

MOLECULAR DIAGNOSTICS OF DUCHENNE/BECKER MUSCULAR DYSTROPHY PATIENTS BY MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION ANALYSIS AND DIRECT SEQUENCING

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ABSTRACT

Duchenne/Becker muscular dystrophy (DMD/BMD), the most common X-linked muscular dystrophy is caused by mutations in the enormously large DMD gene. We screened this gene in 51 unrelated Bulgarian DMD/BMD patients and four families with no living index patient available, by multiplex ligation-dependent probe amplification (MLPA) analysis, which is a powerful tool for detecting deletion/duplication along the DMD gene. This, in combination with direct sequencing, characterized the mutation in all patients, which comprised 42 deletions (82%), six duplications (12%) and three point mutations (6%), and precisely determined all deletion/duplication borders. In all the families with no living index patient available, deletions were

detected by direct analysis on the patients' mothers and sisters, proving the value of MLPA for carrier status determination.

Key words: Duchenne/Becker muscular dystrophy (DMD/BMD), DMD gene, Dystrophin, Deletions, Duplications, Point mutations, Multiplex ligation-dependent probe amplification (MLPA) analysis

INTRODUCTION

Duchenne/Becker muscular dystrophy (DMD/BMD; OMIM#310200; 300376), the most common X-linked muscular dystrophy, is caused by mutations in the enormously large DMD gene (2.4 Mb; 79 coding exons). About 60% of the cases are due to deletions of one or more exons, ~20% to duplications of one or more exons [1], and in 10% to point mutations of different type and location [1]. So far, the deletions have been mainly screened on the hot-spot regions by standard multiplex polymerase chain reaction (mPCR) [2,3]. Precise deletion borders have rarely been clarified by analysis of single exons, and then only for research rather than a diagnostic purpose [4,5].

Recently, MLPA (multiplex ligation-dependent probe amplification) analysis [6] was successfully applied in the study of the DMD gene and resolved many complications of routine diagnostic approaches that are offered to DMD/ BMD families.

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Although not yet approved for diagnostic purposes, MLPA has been broadly applied for analyzing different genes [6]. However, it has been accepted as a standard in analysis of DMD/BMD patients and in preparing them for future gene therapy, and is required for inclusion in the Treat-NMD Network [7]. Here we present our results and experience in use of MLPA/direct sequencing for molecular analysis of the DMD gene in Bulgaria.

MATERIALS AND METHODS

Patients. Fifty-one patients and four families with no living index patient available were analyzed by MLPA/ direct sequencing. All patients were diagnosed clinically only on the basis of clinical symptoms, family history, electromyography (EMG) data and creatine kinase levels. In some, the result of muscular biopsy was also available. Thirty-six patients were severely affected, diagnosed as DMD, 14 had BMD and one patient was considered to represent an intermediate form (IMD). The affected members of the four families with no living index patient were suspected to be severely affected (there was one affected patient in each family).

Method. DNA samples were obtained from peripheral blood, using a DNA extraction kit (QIAamp DNA Mini Kit, QIAGEN, Hilden, Germany). Multiplex PCR of 18 exons for deletions detection along the deletion hot-spot was performed by the classical protocols of Chamberlain *et al.* [2] and Beggs *et al.* [3].

The SALSA MLPA P034/P035 (MRC-Holland, Amsterdam, The Netherlands) kit was used in accordance with the manufacturer's instructions [6]. Fifty to 200 ng of DNA were diluted with TE (Tris/EDTA) (1 M Tris, 0.5M EDTA, pH 8.0) buffer to a volume of 5 μ L. The diluted samples were subjected to hybridization with the DMD gene-specific probes for all 79 exons and to 13 control probes (along Y, Xq and Xp chromosome regions) situated in both sets P034 and P035 [6], at 60°C overnight. The hybridized probes were ligated with a specific ligase mix, provided by the manufacturer. The final step represents PCR amplification of the ligated fragment products. The PCR buffer, PCR primers 6-FAM (6-carboxyfluorescein) labeled, the enzyme dilution buffer and the polymerase were provided in the kit.

The PCR products obtained were analyzed on an ABI PRISM™ 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) in the presence of ROX500 size standard (Applied Biosystems). Each patient sample was analyzed simultaneously with at least two normal male samples. In order to assess copy number changes (duplications) in comparison to the normal controls, MLPA data interpretation was performed by MLPA software - Coffalyser [6].

