Detection of Duchenne/Becker Muscular Dystrophy Carriers
in a Group of Iranian Families by Linkage Analysis

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Abstract- This study determines the value of linkage analysis using six RFLP markers for carrier detection and prenatal diagnosis in familial DMD/BMD cases and their family members for the first time in the Iranian population. We studied the dystrophin gene in 33 unrelated patients with clinical diagnosis of DMD or BMD. Subsequently, we determined the rate of heterozygosity for six intragenic RFLP markers in the mothers of patients with dystrophin gene deletions. Finally, we studied the efficiency of linkage analysis by using RFLP markers for carrier status detection of DMD/BMD. In 63.6% of the patients we found one or more deletions. The most common heterozygous RFLP marker with 57.1% heterozygosity was pERT87.15*Taq1*. More than 80% of mothers in two groups of familial or non-familial cases had at least two heterozygous markers. Family linkage analysis was informative in more than 80% of the cases, allowing for accurate carrier detection. We found that linkage analysis using these six RFLP markers for carrier detection and prenatal diagnosis is a rapid, easy, reliable, and inexpensive method, suitable for most routine diagnostic services. The heterozygosity frequency of these markers is high enough in the Iranian population to allow carrier detection and prenatal diagnosis of DMD/BMD in more than 80% of familial cases in Iran.

Introduction

Muscular dystrophies are a group of neuromuscular disorders characterized by progressive muscle weakness and degeneration of skeletal muscle. The best known muscular dystrophies are the X-linked Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD).

DMD and BMD are the most common muscle disease in children. DMD has an incidence of 1:3500 male newborns (1). Physical signs, which usually begin between the ages of 3-5 years, include trouble walking, running, jumping, calf hypertrophy and lumbar lordosis. Boys with DMD are usually confined to a wheelchair by the age of 12 and die in their third decade of life from cardiac or respiratory failure. In BMD, the variant of DMD with an incidence of approximately 3 per 100,000 male newborns, the clinical course is milder. Symptoms begin at about 11 years of age and progression is slower than DMD (2).

The DMD gene is the largest known human gene, spanning a genomic region of 2.4Mb on chromosomal location of Xp21 and contains 79 exons transcribed in to a 14 Kb mRNA encoding the dystrophin protein. The

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dystrophin protein contains 3,685 amino acids with a molecular weight of about 427 kD (3). The location of the dystrophin protein is on the inner side of the plasma membrane of all myofibers.

Majority of DMD/BMD cases are caused by gene deletions (60%) or duplications (5%) of various sizes. These deletions and duplications are most often located in two major hot-spots: exons 3–7 and exons 44–55. Point mutations and small deletion/insertions account for the rest of the cases (35%) (4,5).

In the past, deletions of the DMD gene were screened by Southern-blot analysis using cDNA probes (4). Now, deletions are screened in many DMD/BMD patients by multiplex PCR assays. About 98% of deletions are determined using primers targeting 18 hot-spot exons in the dystrophin gene (6). DMD and BMD are inherited by an X-linked pattern, and therefore affect mainly males. Although there is no cure for DMD/BMD yet, molecular approaches for carrier detection and prenatal diagnosis offer preventative hope for future generations (7). This is crucial because, heterozygous females are usually without clinical symptoms; creatine kinase is only elevated in nearly 50 to 60% of known carriers.

Up to now, several methods have been developed for carrier detection, including dosage analysis by Southern blots of the dystrophin gene, multiple ligation probe amplification (MLPA), FISH by exon specific probes and quantitative PCR with both traditional and real-time methods (8-12). As mentioned, small mutations, including point mutations or micro-deletions/insertions, are the cause of the disease in 35% of the DMD/BMD cases. Most of these mutations were found to be patient specific.

