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Please cite this article **The importance of direct genetic testing to determine female carriers in dystrophinopathies**

Značaj direktnog genetičkog testiranja za utvrđivanje žena prenosioca kod distrofinopatija

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The importance of direct genetic testing to determine female carriers in dystrophinopathies

Značaj direktnog genetičkog testiranja za utvrđivanje žena prenosioca kod distrofinopatija

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Short title: Carrier detection in dystrophinopathies

Abstract

Background / Aim. Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are caused by mutations in the dystrophin gene. They are X-linked recessive diseases, where males are affected and females are mostly healthy carriers of the mutation. It is estimated that 2/3 mothers of DMD probands are carriers, while 1/3 of patients have *de novo* mutations. The aim was to confirm the carrier status of females in the families of DMD/BMD probands, using direct genetic methods. M**ethods.** We tested 38 females from 31 families of DMD/BMD probands with deletion/duplication in the dystrophin gene. Also, 4 cases of prenatal diagnosis of DMD/BMD were included. We preformed the ***polymerase chain reaction*** (***PCR***) and the multiplex ligation-dependent method (MLPA) for deletion detection, i.e. deletion/duplication in the dystrophin gene. **Results.** In 31 DMD/BMD probands, we identified 87.1% deletions and 12.9% duplications of one or more exons. Of the 29 tested mothers, mutations were found in 17 (14 deletions and 3 duplications). Mutations were found in 57.9% (11/19) mothers of DMD and in 60% (6/10) mothers of BMD, respectively. Also, in probands with deletions 56% (14/25) of mothers were carries and in probands with duplications 3 mothers of 4 (75%). Of the 9 other female relatives, mutations were found in 4. In prenatal diagnosis, we identified deletion in one male and one female foetus of one mother. **Conclusion.** The study showed that mothers were carriers in almost 60% of sporadic cases of DMD/BMD with deletions and duplication. Also, the carrier frequency tended to be higher in mothers of the probands with duplication (75%) then in probands with deletions (56%). In the case of a mother who was confirmed as a carrier, deletion was detected in 2 of 3 foetuses.

Key words: dystrophinopathies, MLPA method, female carriers, prenatal diagnosis.

Apstrakt

Uvod / Cilj. Dišenova mišićna distrofija (DMD) i Bekerova mišićna distrofija (BMD) su uzrokovane mutacijama u genu za distrofin. To su X-vezane recesivne bolesti, gde oboljevaju muškarci a žene su uglavnom zdravi prenosioci mutacije. Procenjeno je da su kod DMD probanada 2/3 majki nosioci, dok 1/3 pacijenata ima novu mutaciju. Cilj rada je bio da se utvrdi status prenosioca kod žena u porodicama obolelih od DMD/BMD, primenom direktne genetičke metode. M**etode.** Testirali smo 38 žena iz 31 porodice DMD/BMD probanada sa delecijama i duplikacijama u genu za distrofin. Takođe, u studiju su bila uključena i 4 slučaja prenatalne DMD/BMD dijagnoze. Primenjene su metoda lančane reakcije polimerizacije (PCR) i metoda višestrukog umnožavanja vezanih proba (MLPA) za detekciju delecija, odnosno delecija i duplikacija u genu za distrofin. **Rezultati.** Kod 31-og DMD/BMD probanda utvrđeno je 87,1% delecija i 12,9% duplikacija jednog ili više egzona. Od 29 testiranih majki probanada, mutacije su nađene kod njih 17 (14 delecija i 3 duplikacije). Mutacije su nađene kod 57,9% (11/19) majki probanada sa DMD fenotipom i kod 60% majki probanada sa BMD. Takođe, kod probanada sa delecijom 56% (14/25) majki su potvrđene kao nosioci, a kod probanada sa duplikacijom 3 od 4 majke (75%). Od preostalih 9 ženskih srodnika, mutacije su nađene kod njih 4. Prenatalnom dijagnostikom utvrđene su delecije kod jednog muškog i jednog ženskog ploda iste majke. **Zaključak.** Istraživanje je pokazalo da su majke bile nosioci u skoro 60% izolovanih DMD/BMD slučajeva sa delecijama i duplikacijama. Takođe, učestalost majki nosioca kod probanada sa duplikaciom (75%) se pokazala većom nego kod majki probanada sa delecijom (56%). U slučaju majke koja je bila potvrđena kao nosilac, delecija je otkrivena kod njena 2 ploda od 3 ispitana.

Ključne reči: distrofinopatije, MLPA metoda, žene prenosioci, prenatalna dijagnoza.

