient, whereas the experimental control had a single band (data not shown). Sequencing across the deletion breakpoint revealed that part of intron 16 is joined to intron 17, corresponding to the loss of ∼5.3Kb that spans exon 17 (Fig. 1B). This novel large mutation, c.2322+259_2450+2037del, predictably causes frameshifting. DNA samples from the patient's parents were unavailable for study.

Patient P2 was referred for molecular study at 3 years of age having a typical MDC1A phenotype with absence of laminin-α2 in IHC analysis of muscle. The heterozygous nonsense mutation c.3085C>T (p.Arg1029*) was the only pathogenic sequence change detected by sequencing the *LAMA2* gene. A large heterozygous duplication encompassing exons 28 and 29 was identified in patient P2 by MLPA (Fig. 2A). cDNA studies performed in the

Patient P1

A- MLPA analysis
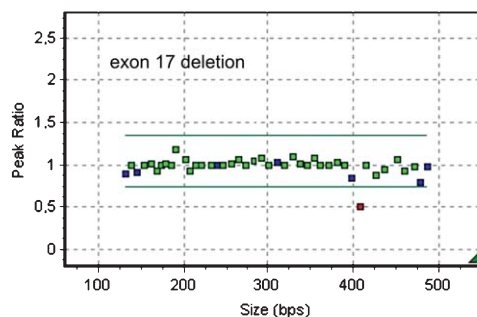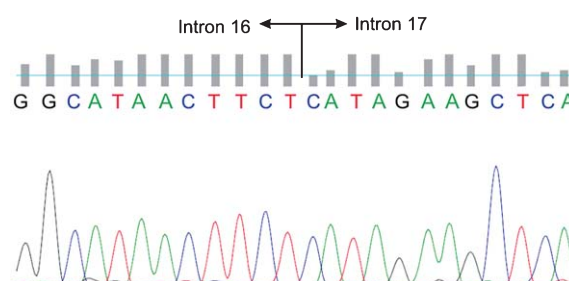

B- Breakpoint sequencing

Fig. 1. Results obtained for patient P1. (A) MLPA technique showing reduction of one probe (0.5 peak ratio) corresponding to exon 17 of the *LAMA2* gene. (B) Sequencing electropherogram of a fragment (obtained from long-range PCR) that enabled the identification of the deletion breakpoint, corresponding to the loss of ∼5.3 Kb.

patient revealed a normal transcript together with other abnormal PCR products (Fig. 2B). These include one out-of-frame transcript resulting from the contiguous duplication of exons 28 and 29 (Fig. 2C).

Patient P3 with an MDC1A phenotype has a novel nonsense mutation in exon 4 (c.497G>A, p.Trp166*) detected in a heterozygous and apparently homozygous state, depending on the primer-pair used to study this region (Fig. 3A). These ambiguous results led us to suspect a possible deletion comprising at least part of intron 3. The application of MLPA confirmed this assumption, since a reduced amplification signal was observed for the exon 3-specific probe (Fig. 3B). For further characterization, and since no RNA was available for study, we performed Southern-blotting and hybridization using a cDNA probe that recognizes exons 1 to 4. This experiment suggested that the genomic deletion originates a new fragment of approximately 6 Kb in the patient, that is absent in the control (Fig. 3C). To delineate the deletion endpoints several primers were tested to perform a deletion-specific PCR. A 42931 bp deletion combined