There are no hot-spot regions and in most routine diagnostic services, these types of mutations remain undefined because sequencing the entire gene is both very expensive and difficult. In families with uncharacterized mutations, carrier detection and prenatal diagnosis depend on linkage analysis using markers such as STRs, VNTRs, or RFLPs (13-15). This method is on the basis of the co-inheritance of the disease gene with those DNA markers known to be located near or within the disease gene. Thus, when the gene mutation of disease is undefined, the linkage technique helps us to follow the mutation through the patient's family and predict the inheritance of the disease. In familial cases, the mother of a patient is an obligatory carrier and linkage analysis can be effectively used.

Carrier detection and prenatal diagnosis by linkage analysis using RFLP markers is a fast, easy, inexpensive and straightforward way for carrier detection in most routine diagnostic services (16). This method can be used effectively for carrier detection and prenatal diagnosis in familial DMD/BMD cases with or without deletion; furthermore, it may help to confirm results of other methods used to detect carrier status of patient's female relatives.

The aim of this investigation is to use six RFLP markers of the DMD gene to determine the carrier status in a group of Iranian families with diagnosis of DMD/BMD and to evaluate the usefulness of this technique as a rapid, inexpensive and easy diagnostic tool in routine diagnostic services.

Material and Methods

Patients

We studied 33 unrelated patients referred for genetic analysis of the dystrophin gene between 2008 and 2009 in Tehran, Iran. Examinations of patients were performed in the Department of Neurology at the Children Medical Center of Tehran. Most patients had a diagnosis of DMD or BMD based on the following findings; proximal muscle weakness beginning in childhood, an unusual waddling gait, pattern of muscle weakness in the pelvic and shoulder girdle muscles, calf enlargement, lumbar lordosis, changed electromyography (EMG) and elevated serum creatine kinase activity. This study was conducted in families of patients with a confirmed exon deletion in the dystrophin gene.

DNA extraction

Four to five ml of peripheral blood from each proband and their family members were collected in autoclaved tubes cantaining sodium EDTA. Genomic DNA was extracted from leukocytes obtained from blood according to the salting out procedure (17).

Multiplex PCR

Analysis of the dystrophin gene deletion was performed by multiplex PCR with primer sets of Beggs et al. and Chamberlain et al. with slight modifications (6, 18).

The analysis included exons 3, 4, 6, 8, 12, 13, 17, 19, 43, 44, 45, 47, 48, 50, 51, 52, 60 and Pm. The PCR products were inspected on a 6% polyacrylamide gel and the bands were detected under U.V. light after ethidium bromide staining. Deletions were diagnosed when one of the bands present in the amplified control DNA was absent from the patient DNA.

Table 1. Six DMD RFLP markers, their locations, and primers used in linkage study.

RFLP Marker	Location	Forward / reverse primers
pERT87-15/ *XmnI*	Intron 17	acaatttccctttcattccag/ Gactggagcaagggtcgcc
pERT87-15/ *BamHI*	Intron 17	tccagtaacggaaagtgc/ ataattctgaatagtcacaaaaag
pERT87-15 / *TaqI*	Intron 16	gactttcgatgttgagattactttccc/ aagcttgagatgctctcaccttttcc
pERT87-8 / *TaqI*	Intron 13	gtcagttggtcagtaaaagcc/ ccaattaaaaccacagcag
pERT 87-1/ *BstNI*	Intron 12	ctatcatgcctttgacattccag/ ctcaataagagttggattcattc
pERT84-1/ *MaeIII*	Intron 1m	cagggatgcaaaggaactggg/ cagtttgtttaacagtcactc

Linkage study

Linkage analysis was performed using six intragenic DMD markers with primers from Leiden Muscular Dystrophy (http://www.dmd.nl, accessed January 2009) and Roberts RG et al. (16, 19). Markers, their locations and primers are summarized in table 1.

PCR was carried out by the following steps: samples were heated to 95°C for 5 minutes, followed by 33 cycles at 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds for pERT87-15/ *BamHI* , pERT87-8 / *TaqI* and pERT84-1/ *MaeIII* loci, and 94°C for 45 seconds, 59°C for 30 seconds and 72°C for 45 seconds for pERT87.15 *Taq1* , pERT87-1/ *BstN1* and pERT87-15/ *XmnI* loci. A final elongation step at 72°C for 7 minutes was done for all of the loci. Fifteen μl of the PCR product was electrophoresed in a 6% polyacrylamide gel stained with ethidium bromide or silver.