Introduction

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are diseases that result from mutations in the dystrophin gene (DMD gene, Xp21.1). Duchenne muscular dystrophy is the most severe form of the dystrophinopathies, because of the missing protein dystrophin. The incidence of DMD is 1:3500 in live-born males and it is characterized by an early onset (about the second year of life), with the progressive weakness of skeletal and cardiac muscles, with a fatal outcome in the early twenties [1]. Becker's muscular dystrophy occurs 10 times more rarely and represents a milder form of the disease that manifests mostly around the age of 10, with variability in the clinical features - from practically asymptomatic forms to severe forms such as DMD.

The DMD gene is the largest human gene, its 2.4MB in size, and shows a high rate of spontaneous mutations [2]. The changes that occur in the DMD gene are deletions/duplications of one or more exons in 80% of patients, while mutations of less than one exon are present in 20% of patients (point mutations, microdeletions, microinsertions, and splice site mutations) [3]. The most common mutations in the DMD gene are deletions (65-75%) that show specific distribution in the gene, appearing in the so-called “hot spots”, exons 2-20 (the proximal part of the gene) and exons 45-55 (the distal part of the gene) [4]. Duplications are present in 5-15% of cases and can affect any part of the gene, more often the proximal part [5]. It has been estimated that two-thirds of DMD are familial cases, while one-third of the patients have *de novo* mutations [6]. Also, duplications and point mutations are more likely to be family cases [7].

Duchenne and Becker muscular dystrophies are monogenic diseases, where males as hemizygous are affected, while female are generally phenotypically healthy heterozygous carriers of the mutation. According to the X-linked recessive inheritance model, the affected father passes the mutated allele to his daughters, who will be phenotypically healthy carriers of the disease. A woman, who is a heterozygous carrier of a mutation, has a 50% chance that her daughter inherits a risky X-chromosome and becomes a carrier, or 50% chance that her sons will inherit that chromosome and become ill. In classical literature, based on family history, women are defined as obligatory, probable and possible

carriers of the mutation [8]. According to the risk we have described, it is important to detect women who are phenotypically healthy carriers of the mutation, using molecular genetic testing. The multiplex ligation-dependent method (MLPA) is a direct molecular genetic method that allows a quantitative analysis of all 79 exons of DMD gene and the detection of deletions and duplications in the probands as well as the female carriers of the mutations [9-12]. In addition to direct gene analysis, in cases without deletion or duplication in the probands, an indirect method of gene analysis is applied, which is important in 20-30% of cases resulting in point mutations. Indirect analysis is based on a linkage analysis and implies that it follows the inheritance of polymorphic DNA markers, located within or near the DMD gene, indirectly determining whether a particular family member has inherited a mutation or not. However, indirect detection of mutations has its own limitations, which relate to the possibility of recombination within the gene, i.e. within the analysed region, as well as on the limited information markers [13-16].

The aim of the work

The aim of this paper was to confirm the carrier status of females in the families of DMD/BMD probands, using direct genetic methods.

Methods

Patient samples

The sample consisted of 38 female members (29 mothers and 9 other female relatives) from families of 19 DMD and 12 BMD probands with confirmed deletion/duplication in the dystrophin gene. All the cases of those affected were sporadic, except for one family with two affected sons (only one was analysed in the study). In 25 cases, only the mother of the patient was examined, and in 6 cases the mothers and/or other female relatives of the patient were examined. In addition, 4 cases of prenatal diagnosis of DMD/BMD were included in the study.

The study was approved by the Ethics Committee of the Medical School, University of Belgrade. The study was carried out at the Neurology Clinic, KCS, Belgrade, and at the Institute of Human Genetics, School of Medicine, University of Belgrade. Patients were selected based on clinical parameters: the onset of the disease, the clinical features, EMG findings and elevated levels of keratin-phospho kinase. The genomic DNA for analysis was

isolated from the peripheral blood lymphocytes of the subjects, using the isolation method according to standard procedure [17]. For prenatal diagnosis, the DNA was isolated from chorionic samples using a commercial kit (Qiagen DNA mini kit).

Genetic analysis of the patient and their female relatives

In the probands, we previously applied the ***polymerase chain reaction*** (***PCR***) and/or the multiplex ligation-dependent method (MLPA) for deletion detection, i.e. deletion/duplication in the dystrophin gene [18, 11]. Also, MLPA method was applied for detection of deletion/duplication in female carriers. For prenatal diagnosis of DMD/BMD mutations, after the determination of the sex, the PCR method was used on two male and one female foetus, while the MLPA method was used on one female foetus.

MLPA: In the MLPA analysis, two complementary SALSA MLPA kits P034 and P035 (MRC Holland, the Netherlands) were used, according to the protocol recommended by the manufacturer [19]. The analysis was carried out using an ABI Thermal Cycler Verity and an ABI 3500 Genetic Analyser, and the software was processed using the Coffalyser.Net program (MRC Holland).