Sequencing. The DNA from patients who tested negative by MLPA were sequenced for the entire coding sequence of the DMD gene, including exon/intron borders. Each exon was amplified by primers, designed for sequencing in our laboratory (the primer sequence is available upon request). The PCR product was purified by PCR Product Pre-Sequencing Kit [United States Biochemicals (USB); Affymetrix Inc., Santa Clara, CA, USA] and sequenced by the BigDye Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems). The sequenced sample were subjected to a standard ethanol precipitation. The pellet was air-dried and kept at room temperature in the dark. Before run, the pellet was resuspended in 13 μ L Hi-Di Formamide (Applied Biosystems) and loaded into the ABI PRISM™ 310 genetic analyzer (Applied Biosystems). Sequencing results were interpreted by Sequence Scanner v.1 (Applied Biosystems).

RESULTS AND DISCUSSION

The mutations we detected and clinical data are listed in Tables 1a and 1b. Some of these patients were reported in our previous paper [8]. The creatine kinase levels were highly elevated in all cases, with the exception of creatine kinase levels in handicapped patients, where the destroyed muscle leads to creatine kinase level normalization. The main group of patients started walking later than normal children (after 12 months). In two cases: deletion patient #32 and duplication patient #2, a serious delay in starting to walk was registered at around or after second year of age. The duplication patient #2 had very severe symptoms with an early involvement of limbs, limb-girdle and neck muscles and fast disease progression.

The early symptoms in all patients have been very similar: walking on toes, difficulties in climbing stairs, running, getting up. Family history (the

Table 1. Mutations detected in Bulgarian DMD/BMD patients and four families with no living DMD patients (DMD: Duchenne muscular dystrophy; BMD: Becker muscular dystrophy; IMD: intermediate muscular dystrophy; n.d.: no data.).

Diagnosis	Mutations	Creatine Kinase	Clinical Symptoms			Family History
	a) DELETIONS		First Walked at (months):	First Symptoms at (years):	Special Clinical Findings	
1. BMD	ex45ex48del>c.6439-?_7098+?del in-frame	high	n.d.	n.d.	n.d.	no
2. DMD	ex53ex54del>c.7661-?_8027+?del out-of-frame	high	14	3	wheelchair bound	yes
3. DMD	ex48ex52del>c.6913-?_7098+?del out-of-frame	1238	14	3	wheelchair bound, absent tendon reflexes in lower limbs, contractures	no
4. BMD	ex45ex48del>c.6439-?_7098+?del in-frame	n.d.	12	10	able to walk (age 33), difficulties in walking and climbing stairs	yes
5. BMD	ex45ex48del>c.6439-?_7098+?del in-frame	1664	14	12	able to walk (age 21)	no
6. DMD	ex79del>c.11047-?_22064+?del does not encode to a functional protein?	14390	13	6	muscle hypotonia, profound lordosis	no
7. DMD	ex49ex52del>c.7099-?_7660+?del out-of-frame	931	13	3.5	wheelchair bound, contractures, absent reflexes in lower limbs	no
8. BMD	ex45ex48del>c.6439-?_7098+?del in-frame	4845	12	12	able to walk (age 23), difficulties in climbing stairs	no
9. DMD	ex46ex50del>c.6616-?_7309+?del out-of-frame	15600	18	<2	wheelchair bound, contractures, scoliosis	no
10. BMD	ex45ex53del>c.6439-?_7872+?del in-frame	413	12	11	able to walk (age 19)	no
11. DMD	ex55del>c.8028-?_8217+?del out-of-frame	5979	13	3	n.d.	no
12. DMD	ex58del>c.8548-?_8668+?del	14548	14	5	wheelchair bound, scapula alatae, lumbar lordosis, contractures of Achilles tendons	no
13. DMD	ex46ex50del>c.6615-?_7309+?del out-of-frame	6000	14	3	able to walk (age 10), contractures, scapula alatae, absent patella reflexes	no
14. IMD	ex49ex50del>c.7099-?_7309+?del out-of-frame	9212	13	9	able to walk (age 17), pronounced lordosis, scapula alatae, diminished patellar reflexes	no
15. DMD	ex18ex37del>c.2169-?_5325+?del out-of-frame	13462	n.d.	6	Wheelchair bound, contractures, reduced tendon reflexes in lower limbs	no
16. DMD	ex46ex50del>c.6615-?_7409+?del out-of-frame	12000	16	4	waddling gait, pronounced lordosis, hyporeflexia, contractures	no
17. BMD	ex03ex07del>c.94-?_649+?del out-of-frame	2355	12	10	able to walk (age 18), contractures, muscle hypotonia, absent patellar reflexes	no
18. BMD	ex45ex47del>c.6439-?_6912+?del in-frame	7979	12	11	able to walk (age 19), at age 15 myocardial infarction, no muscle involvement at this age, active in sports	no
19. DMD	ex46ex50del>c.6615-?_7309+?del out-of-frame	9319	14	<2	able to walk (age 13), pronounced lordosis, scapula alatae, absent patellar reflexes, contractures	no
20. BMD	ex45ex47del>c.6439-?_6912+?del in-frame	2733	12	11	able to walk (age 20), difficulties in walking and climbing stairs	no
21. DMD	ex51del>c.7310-?_7542+?del out-of-frame	11670	12	2	wheelchair bound, hypotonia, hyporeflexia	no

