DE GRUYTER OPEN

Research article

DOI: 10.2478/rrlm-2014-0038

Identification of exonic copy number variations in dystrophin gene using MLPA

Identificarea variaţiilor numărului de copii în gena distrofinei folosind metoda MLPA

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Abstract

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked progressive muscle disorders determined by mutations of the dystrophin (DMD) gene. Multiplex Ligation - Dependent Probe Amplification (MLPA) is a simple, inexpensive and reliable test for molecular diagnosis of DMD gene mutations. It identifies exonic copy number variations in the DMD gene, but the test should be completed with sequencing analysis in case of single exon deletions/duplications. The aim of this study was to evaluate the efficiency of MLPA as a DMD mutation screening tool in affected males and carrier females, as well as to appreciate the frequency of different types of mutations and to check the validity of the "reading frame rule". We have used MLPA for the detection of deletions/duplications in DMD gene in 53 individuals (30 affected males and 23 asymptomatic female relatives) referred for evaluation and genetic counseling due to the clinical suspicion of DMD/BMD. In the affected males (21 DMD and 9 BMD) MLPA had a detection rate of 63.5% (53.5% deletions and 10% duplications). The most frequently deleted exon was exon 45 and the most frequent duplication involved exons 3-5, confirming the presence of the two hotspot mutation regions reported in the literature. Mutations detected in our study have a slightly different location compared to literature data. Reading frame rule was valid in 84% of our cases.

***Keywords**: Duchenne and Becker muscular dystrophies;dystrophin;MLPA;deletions/duplications*

Rezumat

Distrofiile musculare Duchenne şi Becker (DMD/BMD) sunt boli musculare progresive legate de X determinate de mutaţii în gena distrofinei (DMD). Multiplex Ligation - Dependent Probe Amplification (MLPA) este o metodă simplă, necostisitoare şi precisă pentru diagnosticul molecular al mutaţiilor genei DMD. Ea identifică variaţiile numărului de copii în gena distrofinei, dar trebuie completată cu secvenţierea genei în cazul identificării deleţiilor/duplicaţiilor unui singur exon. Scopul acestui studiu a fost de a evalua eficienţa MLPA ca test de screening al mutaţiilor în gena DMD la indivizii afectaţi şi femeile purtătoare, dar şi de a aprecia frecvenţa diferitelor tipuri de

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mutaţii şi de a verifica valabilitatea "regulii cadrului de lectură". Am folosit MLPA pentru detectarea deleţiilor/duplicaţiilor în gena DMD la 53 indivizi (30 băieţi afectaţi şi 23 rude asimptomatice de sex feminin) trimişi pentru evaluare şi sfat genetic datorită suspiciunii clinice de DMD/BMD. La băieţii afectaţi (21 DMD şi 9 BMD) MLPA a avut o rată de detecţie de 63,5% (53,5% deleţii şi 10% duplicaţii). Cel mai frecvent deletat exon a fost exonul 45 şi cea mai frecventă duplicaţie a implicat exonii 3-5, confirmând prezenţa celor două regiuni critice mutaţionale raportate în literatură. Mutaţiile detectate în studiul nostru au avut o localizare uşor diferită comparativ cu datele din literatură. Regula cadrului de lectură a fost valabilă în 84% din cazuri.

***Cuvinte cheie**: distrofii musculare Duchenne şi Becker;distrofină;MLPA;deleţii/duplicaţii*

Received: 8th July 2014; Accepted: 28th October 2014; Published: 28th November 2014.

Introduction

Dystrophinopathies include a spectrum of neuromuscular disorders, caused by mutations in the DMD gene that encodes for dystrophin, which is a key element for sarcolemma stability during muscle contraction (1). Duchenne muscular dystrophy (DMD; MIM# 310200) is the most severe form, being characterized by progressive symmetrical muscle weakness, calf pseudohypertrophy (before age 5), wheelchair dependency by age 12, and death in the 2nd-3rd decade due to heart or respiratory failure. Becker muscular dystrophy (BMD; MIM# 300376) is a less severe allelic form of DMD, affected individuals surviving till the 7th decade (2). As X-linked recessive disorders, DMD and BMD fully express in males and carrier females rarely present features, the clinical expression becoming evident in very specific situations (3) and being usually mild. Typical dystrophin isoform involved in DMD/BMD has 4 main domains – actin-binding NH_2- terminal, rod domain, cysteine-rich domain and COOH- terminal. Most DMD mutations occur in the central rod domain and have different consequences depending if they affect or not the reading frame ("reading frame rule") (4). In DMD cases the mutation alters the reading frame (out-of-frame mutation), the result being a severely truncated, nonfunctional or even absent dystrophin, while in BMD cases the mutation doesn't alter the reading frame (in-frame mutation), the result being a partly functional dystrophin (5). Different methods have been used for DMD mutation detection (2, 6-12). However, the molecular genetic workup can be performed in two steps: MLPA, followed by sequencing of the coding regions and splice sites (13). For the approximately 2% of the cases in which both MLPA and sequencing fail to identify a mutation, a muscle biopsy with immunohistochemical staining of dystrophin or Western blotting is recommended (14).