PCR analysis: For the PCR analysis of 26 exons of the dystrophin gene, three sets of primers, A, B, and C, were used [18]. The PCR products were analysed using 8% polyacrylamide gel and the nucleic acid electrophoresis standard procedure.

Statistical analysis: For statistical analysis, frequencies and percentages were used as descriptive statistics.

Results

Results of genetic analysis in DMD/BMD probands:

In 19 DMD and 12 BMD (N = 31) probands with major changes in the DMD gene, deletions were identified in 27 (87.1%), and duplications in 4 (12.9%). The most frequent localization of deletion/duplication was in the “hot spots” of the DMD gene, 22.6% in the

proximal part of the gene (exons 2-20), and 61.3% in the distal part of the gene (exons 45-55), while 5 (16.1%) mutations were outside the predilection regions of the gene. In patients with the DMD phenotype, 15 deletions and 4 duplications were identified, while deletions were found in all patients with the BMD phenotype (Table 1).

Results of genetic analysis in mothers of DMD/BMD probands:

Of the 29 tested mothers of 19 DMD and 10 BMD probands, mutations in the DMD gene were found in 58.6% (17/29). Deletions were found in 82.4% (14/17) and duplications in 17.6% (3/4). All the mutations found in mothers who were carriers were the same as in their affected sons, except in two cases where one mutation was larger, and the other smaller (Table 2).

In relation to the phenotype of the probands, mutations were found in 57.9% (11/19) of mothers whose sons had a DMD phenotype, and 60% (6/10) of mothers whose sons had a BMD phenotype.

In relation to the type of mutation, in probands with deletions, mothers were carries in 56% (14/25) of cases, and in probands with duplications, in 75% (3/4) of cases.

Results of genetic analysis in other female relatives:

Of the 9 other female relatives of the DMD/BMD probands (5 sisters of 2 DMD and 1 BMD probands, 1 grandmother of a DMD proband, 1 niece whose uncle was a BMD proband, and 2 daughters whose father was a BMD proband), mutations were found in 4 (Table 2):

Case no. 5: DMD grandson/grandmother (mother's line) (deletions in exons 49, 50).
Case no. 20: BMD uncle/niece (deletions in exons 45-47)
Case no. 31: BMD father/two daughters (deletions in exon 13); the affected father has a third daughter who was not tested for carrier status.
From the tested sisters of the probands, not one was confirmed as a carrier.

Results of prenatal testing:

A prenatal diagnosis was performed in two of the three daughters whose father suffered from BMD (deletion of exon 13). A prenatal diagnosis was performed in one daughter, tested and confirmed as a carrier (case 31, Table 2), and in another daughter not previously tested on the carrier status.

In the first case, in three pregnancies the PCR method confirmed the deletion of exon 13 in one male foetus, while there was no deletion in the other male foetus. In the third – a female foetus, the MLPA method confirmed heterozygous deletion in exon 13 (carrier).

In the second case, the PCR method did not confirm homozygous deletion in the female foetus, but it is still possible that the female foetus is a heterozygous carrier of the deletion.

Discussion

Our study included 19 DMD (mean age 8.5) and 12 BMD probands (mean age 28.5), where deletions or duplications were found in the DMD gene, using PCR and/or MLPA methods. The incidence of 87.1% deletions and 12.9% duplications, which were established in our sample, correspond to data in literature [3]. In relation to the PCR method, the MLPA method revealed 3 larger deletions, 4 deletions outside of the "hot spots" in the gene and 4 duplications in the probands, which confirms the effectiveness of the MLPA method in the detection of deletions/duplications in the dystrophin gene [11, 20-22]. The largest number of mutations was localized in the distal part of the gene (exons 45-55) in 61.3% (19/31) of cases, which is the most common localisation of deletions/duplications in sporadic DMD/BMD cases [23].

In order to analyse the status of the carriers, a total of 38 female members from the proband families were tested. Of the 29 DMD/BMD tested mothers, a mutation of the DMD gene was found in 58.6% (17/29) of cases, which is similar to the results of other authors [24]. In

82.4% (14/17) of mothers, we discovered deletion, and in 17.6% (3/17) duplication. Mutations found in mothers were the same as their affected sons, except in two cases.