Patient P2

A- MLPA analysis

B- cDNA analysis
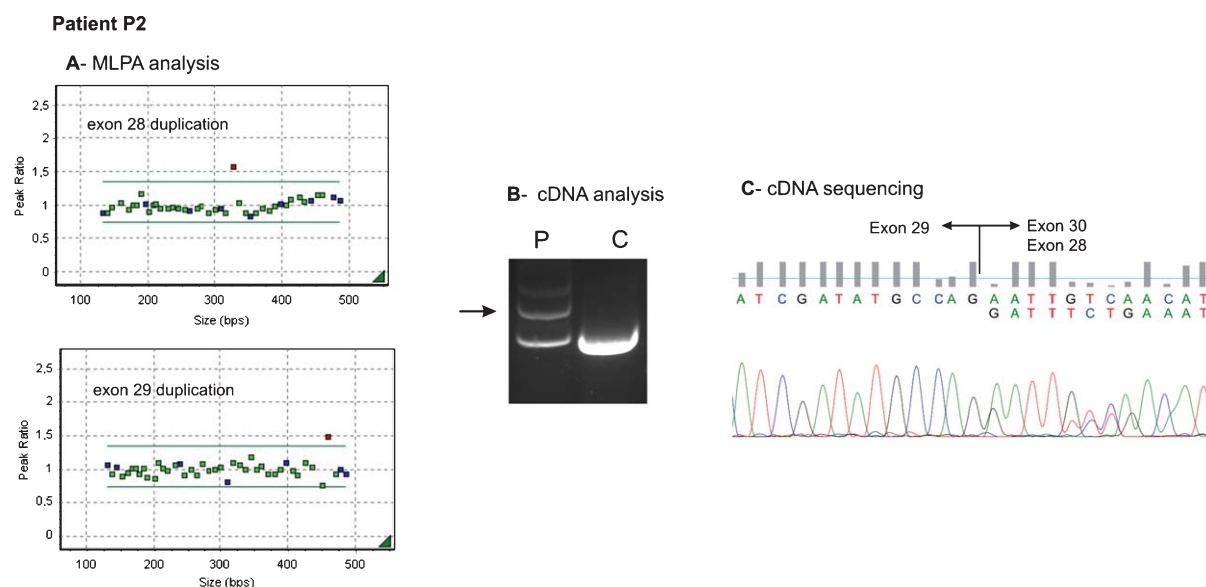
C- cDNA sequencing

Fig. 2. Duplication identified in patient P2. (A) Increased signal for MLPA probes (peak ratios around 1.5) recognizing exon 28 (upper panel) and exon 29 (lower panel). (B) cDNA analysis of *LAMA2* transcripts revealed the presence of abnormal PCR products in the patient that were not detected in the control. (C) Sequencing electropherogram of the cDNA PCR product (indicated by arrow) reveals the adjacent duplication of exons 28 and 29. C- control; P- patient.

with the insertion of three nucleotides was identified, annotated as c.284-4685_397-146delinsATA (Fig. 3D). Compound heterozygosity for these two mutations was confirmed by the analysis of patient's parents.

Lastly, patient P4 has also an MDC1A phenotype but partial laminin-α2 absence in muscle. Besides one heterozygous 8 bp duplication in exon 13 (c.1854_1861dup) previously reported in the literature [9], we were able to identify a large heterozygous deletion involving exons 3 to 10 by MLPA (Fig. 4). This deletion is predicated to be out-of-frame. A more extensive characterization was not possible since no RNA sample was available for study. Compound heterozygosity was verified since each parent carried a different mutation.

Novel point mutations in LAMA2

Fifty-two patients with *LAMA2* mutations have been characterized until now: 26 of which were previously reported in 2008, and another two more recently in a publication describing their atypical phenotype associated with novel missense mutations [19]. In all cases mutations have been identified in both disease alleles. A list of 10 novel mutations is shown in supplementary data II. These include four nonsense mutations (c.3520C>T, c.5263A>T, c.6501C>G e c.6979G>T), four changes affecting splice sites (c.396+1G>T, c.2450+4A>G, c.6708-1G>T and c.8988+1G>A), one

single nucleotide duplication (c.2350dupT) and a missense mutation (c.3235T>G, p.Cys1079Gly).

Reviewing large deletions and duplications in LAMA2

As part of this work we reviewed all large deletions and duplications reported in the literature or in the *locus* specific database for the *LAMA2* gene (http://www.lovd.nl/LAMA2, information last accessed in May 2014). In addition to the mutations presented in this publication, 12 different large deletions and a single duplication were previously described (Table 2).

LAMA2 large deletions ($n = 15$, reported in 35 patients) are apparently dispersed throughout the gene, but two possible mutational "hotspots" may exist: i) one includes exons 3 and 4 (5 different deletions) and ii) in the 3' region of the gene (from exons 56 to 65). The largest *LAMA2* deletion, encompassing exons 23 to 56, was detected by the protein truncation test [27] and comprehends more than half of the gene's coding regions. At least six different deletions affect single exons and were confirmed by a second technique in order to exclude false positive results. The majority of deletions are predicted to cause frameshifting (out-of-frame deletions). Still, only two cases were further characterized at the cDNA level which limits the accuracy of this data. Considering deletions

Patient P3

A- gDNA sequencing

c.497G>A
Hom.

c.497G>A
Het.