For RFLP analysis, 10 μl of amplification product was digested with 5-10 units of appropriate enzyme, electrophoresed on a 6% polyacrylamide vertical gel, and visualized by silver or ethidium bromide staining. A positive allele of every RFLP marker was used as control in this study.

Results

Deletions removing portions of the DMD gene were found in 21 index cases (63.6%); 17 deletions (80.9%) occurred in the central region of the gene and 4 (19.1%) at the 5' region. In 12 patients (36.4%) we didn't find any deletion.

Analysis of family pedigrees in patients with DMD gene deletions showed that 52.38% of mothers were obligate DMD/BMD carriers and the remainders of cases (47.62%) were isolated or sporadic.

The PCR product length of each RFLP marker and their respective digested fragments of negative and positive alleles are presented in table 2.

Heterozygosity frequency rate of the six RFLP markers in all 21 mothers are shown in table 3. As it is shown, RFLP marker pERT87.15 Taq1 had the most heterozygosity among these markers.

The results showed that 80.9% of all mothers and 81.8% of obligate carrier mothers were heterozygous for at least two of the RFLP markers.

Carrier detection for 11 daughters in 9 families with obligate carrier mothers, which were heterozygous for at least two of these RFLP markers, was performed and linkage analysis was informative in all of these families. The results showed that 7 out of 11 of the daughters of these families were carriers. Examples of linkage analysis performed are presented and explained in figures 1, 2, 3, and 4.

The mother of family 3 was heterozygous for two RFLP markers and linkage analysis showed that one of the daughters in this family is a carrier and the other is not a carrier.

Table 2. PCR products length and their respective digested fragments products of studied RFLP markers

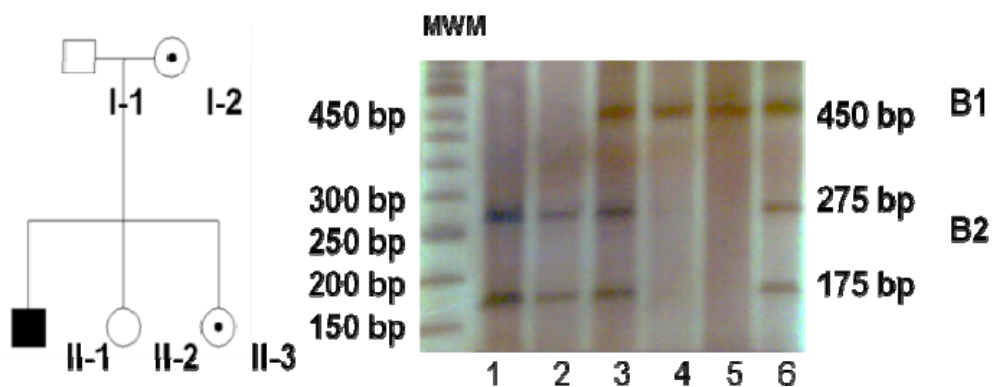| RFLP Marker	PCR products length in bp	Fragments of negative allele in bp	Fragments of Positive allele bp
pERT87-15/ *XmnI*	740	730+10 (X1)	520+210+10 (X2)
pERT87-15/ *BamHI*	226	216+10 (D1)	166+50+10 (D2)
pERT87-15 / *TaqI*	415	415 (E1)	231+184 (E2)
pERT87-8/*TaqI*	155	145+10 (T1)	74+71+10 (T2)
pERT 87-1/ *BstNI*	450	450 (B1)	275+175 (B2)
pERT84-1/ *MaeIII*	252	236+16 (M1)	128+108+16 (M2)