Continue ➡

Table 1 continue

Diagnosis	Mutations	Creatine Kinase	Clinical Symptoms			Family History
22. BMD	ex45ex47del>c.6439-?_6912+?del in-frame	1758	n.d.	8	able to walk (age 35), pronounced lordosis, absent patellar reflexes	yes
23. BMD	ex45ex47del>c.6439-?_6912+?del in-frame	12000	12	11	wheelchair bound, elbow contractures	no
24. DMD	ex46ex55del>c.6615-?_8217+?del out-of-frame	2310	14	7	wheelchair bound, lower limb areflexia, scoliosis, contractures	no
25. DMD	ex45ex50del>c.6439-?_7309+?del out-of-frame	9191	12	4	able to walk (age 7), diminished patellar reflexes, absent ankle reflexes, scapula alatae, pronounced lordosis	yes
26. DMD	ex08ex11del>c.650-?_1331+?del out-of-frame	18352	12	2	able to walk (age 10), scapula alatae, pronounced lordosis, contractures, diminished patellar reflexes	no
27. DMD	ex44del>c.6291-?_6438+?del out-of-frame	19670	13	6	able to walk (age 9), diminished reflexes in lower limbs, contractures	no
28. DMD	ex08ex09del>c.650-?_960+?del out-of-frame	14228	14	5	able to walk (age 10), scapula alatae, contractures	no
29. DMD	ex45ex79del>c.6439-?(*2691_?)del unexpected problem?	1919	13	3	unable to walk independently, able to sit independently, muscle hypotonia	no
30. BMD	ex03ex07del?c.94-?_649+?del out-of-frame	2602	13	5	unable to walk independently, able to sit independently, pronounced lordosis, diminished or absent reflexes	no
31. DMD	ex45del>c.6439-?_6614+?del out-of-frame	298	18	4	wheelchair bound, absent reflexes in lower limbs, scoliosis, contractures	no
32. DMD	ex53del>c.7661-?_7872+?del out-of-frame	13182	20	4	able to walk (age 5), contractures	yes
33. DMD	ex45ex54del>c.6439-?_8027+?del out-of-frame	15395	14	3	able to walk (age 5), lordosis	no
34. DMD	ex45ex53del>c.6439-?_7872+?del in-frame	22394	n.d.	3	Able to walk (age 8), scapula alatae	yes
35. BMD	ex45ex47del>c.6439-?_6912+?del in-frame	1240	n.d.	12	able to walk (age 28)	no
36. DMD	ex42ex45del>c.5932-?_6614+?del out-of-frame	high	14	2	n.d.	No
37. DMD	ex44del>c.6291-?_6438+?del out-of-frame	12460	14	2	muscle hypotonia	no
38. DMD	ex46ex50del>c.6615-?_7309+?del out-of-frame	3982	14	2	n.d.	No
39. DMD	ex44del>c.6291-?_6438+?del out-of-frame	2280	14	3	profound lumbar lordosis, scapula alatae	no
40. DMD	ex16ex17del>c.1813-?_2168+?del out-of-frame	high	15	3	wheelchair bound	yes
41. BMD	ex45ex48del>c.6439-?_7098+?del in-frame	800	12	13	muscle hypotonia	no
42. DMD	ex45ex52del>c.6439-?_7660+?del out-of-frame	420	14	3	wheelchair bound	yes