C- Southern Blot

B- MLPA analysis
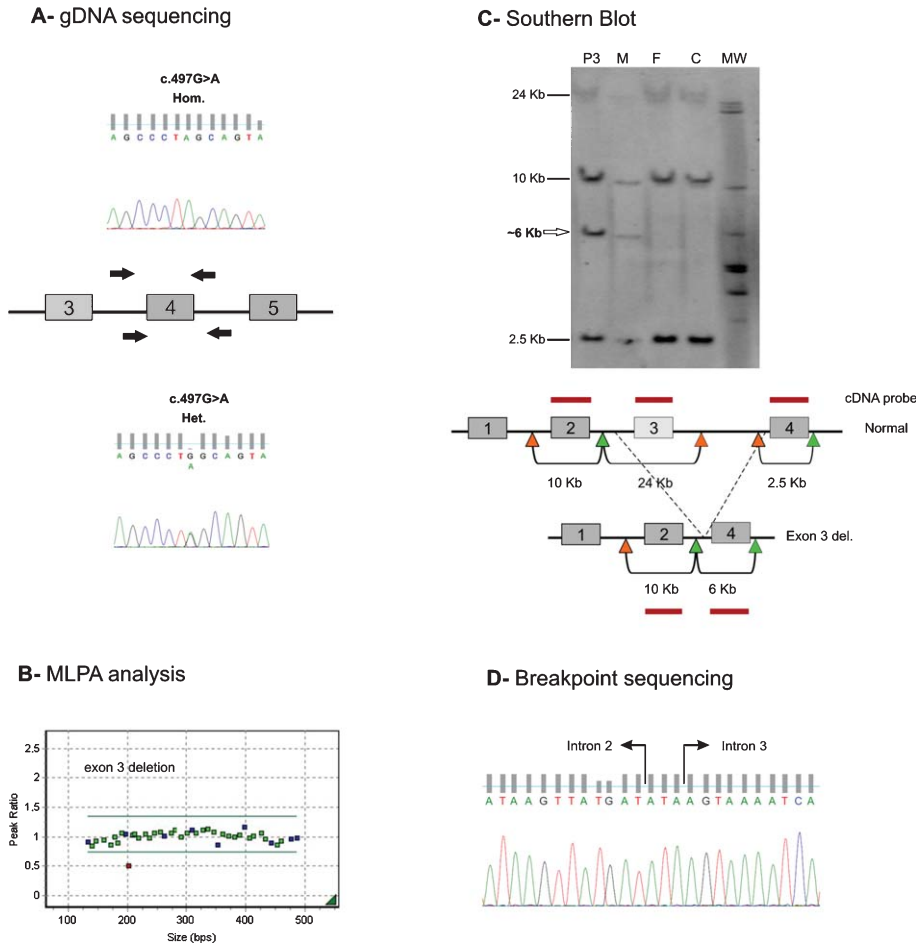

D- Breakpoint sequencing

Fig. 3. Characterization of a heterozygous deletion in patient P3. (A) The initial suspicion of a heterozygous deletion encompassing intron 3 derived from the genomic sequencing data. The nonsense mutation c.497G>A located in exon 4 was detected both in heterozygosity and homozygosity depending upon the primers used. (B) Confirmation by MLPA, with reduced amplification signal for the exon 3 probe. (C) Southern-blot followed by hybridization using a cDNA probe that recognizes exons 1 to 4, revealing a 6 Kb fragment in the patient and in his mother, but not in the father nor the control. (D) Deletion breakpoint identified by deletion-specific PCR followed by sequencing. Sequencing electropherogram revealed a 42.9 Kb deletion combined with the insertion of three nucleotides. C- control; F- father; M- mother; MW- molecular weight marker; P3- patient 3.

predicted to be in-frame, all except one [31] were reported in combination with a second truncating mutation (causing a frame-shift or a nonsense mutation). All of these patients have typical MDC1A phenotypes, except the patient with the deletion of exons 41 to 48, which remains ambulant at the age of 10 years (patient #6, reported by [32]). Six patients have been described with large homozygous deletions which may be explained by a higher frequency of a particular mutation within the population (deletion of exon 56 in Portugal and exon 4 deletion in Chinese patients) or due to consanguinity in individual sporadic cases. Patients with homozygous deletions are usually detected by

genomic sequencing, since the affected regions will fail to amplify during PCR. Until now, no patients have been reported with compound heterozygosity between two different large rearrangements.

LAMA2 duplications are even rarer mutational events; besides our report of a novel duplication encompassing exons 28 and 29 detected in patient P2, only one other heterozygous in-frame duplication involving exons 5 to 12 has been documented in a patient presenting muscular dystrophy [31]. However, these authors did not identify a second mutation in the patient, which might have explained an autosomal recessive LAMA2-RD.