Figure 1. Partial pedigree and linkage analysis of family 3 (locus: pERT87-1/ *BstN1*). One of the proband's sister is not a carrier and the other one is carrier (B1 for the alleles lacking the restriction sites or negative allele and B2 for the alleles containing the restriction sites or positive allele). Lane**1:** the control [++] or B2B2 allele, **2:** Proband, **3:** Proband's mother, **4:** Proband's father, **5:** Proband's sister II-2 (non carrier), **6:** Proband's sister II-3 (carrier).
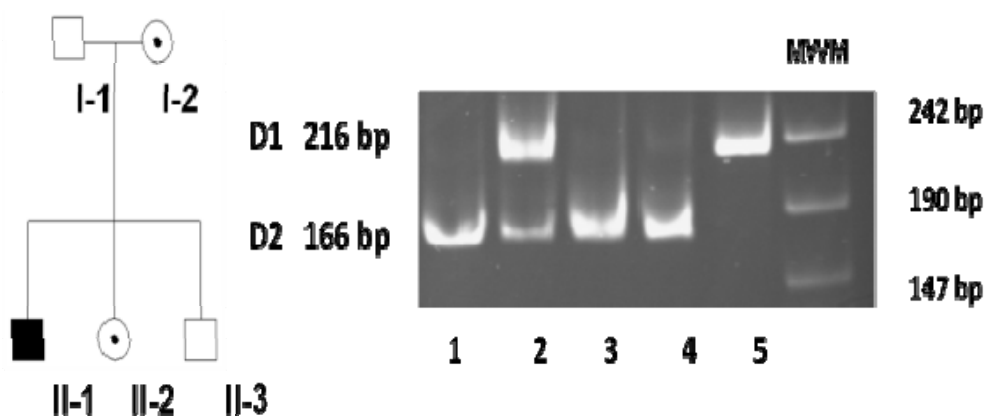

Figure 2. Partial pedigree and linkage analysis of family 7 (locus: pERT87-15/ *BamHI*). The proband's sister II-2 is a carrier (D1 for the alleles lacking the restriction sites or negative allele and D2 for the alleles containing the restriction sites or positive allele). Lane **1:** Proband **2:** Proband's mother **3:** Proband's father **4:** Proband's sister, **5:** Proband's normal brother.
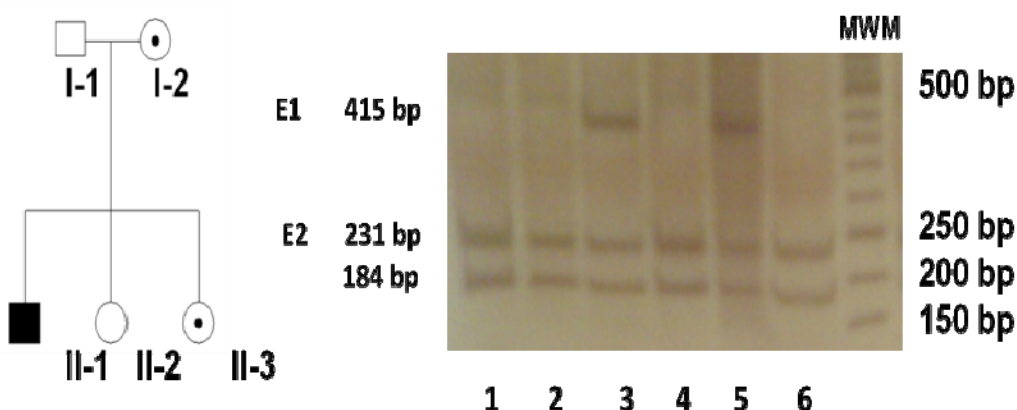