The aim of this study is to evaluate the efficiency of MLPA as a DMD mutations screening tool (both for patients and carriers), as well as to appreciate the frequency of different types of mutations, and to check the validity of the "reading frame rule". To our knowledge this is the first full-text study regarding the efficiency of MLPA for the screening of exonic copy number variations in dystrophin gene in Romania.

Material and methods

This study included 53 individuals (30 affected males and 23 asymptomatic female relatives) referred to the Medical Genetics Unit of „Sfânta Maria" Children's Hospital in Iaşi for evaluation and genetic counseling due to the clinical suspicion of DMD/BMD. The study was approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy from Iaşi and informed consent was signed by the parents or by the patient. The diagnosis was based on physical examination, serum CK levels and family history. For asymptomatic carriers (mothers/sisters of affected males) we asked about symp-

toms related to exercise and checked serum CK level. Genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen). The standard MLPA analysis was performed according to the manufacturer's instructions using SALSA MLPA probemixes P034-A2 (dystrophin gene exons 1-10, 21-30, 41-50, and 61-70) and P035-A2 (dystrophin gene exons 11-20, 31-40, 51-60, and 71-79). Briefly, 200 nanograms of genomic DNA was denatured and then hybridized with SALSA probemixes. Following ligation, PCR was performed in a Gradient Palm-Cycler (Corbett Research, Mortlake, NSW, Australia), using Cy5 universally labeled primers. Fluorescent amplification products were subsequently separated by capillary electrophoresis on a CEQ 8000 GeXP Genetic Analysis System (Beckman-Coulter) and the data obtained were analyzed with the Coffalyser. Net software, which uses block normalisation in order to assess copy numbers of the target sequences. The deletions/duplications obtained by MLPA were subjected to the reading-frame checker from Leiden Database Muscular Dystrophy (15), which generates a prediction of the effect of whole-exon changes upon the reading frame.

Results

Out of the 30 affected males referred for evaluation, 21 had the clinical diagnosis of DMD, whereas 9 were diagnosed with BMD. MLPA analysis detected abnormalities in 63.5% (19/30) of male patients (DMD 71.5% - 15/21 and BMD 44.5% - 4/9). Deletions were detected in 53.5% of cases (16/30) and duplications in 10% (3/30). Deletions account for 84% of mutated cases (16/19) and duplications account for 16% (3/19). Out of the 16 males with deletion, 4 had deletion of a single exon. Deletions were grouped in the actin-binding (NH_2- terminal) domain and rod-like domain, none of the mutations affecting the cysteine-rich or COOH- terminal domains. The most frequently deleted exons were exons 45, 46, 47, 8 and 9, and the most frequent duplication involved exons 3-5 (see Figure 1). The most frequent breakpoints were recorded in introns 44 and 47, intron 2 breakpoint being involved both in deletions and duplications.

Out of the 23 females referred for evaluation and/or genetic counseling, none was symptomatic. The mutation rate in females was 43.5% (10/23), deletions affecting 35% of cases (8/23) and duplications 9% (2/23). Most of the mutated cases (80%, 8/10) were deletions. Unexpectedly, MLPA proved helpful in detecting numerical chromosomal abnormalities. The mother of a child with normal MLPA result had 35-50% increased relative peak area for all probes, suggesting the presence of triple X syndrome, situation confirmed by a karyotype performed afterwards.