Patient P4

Deletion of exons 4, 7, 9

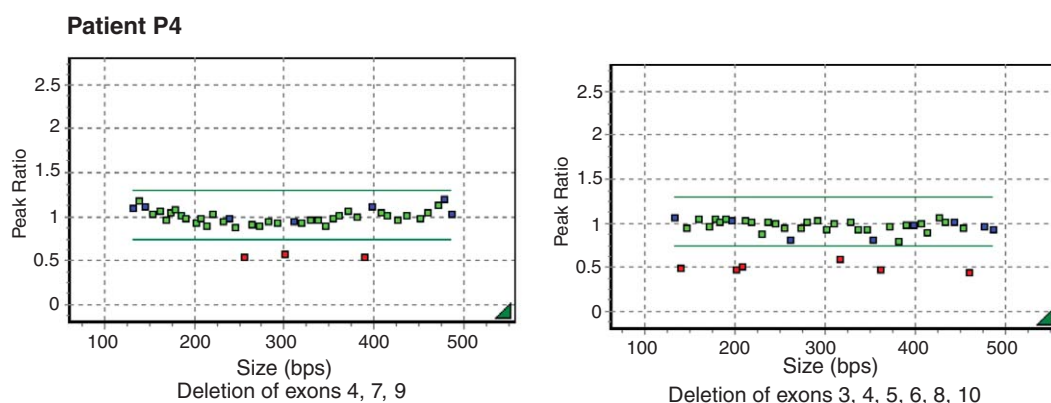Deletion of exons 3, 4, 5, 6, 8, 10

Fig. 4. MLPA results for patient 4. Several *LAMA2* probes with decreased peak ratios (0.5), corresponding to exons 3 to 10, compatible with a large heterozygous deletion.

The frequency of large deletions and duplications in *LAMA2* may be estimated based on the two largest patient cohorts reported and that employed quantitative techniques: 18/104 alleles from our patient cohort of 52 patients and 17/86 alleles from the recent work of Xiong and collaborators that studied 43 patients [32]. The overall frequency of these mutations is thus around 18.4% (35/190 alleles).

DISCUSSION

This work describes the detailed genetic characterization of four patients with compatible features with a MDC1A phenotype, but with only one heterozygous pathogenic sequence variant detected upon complete *LAMA2* sequencing. Three novel large deletions in the *LAMA2* gene and the first pathogenic large duplication were successfully identified in this group of patients, allowing a definitive molecular diagnosis, carrier screening and prenatal diagnosis. Characterization of these mutations implied the use of a variety of techniques such as long-range PCR, cDNA and Southern-blot analysis. These methods are not generally used in the routine genetic diagnosis of this disease, but are essential to obtain accurate genotype-phenotype correlations.

Up to now reports of large deletions and duplications in *LAMA2* are very rare; only three publications have referred this type of mutation [28, 31, 32]. The work of the Italian group included a more heterogeneous patient cohort and a broader technical approach. An array-based comparative genomic hybridization (array-CGH) developed to screen genes implicated in neuromuscular diseases, enabled the identification of several novel copy number variants (CNVs) includ-

ing two present in the *LAMA2* gene [31]. Based on the data from a total of 95 fully genotyped LAMA2-RD patients, from two large cohorts, we estimate that the frequency of large deletions and duplications in *LAMA2* may be as high as 18.4%. Considering this relatively high frequency, it is important to include screening techniques such as MLPA or array-CGH in the molecular diagnostic work-up. Here, laboratories should consider the variety of equipments required, running costs and sensitivity of these two approaches to screen this type of rearrangement. The presence of a single heterozygous large deletion or duplication, especially when in-frame, should be carefully evaluated. It is conceivable that the presence of a non-pathogenic CNV in a CMD patient may not necessarily explain the clinical phenotype.

Readers should also be aware that genomic sequencing is the technique with the highest sensitivity to screen for *LAMA2* mutations (>80%), especially in CMD cases with laminin-α2 deficiency. Our current strategy for *LAMA2* genetic analysis is sub-divided in three tiers: i) the first level comprising a selected number of exons (namely: 3, 13, 22, 27, 33, 36, 54, 58, and 61) corresponding to the genomic regions where the majority of point mutations in our population are located, together with the screening of exon 56 deletion; ii) the second tier includes the remaining *LAMA2* exons; and finally iii) MLPA analysis (two panels). Until now, seventeen patients have been analyzed in this manner. In 35% of patients both mutated alleles were identified using tier 1, and in 82% at least one heterozygous mutation was detected. We consider feasible in our population to screen these *LAMA2* regions in patients with compatible features of CMD (such as white matter changes in brain MRI), even before performing a muscle biopsy.