Figure 3. Partial pedigree and linkage analysis of family 11 (locus: pERT87-*15/Taq1*). The proband's sister is a carrier (E1 for the alleles lacking the restriction sites or negative alleles and E2 for the alleles containing the restriction sites or positive alleles). Lane **1:** The control [++] or E2E2 allele **2:** Proband, **3:** Proband's mother, **4:** Proband's father, **5:** Proband's sister II-2 (non carrier), **6:** Proband's sister II-3 (carrier).
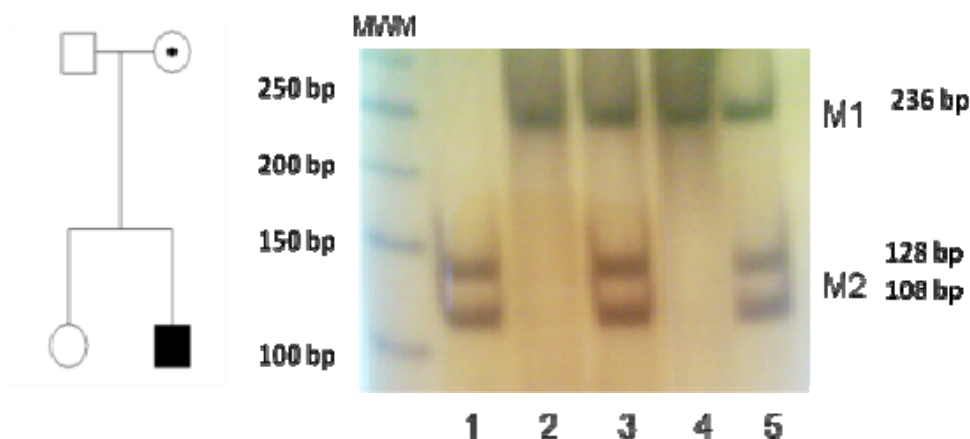
Figure 4. Partial pedigree and linkage analysis of family 20 (locus: pERT84-1/ *MaeIII*). The proband's sister is not a carrier (M1 for the alleles lacking the restriction sites or negative allele and M2 for the alleles containing the restriction sites or positive allele). Lane **1:** The control [++] or M2M2 allele, **2:** Proband, **3:** Proband's mother, **4:** Proband's father, **5:** Proband's sister (non carrier).

The pedigree and linkage analysis of family 3 for RFLP marker pERT87-1/ *BstN1* are shown in figure1. As demonstrated, B2 allele is linked to the disease and the daughter II-3 (lane 6) has B1B2 genotype so she has inherited B2 allele from her mother and is a carrier. Daughter II-2 (lane 5) with B1B1 genotype has inherited non-carrier X chromosome from her mother, thus she is not a carrier (Figure 1).

The mother in family 7 was heterozygous for two RFLP markers and linkage analysis showed that the daughter of this family is a carrier. In figure 2; the pedigree and linkage analysis of family 7 for RFLP marker pERT87-15/*BamH1* are shown.

In family 11, the mother was heterozygous for three RFLP markers and linkage analysis showed that one of the daughters in this family is a carrier and the other daughter is not a carrier. The pedigree and linkage analysis of family 11 for RFLP marker pERT87-15/*Taq1* are shown in figure 3.

In family 20, the mother was heterozygous for all six RFLP markers. Linkage analysis was informative in this family and showed that the daughter of this family is not a carrier. The pedigree and linkage analysis of family 20 for RFLP marker pERT84-1/ *MaeIII* are shown in Figure 4.

Discussion

DMD/BMD is the most common progressive neuromuscular genetic disorder in males. There is no cure for the disease; therefore, carrier detection and prenatal diagnosis are very important for genetic counseling. The lack of any cure for this disease requires absolute accuracy in genetic test results. This is especially important during prenatal diagnosis, when leaving a residual risk of disease for the fetus could be critical in deciding to continue the pregnancy. However, carrier designation in female relatives of DMD/BMD families is still difficult due to absence of any clinical signs and the presence of both normal and mutant copies of the gene.

So far, several methods have been proposed for the molecular identification of DMD/BMD carriers. The direct determination of deletions in carriers by detection of a transcript with altered length (20) is difficult to perform and requires special equipment.

Table 3. Frequency of heterozygosity in mothers of patients with DMD gene deletion.