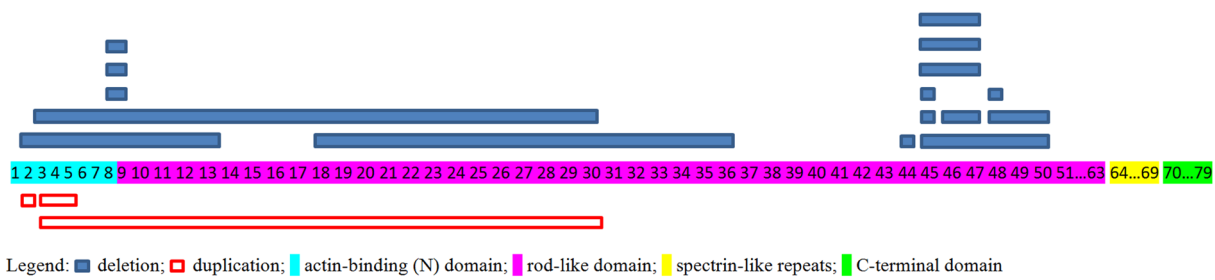

Figure 1.Mutations identified within DMD gene

Table 1: Abnormal MLPA results in the patients studied

Nr.	Clinical diagnosis	Serum CK level (x normal)	MLPA result in proband	FS	Potential carrier	MLPA result in potential carrier	FH*
1	DMD	27	dup 2	Yes	mother	N	-
2	DMD	29	del 2-13	Yes	mother	del 2-13	+
3	DMD	68	del 8-9	Yes	mother	del 8-9	+
4	DMD	48	del 8-9	Yes			
5	DMD	42	del 8-9	Yes	mother	del 8-9	+
6	DMD	28	del 44	Yes	mother	del 44	+
7	DMD	45	del 45	Yes	mother	del 45	+
8	DMD	51	del 45	Yes	mother	NT	+
9	DMD	22	del 48	No	mother	NT	-
10	DMD	29	del 45-50	Yes	sister	del 45-50	+
11	DMD	72	del 46-47	Yes	mother	N	-
12	DMD	67	del 48-50	Yes	mother	N	-
13	DMD	18	del 3-30	No	mother	del 3-30	-
14	DMD	24	del 18-36	Yes	mother	NT	-
15	DMD	18	dup 3-5	No	mother	dup 3-5	+
16	BMD	17	del 45-47	No	mother	del 45-47	+
17	BMD	11	del 45-47	No			
18	BMD	9	del 45-47	No	mother	NT	-
19	BMD	7	dup 3-10	No	mother	dup 3-10	+

FS – frame-shift, FH* – family history, more than one affected male; DMD – Duchenne muscular dystrophy; BMD – Becker muscular dystrophy; N- normal, NT – not tested.

Discussion

Efficiency of DMD gene mutation screening

Various studies reported a detection rate of 60-70% for deletions and approximately 10% for duplications (see Table 2). In our study, by selecting patients based on detailed physical examination and increased plasma CK levels, the detection rate has been 53.5% for deletions and 10% for duplications. We expect the difference to be due to a geographic variation, such differences being cited in the literature. After performing MLPA, 11/30 affected males received a normal result, in spite of the typical features of DMD/BMD. These cases are further candidates for gene sequencing in order to identify point mutations.

Table 2: DMD/BMD mutation frequency in the literature

Type of mutation	DMD	BMD	References
Deletion (1/ more exons)	60-65%	65-70%	Yan et al., 2004 (16); Dent et al., 2005 (17); Dolinsky et al., 2002 (18); Takeshima et al., 2010 (19)
Duplication	5-10%	10-20%	White et al., 2002 (20); White et al., 2006 (21); Flanigan et al., 2009 (22); Takeshima et al., 2010 (19)
Point mutation (small deletion/ insertion, single base change or splicing mutation)	25-35%	10-20%	Dolinsky et al., 2002 (18); Hofstra et al., 2004 (23); Takeshima et al., 2010 (19)

An important aspect is that MLPA is an inexpensive, rapid and reliable technique that proved repeatedly to be useful for DMD diagnosis (Table 2). Moreover, MLPA can be performed on DNA samples extracted from paraffin embedded muscle biopsies and this could be a valid solution for the specific situation when blood samples are not available and a person with positive family history is asking for genetic counseling. An alternative to the combined use of MLPA and sequencing analysis can be the use of a complex MLPA variant that checks in the same time for large exon deletions/duplications and for the most common point mutations in DMD gene (24).