Continue ➡

Table 1 continue

Diagnosis	Mutations	Creatine Kinase	Clinical Symptoms			Family History
	DUPLICATIONS					
1. DMD	ex48ex50dup>c.6913-?_7309+?dup out-of-frame	4500	14	3	wheelchair bound, lordosis	yes
2. DMD	ex02ex33dup>c.32-?_4674+?dup out-of-frame	1521	29	1	cannot sit up, twist, or keep the head upright at 10 months of age	no
3. DMD	ex08ex11dup>c.650-?_1331+?dup out-of-frame	9000	15	5	walks on toes, cannot climb stairs alone	no
4. BMD	ex13ex40dup>c.1483-?_5739+?dup in-frame	1107	12	12	profound lordosis	yes
5. DMD	ex02ex10dup>c.32-?_1149+?dup out-of-frame	409	15	<2	wheelchair bound, hyporeflexia, contractures, scoliosis	yes
6. DMD	ex51dup>c.7310-?_7542+?dup out-of-frame	4500	16	3	wheelchair bound	yes
	POINT MUTATIONS					
1. DMD	exon 7 c.583C>T, p.Arg195X	12000	16	2	hyporeflexia in Achilles tendons and knees	no
2. DMD	exon 23 c.2991C>G, p.Tyr997X	17100	13	3	died at age 17	no
3. DMD	exon 59 c.8776C>T, p.Gln2926X	9000	15	2	hyporeflexia in lower limbs	yes
	b) NO INDEX PATIENT AVAILABLE					
1. DMD	ex46ex52del>c.6913-?_7660+?del out-of-frame	–	–	–	–	no
2. DMD	ex48ex50del>c.6913-?_7309+?del out-of-frame	–	–	–	–	no
3. DMD	ex45ex50del>c.6439-?_7309+?del out-of-frame	–	–	–	–	no
4. DMD	ex49ex54del>c.7099-?_8027+?del out-of-frame	–	–	–	–	no

presence of other affected family members) was positive in 13 cases.

The deletion patient #23 had been clinically diagnosed as Emery-Dreifuss muscular dystrophy (OMIM#181350; OMIM#310300). The precise diagnosis of BMD was established during the present study.

Of interest is the deletion of the very last exon of the DMD gene (exon 79), detected in deletion patient #6. It is not clear where the deletion ends, but the clinical picture was compatible with DMD phenotype (early onset and fast progression). Deletion patient #29 also had a deletion covering exon 79. He was also severely affected with a fast disease progression. A similar mutation has been described earlier in a BMD patient as a part of a contiguous gene deletion syndrome [9].

Two duplications of exons 2-33 and 13-40 (duplication patients #2 and #4) cover more than one-third of the gene sequence, and result in DMD and

BMD phenotypes, respectively. Most probably, the duplication situated close to the N-terminus of the dystrophin is associated with a severe phenotype, while the one in the rod-domain leads to the BMD phenotype [8].

Some patients were pre-screened for deletions in the DMD gene by a standard multiplex set of primers. In deletion patient #14 the deletion of exons 49-50 was detected in this way, but the family requested confirmation with an alternative method. In several cases the deletions were detected by mPCR, but deletion borders were not precisely determined, or carrier status determination in these families required MLPA. In deletion patients #37 and #39, the deletion of exon 44 was not detected by multiplex PCR (most probably because of PCR unspecificity or contamination) and they were not considered as deletions. A number of index patients were classified as unknown mutations after mPCR.