Marker	pERT 87.15 BamHI	pERT 87.8 Taq1	pERT 87.15 Taq1	pERT 87.1 BstNI	pERT 84.1 MaeIII	pERT87.15 XmnI
HT%	38%	33.3%	57.1%	47.6%	33.3%	42.8%

The gene dosage protocol is expensive and difficult due to the presence of both normal and aberrant X chromosomes, and also due to high proportion of patients with an uncharacterized mutation. Recent carrier detection by real- time PCR and MLPA (multiple ligation probe amplification) has been used successfully (10, 12), but these methods cannot be used for the non-deletional form of the disease.

Linkage analysis is an alternative approach for carrier detection, but there are limitations of its use due to high recombination rate (12%) within the DMD gene (21) and designation of family members due to death. However, in familial cases of disease with or without a defined deletion, linkage methods can be effectively used for carrier detection and prenatal diagnosis (13).

Indirect diagnosis of carriers and prenatal diagnosis by restriction fragment length polymorphism (RFLP) (16, 19 and 22-25) may be easier, rapid and less expensive compared to short tandem repeats (STRs) analysis. The RFLP method does not require end-labeled primers, radiolabeled deoxynucleotids, or sequencing gels. There is also less complexity in the electrophoretic band pattern compared to short tandem repeats (STRs) linkage analysis (13). Use of an additional RFLP marker located on exon 48 of the dystrophin gene (*Msel* polymorphism) may reduce the recombination error rate of this method and increase overall heterozygosity rate (26). To increase the reliability of linkage analysis and to rule out recombination error, it is necessary that the proband mothers be heterozygous in at least two RFLP markers as we have seen in more than 80% of our cases. By using more RFLP markers like Msel polymorphism on exon 48, the reliability of our linkage analysis may be increased to more than 90%.

In conclusion, it should be said that linkage analysis by using RFLP markers is possible in many routine diagnostic settings and may be a method of choice in Iran and similar countries for carrier detection and prenatal diagnosis in deletional and non-deletional familial cases of DMD/BMD. This method, if combined with short tandem repeats (STRs) linkage analysis, may overcome linkage analysis limitations caused by high recombination rate within the dystrophin gene.

Acknowledgments

References

1. Emery AE. The muscular dystrophies. Lancet 2002;359(9307):687-95.
2. Blyth H, Pugh RJ. Muscular dystrophy in childhood; the genetic aspect; a field study in the Leeds region of clinical types and their inheritance. Ann Hum Genet 1959;23(2):127-63.
3. Worton R. Muscular dystrophies: diseases of the dystrophin-glycoprotein complex. Science 1995;270(5237):755-6.
4. Den Dunnen JT, Grootscholten PM, Bakker E, Blonden LA, Ginjaar HB, Wapenaar MC, van Paassen HM, van Broeckhoven C, Pearson PL, van Ommen GJ. Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. Am J Hum Genet 1989;45(6):835-47.
5. Hu XY, Burghes AH, Ray PN, Thompson MW, Murphy EG, Worton RG. Partial gene duplication in Duchenne and Becker muscular dystrophies. J Med Genet 1988;25(6):369-76.
6. Beggs AH, Koenig M, Boyce FM, Kunkel LM. Detection of 98% of DMD/BMD gene deletions by polymerase chain reaction. Hum Genet 1990;86(1):45-8.
7. Laing NG. Molecular genetics and genetic counselling for Duchenne/Becker muscular dystrophy. Mol Cell Biol Hum Dis Ser 1993;3:37-84. Review.
8. Bakker E, Hofker MH, Goor N, Mandel JL, Wrogemann K, Davies KE, Kunkel LM, Willard HF, Fenton WA, Sandkuyl L, et al. Prenatal diagnosis and carrier detection of Duchenne muscular dystrophy with closely linked RFLPs. Lancet 1985;1(8430):655-8.
9. Ligon AH, Kashork CD, Richards CS, Shaffer LG. Identification of female carriers for Duchenne and Becker muscular dystrophies using a FISH-based approach. Eur J Hum Genet 2000;8(4):293-8.
10. Gatta V, Scarciolla O, Gaspari AR, Palka C, De Angelis MV, Di Muzio A, Guanciali-Franchi P, Calabrese G, Uncini A, Stuppia L. Identification of deletions and duplications of the DMD gene in affected males and carrier females by multiple ligation probe amplification (MLPA). Hum Genet 2005;117(1):92-8. Epub 2005 Apr 20.
11. Abbs S, Bobrow M. Analysis of quantitative PCR for the diagnosis of deletion and duplication carriers in the dystrophin gene. J Med Genet 1992;29(3):191-6.
12. Joncourt F, Neuhaus B, Jostarndt-Foegen K, Kleinle S, Steiner B, Gallati S. Rapid identification of female carriers of DMD/BMD by quantitative real-time PCR. Hum Mutat 2004;23(4):385-91.