Frequency of different types of mutations

The precise identification of the mutation is essential in DMD, since clinical trials developed recently are personalized (therapy aimed to a specific mutation only) (25). Most of the deletions and duplications cluster in two hotspot regions of the dystrophin gene (exons 2-20 and 44-53) (26), unlike small deletions and point mutations that seem to be evenly distributed throughout the gene (2). The mutations we have detected are indeed located in those areas, but slightly different – the first region covered exons 2-36, whereas the second region covered exons 44-50 (see Figure 1). If we consider the areas covered by multiple mutations, we can narrow these intervals to exons 3-30 for the first hotspot and 45-48 for the second hotspot. To evaluate if this is a particularity of the local population, further studies should be performed. We have found that the major deletion breakpoint was in intron 44, similar to literature data (27) (see Figure 1).

Deletions of a single exon should be differentiated by small mutations or polymorphisms situated close to the probe ligation site, which can influence the peak area of the amplification product of that probe (28). We have identified 4 deletions of a single exon (cases 6-9), the most frequently involved (2/4) being exon 45, as reported also in Leiden database (15). These deletions were associated with a DMD phenotype, so we don't expect this to be due to a polymorphism, but sequencing should be performed to confirm the diagnosis.

The most frequent deletions in the literature are those involving exons 45-47 (7% of cases), 45 (5.3%), 48-50 (5.1%) and for duplications the most common one is that involving exon 2 (8.6% of cases) (21). We have found all these types of defects in our patients. However, besides the common mutations, we have also found mutations that are rarely cited. Deletion 18-36 (case 14) is the rarest mutation identified in our group, being reported only once in the literature (7). Other rare deletions found (2-13 and 3-30) are probably related to the common breakpoint in exon 2 (involved mainly in duplications). Deletion 8-9 was found in two different families, living in remote areas and to exclude their relatedness we shall perform a linkage study.

According to the literature data the duplications are frequently located near the 5' end of the gene, the most common one is exon 2 duplication and most of them have grandpaternal origin, with a high recurrence risk (21). The mechanism for exon 2 duplication seems to be nonhomologous end joining and not unequal crossing over (21). For duplication cases the genotype – phenotype relationship is difficult to evaluate because the reading frame rule is not always valid. Tandem duplications are common, but non-tandem duplications may also occur and the orientation of the duplicated fragment is very important (22). All duplications identified in our study (exon 2, 3-5, 3-10) were close to the 5' end of the gene, and involved a breakpoint in intron 2.

Validity of "reading frame rule"

Most of the phenotypic differences between BMD and DMD cases can be explained by the "reading frame rule", meaning that out-of-frame mutations are associated with a severe pheno-

type (DMD), whereas in-frame mutations are associated with a milder phenotype (BMD) (5). The rule has been valid in 84% of our mutated cases (16/19), except cases 9, 13 and 15. We appreciate that in these cases, even if we have detected a defect, DMD gene sequencing should be performed to look for associated defects that could explain these particularities.

An important issue when analyzing genotype – phenotype relationship is to keep in mind that in advanced stages of disease the plasma CK level decreases, as a result of the progressive elimination of dystrophic muscle fibers (29). We have identified this particularity in two adult patients (cases 10, 18).

Carriers

Carriers' identification is an essential issue for genetic counseling in a DMD family, especially in cases with a single affected male in the family. In such a situation the mother could be carrier, gonadal mosaic or normal (the child being affected due to a new mutation). In our study MLPA identified heterozygous deletions in 10/23 women, enabling an appropriate genetic counseling, especially in case 13, where family history was negative.

Conclusions

In our study, the use of MLPA in patients with suspicion of DMD/BMD had a detection rate of 63.5%, similar with the average value reported in the literature. The identified mutations cover the two hotspots described in the literature, but with slightly different limits. The most frequently deleted exon was exon 45 and the most frequently duplicated exons were exons 3-5, confirming the presence of both hotspots. The reading frame rule was valid in 84% of our cases, allowing both for diagnostic confirmation and for differential diagnosis of DMD versus BMD. MLPA is an accurate, reliable method for the detection of deletions/duplications in all dystrophin gene exons in carriers and affected males, and also has the ability to determine the size of the abnormality, which is critical for personalized gene therapy strategies.

Abbreviations

MLPA– Multiplex Ligation - Dependent Probe Amplification
DMD – Duchenne muscular dystrophy
BMD – Becker muscular dystrophy
CK – creatine kinase
FS – frame-shift
FH – family history
N – normal
NT – not tested

Conflict of interest

We declare no conflict of interest and no sources of funding.

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