Table 2
Review of large deletions and duplications reported in the *LAMA2* gene

Affected gene regions (exons)	Nr. of affected exons	Mutation description gDNA	RNA	Impact on reading frame (prediction)	Nr. of patients reported	Zygosity // other mutation type	Phenotype	References
Deletions								
2–3	2	c.113-?_396+?del	r.(del)	OF	2	het. (n = 2) // splice-site mutation	MDC1A; MDC1A (ambulant at 5yr)	[32]
3	1	c.284-4685_397-146delinsATA	r.(del)	OF	1	het. // nonsense mutation	MDC1A	P3, this paper
3–4	2	c.284-?_639+?del	r.(del)	OF	1	hom.	MDC1A	[32]
3–10	8	c.284-?_1467+?del	r.(del)	OF	1	het. // 8 bp OF duplication	MDC1A	P4, this paper
4	1	c.397-?_639+?del	r.397_639del	IF	5	hom. (n = 2), het. (n = 3) // several	MDC1A	[32]
5	1	c.640-?_819+?del	r.(del)	IF	2	het. (n = 2) // 2 bp OF deletion	MDC1A	[32]
10–12	3	c.1307-?_1782+?del	r.(del)	OF	1	het. // 4 bp OF duplication	MDC1A	[32]
13–37	15	c.1783-19594_5445+1681del	r.(del)	IF	1	het. // unknown	myopathy	[31] Db
17	1	c.2322+259_2450+2037del	r.(del)	OF	1	het. // 3 bp IF deletion	MDC1A (ambulant at 17yr)	P1, this paper P19, [28]
23–56	34	c.(?_3175)_(7898_?)del	r.(del)	OF	1	het. // 1 bp OF deletion	MDC1A	[27]
41–48	8	c.5866-?_6867+?del	r.(del)	IF	1	het. // 4 bp OF duplication	MDC1A	[32]
56	1	c.7750-1713_7899-2154del	r.7750_7898del	OF	14	hom. (n = 2), het. (n = 12) // several	MDC1A; LAMA2-related MD	[28] Db
57–3UTR	9	c.7899-?_(*219_?)del	r.(?)	?	2	hom. (n = 2)	MDC1A	Db
59–63	5	c.8245-?_8988+?del	r.(del)	IF	1	het. // nonsense mutation	MDC1A	[32]
63	1	c.8858-?_8988+?del	r.(del)	OF	1	het. // nonsense mutation	MDC1A	[32]
Duplications								
5–12	8	c.640-?_1782+?dup	r.(dup)	IF	1	het. // unknown	muscular dystrophy	[31] Db
28–29	2	c.4059-?_4311+?dup	r.4059_4311dup	OF	1	het. // nonsense mutation	MDC1A	P2, this paper P24, [28]

Mutations described according to HGVS nomenclature using cDNA reference sequence with accession number NM_000426.3. bp- base pairs; Db- *locus*-specific mutation database for *LAMA2* gene (http://www.lovd.nl/LAMA2); het.- heterozygous; hom.- homozygous; IF- in-frame; OF- out-of-frame; MD- muscular dystrophy; Nr.- number; yr- years.

Large deletions and duplications detected in CMD patients are not confined to *LAMA2* gene; in fact we have recently reported a patient with a Fukuyama CMD caused by a multi-exonic duplication in *FKTN* (fukutin) [33]. There are additional reports of other pathogenic CNVs in CMD genes, such as: *ISPD* (isoprenoid synthase domain containing) [34], *LARGE* (like-glycosyltransferase) [35, 36] and *POMGNT1* [protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-)] [37].

New mutation screening methods are currently being developed based on next-generation sequencing (NGS) technology, which will contribute to establish the genetic causes of hereditary myopathies that remain unsolved. However, prior to its application, it is important to exclude large deletions and duplications as a cause of these diseases. Bioinformatic pipelines for NGS usually do not incorporate algorithms that enable their automatic detection, but we have previously shown that, when properly applied, this technology can help delineate large genomic rearrangements [38].

In summary, we have reassessed the impact of large deletions and duplications in LAMA2-RD and emphasize the importance of including screening for these rearrangements as part of the diagnostic strategy, especially in patients where a single heterozygous mutation has been detected.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

SUPPLEMENTARY MATERIAL

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