13. Clemens PR, Fenwick RG, Chamberlain JS, Gibbs RA, de Andrade M, Chakraborty R, Caskey CT. Carrier detection and prenatal diagnosis in Duchenne and Becker muscular dystrophy families, using dinucleotide repeat polymorphisms. Am J Hum Genet 1991;49(5):951-60.

14. Darras BT, Harper JF, Francke U. Prenatal diagnosis and detection of carriers with DNA probes in Duchenne's muscular dystrophy. N Engl J Med 1987;316(16):985-92.

15. Lindlöf M, Kääriäinen H, Davies KE, de la Chapelle A. Carrier detection and prenatal diagnosis in X linked muscular dystrophy using restriction fragment length polymorphisms. J Med Genet 1986;23(6):560-72. Erratum in: J Med Genet 1987;24(3):171.

16. Roberts RG, Cole CG, Hart KA, Bobrow M, Bentley DR. Rapid carrier and prenatal diagnosis of Duchenne and Becker muscular dystrophy. Nucleic Acids Res 1989;17(2):811.

17. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215.

18. Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res 1988;16(23):11141-56.

19. Roberts RG, Bobrow M, Bentley DR. A MaeIII polymorphism near the dystrophin gene promoter by restriction of amplified DNA. Nucleic Acids Res 1990;18(5):1315.

20. Roberts RG, Barby TF, Manners E, Bobrow M, Bentley DR. Direct detection of dystrophin gene rearrangements by analysis of dystrophin mRNA in peripheral blood lymphocytes. Am J Hum Genet 1991;49(2):298-310.

21. Abbs S, Roberts RG, Mathew CG, Bentley DR, Bobrow M. Accurate assessment of intragenic recombination frequency within the Duchenne muscular dystrophy gene. Genomics 1990;7(4):602-6.

22. González-Herrera L, Gamas-Trujillo PA, García-Escalante MG, Castillo-Zapata I, Pinto-Escalante D. Identifying deletions in the dystrophin gene and detecting carriers in families with Duchenne's/Becker's muscular dystrophy. Rev Neurol 2009;48(2):66-70.

23. Alcántara Ortigoza MA, Aguinaga Ríos M, González del Angel A, Zavaleta Abreu Mde J, Acevedo Gallegos S, Mayén Molina DG, del Castillo Ruíz V. Prenatal molecular diagnosis of a DMD carrier female fetus by chorionic villus sampling and linkage analysis. Ginecol Obstet Mex 2009;77(2):103-9.

24. Percesepe A, Ferrari M, Coviello D, Zanussi M, Castagni M, Neri I, Travi M, Forabosco A, Tedeschi S. Detection of a novel dystrophin gene mutation through carrier analysis performed during prenatal diagnosis in a case with intragenic recombination. Prenat Diagn 2005;25(11):1011-4.

25. Sacare VK. Molecular genetic characteristics of Duchenne-Becker muscular dystrophy in the Republic of Moldova. Genetika 2008;44(10):1404-9.

26. Yau SC, Roberts RG, Bentley DR, Mathew CG, Bobrow M. A MseI polymorphism in exon 48 of the dystrophin gene. Nucleic Acids Res 1991;19(20):5803.