The MLPA analysis/direct sequencing charac-

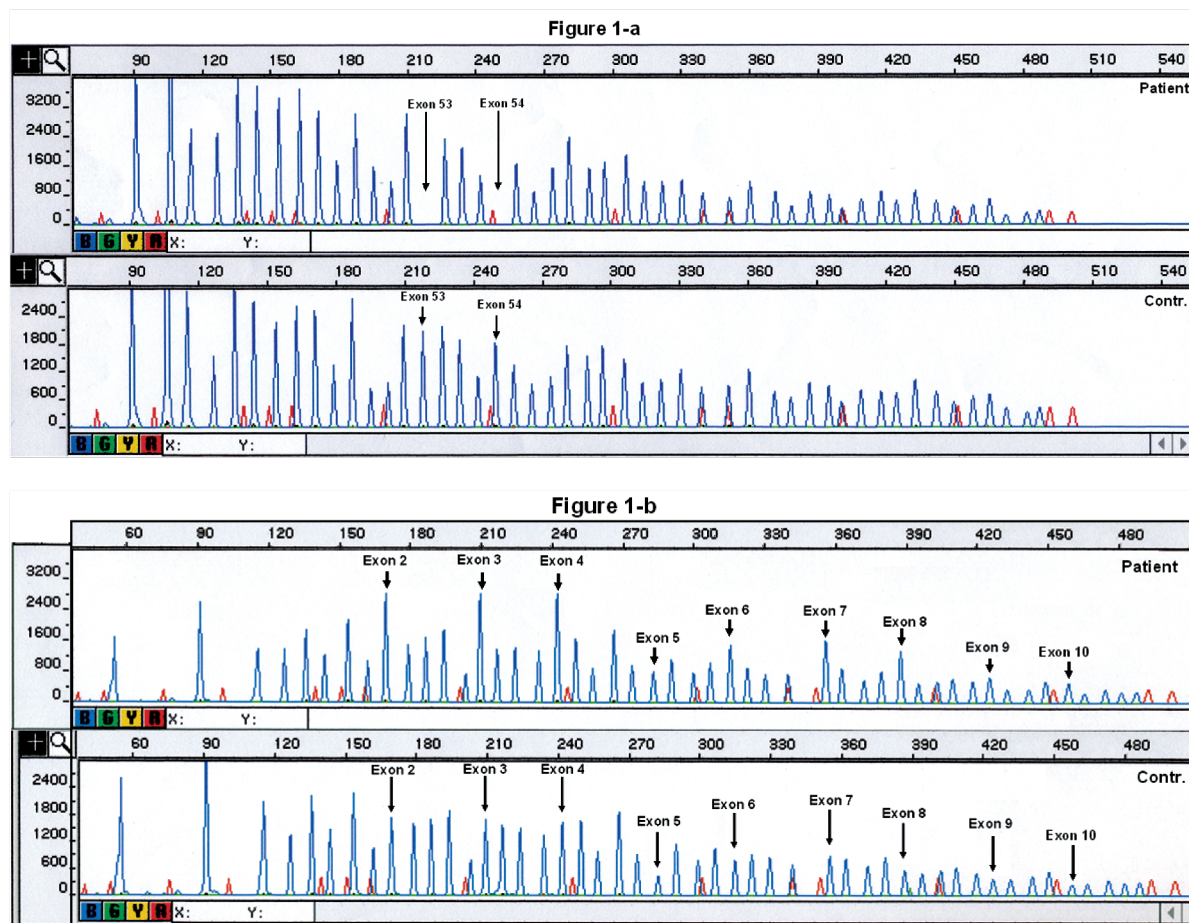


Figure 1. Capillary electrophoresis pattern (a) in patient #2 with deletion of exons 53-54 and (b) in patient #5 with duplication of exons 2-10, compared to a control sample. The deleted or duplicated fragments are indicated by arrows.

terized mutation in all cases, which comprised 42 deletions (82%), six duplications (12%) and three point mutations (6%) (see Table 1a). In addition, this analysis in the four families with no living index patient revealed deletions in all families of dead patients diagnosed with DMD, using the DNA samples of their mothers and sisters (see Table 1b).

All deletion/duplication borders were precisely determined by this method as required for adequate gene therapy in the future. The deletion and duplication electrophoretic profiles are presented on Figure 1a and 1b. All deletions were confirmed by PCR amplification.

In our hands, the combination of the MLPA test with direct sequencing of the entire DMD gene, proved to be a powerful diagnostic approach in DMD/BMD families. In point mutation patient #2 only exon 23 was missing on the MLPA electropherogram. The single amplification of exon 23 showed

its presence in the patient, but the point mutation c.2991C>G, p.Tyr997X was detected by sequencing of the exon [8]. Point mutation patients #1 and #3 tested negative by MLPA, but sequencing of the entire coding sequence of the DMD gene revealed the nonsense mutations c.8776C>T, p.Gln2926X and c.583C>T, p.Arg195X, respectively [8].

The classical mPCR of 18 exons in the deletion hot-spot region of the DMD gene [2,3] could detect 36 deletions in the present sample (86% of deletions), 15 of which (42%) were only partial, with no clear deletion borders. The mPCR could only detect deletions, and it is applicable only when the index patient is available for study. Moreover, the presented MLPA analysis was successful not only in all the patients tested, but also in all four families with no index patient. We identified four deletions in these families (see Table 1b); all four mothers were deletion carriers, one sister was genetically proved to

be a carrier and two were not carriers. The MLPA data is easily interpreted also for direct carrier status determination.

The application of the most recent genetic tests in our routine diagnostic studies, gave us the possibility to genetically clarify a number of Bulgarian DMD/BMD patients. A national web site for clinically and genetically well described neuromuscular cases is available [10], according to the requirements of the Treat-NMD Network.

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