In sporadic DMD cases, it was estimated that 2/3 mothers were carriers of the mutation, 5-10% had gonadal mosaicism, while 25-30% had no mutation. In our study, in sporadic DMD cases, the mother was confirmed as carrier in 57.9% (11/19) of cases, a lower value than the estimated 2/3. Other authors also found this percentage to be lower than expected and that the detection of female carriers of the mutation, as well as the possibility of prenatal diagnosis, has led to a reduction in the number of children born from carrier mothers [25, 26]. In the sporadic BMD cases of our sample 60% (6/10) of the mothers were carriers, which is lower than expected, since the midler form of disease in BMD patients allows them to have offspring and transfer the risky X chromosome to their daughters, who transfer it to the next generation. For example, Lee et al. found that the mothers of BMD probands were carriers in 89.5% of cases [26]. In relation to the type of mutation in the probands, for deletions we established that mothers were carriers in 56% (14/25) of cases, and for duplications in 75% (3/4) of cases. This finding is in accordance with the above, i.e. the risk of a mother being a carrier is greater for certain types of mutations [7, 27].

Of the other female relatives of the probands, mutations were present in 4 out of 9 cases. As there were known mutations in the probands, based on MLPA findings in female relatives, we could conclude if they are carriers or not. However, in familial cases when the mutation cannot be identified, for a mother who is an obligatory carrier there is a 50% risk that her daughter is also a carrier. A negative MLPA finding reduces the risk of the daughter being a carrier to 26.5%, and if the further analysis of gene sequencing is also negative, her risk of being a carrier is 3% [28].

Also, in our study, a prenatal diagnosis was performed on two of the three daughters whose father had BMD (Table 2, Case 31). Two daughters were tested for carrier status and were positive, while one was not tested. A prenatal diagnosis in three pregnancies was carried out on one daughter (confirmed carrier) - a deletion was found in one male foetus and one female foetus (carrier), while one male foetus was healthy. In the case of a daughter who was not tested, but also considered to be a obligatory carrier, homozygous deletion was not

confirmed in the female foetus (Figure 1), which does not exclude the possibility of being a heterozygous carrier.

In some countries it is recommended that testing for carrier status is performed when the female child reaches the age to decide on testing independently, which is in line with ethical principles. However, experience shows that in the Netherlands, for example, 78% of girls over 16 years of age have not yet been tested, and the probable cause is that the average age of motherhood is about 28 years of age, so they are tested later [29], while 1/3 of potential carriers are not tested at all [30]. It has been found that only 52.7% of women at risk for DMD/BMD were tested for carrier status before conception [31]. It should be kept in mind that, in addition to the risk of bearing affected offspring, 10% of female carriers develop cardiomyopathy [32,33], so early detection of carrier status would allow adequate cardiac monitoring of these women, which is recommended from the age of 16 [34]. In our environment, it is practice for parents to decide on testing carriers even prenatally. It is therefore of great importance to inform members of families affected by DMD/BMD about the nature of the disease, its inheritance and possible risks, as well as about the method of testing and possible prevention.

In our sample, 41.4% (12/29) of mothers were not confirmed as carriers, suggesting that mutation in the probands was new. Previously it was mentioned that 1/3 mutations in the DMD probands were *de novo*. This occurrence is explained by an early fatal outcome in DMD patients, which leads to the elimination of 1/3 mutations from the population, but this number is offset by the emergence of new mutations in the next generation [2]. It was found that *de novo* mutations are the most common deletions originating in oogenesis, while duplications and point mutations are mainly due to events during spermatogenesis [35, 28]. The problem is that *de novo* mutation in the DMD gene is clinically diagnosed only after the child experiences symptoms (about the second year at DMD, later at BMD), and at a molecular level, sometimes significantly later - as our sample shows. It is particularly difficult when a second male child is born before the diagnosis of an older brother is confirmed.

Modern molecular genetic tests have enabled the reliable detection of carrier status. In familial cases of DMD/BMD, detection of the mutation in probands and testing for carrier status in female members in the family, enables the determination of risk in the progeny and the provision of adequate genetic advice. In sporadic cases, when a mother has one affected son, the risk of recurrence depends on whether she is a carrier of the mutation or not. If molecular analysis of the mother does not determine the presence of mutation in somatic cells, the risk of recurrence is significantly reduced. However, when giving genetic advice, it should be noted that in 10-15% of cases there may be gonadal mosaicism in the mother, and the smallest calculated risk is 4.3% [36]. When it comes to *de novo* mutations, they remain the biggest problem. The solution could be the introduction of screening for prenatal detection of mutations in the DMD gene in the male foetuses, but there is still no consensus in the literature for this.

Conclusion

In 31 DMD/BMD probands, we identified 87.1% deletions and 12.9% duplications of one or more exons. Mothers were confirmed as carriers in almost 60% of sporadic DMD/BMD cases with deletions and duplications (mutations were found in 57.9% mothers of DMD probands, and in 60% mothers of BMD). Also, the carrier frequency tended to be higher in mothers of the probands with duplication (75%) then in mothers of the probands with deletions (56%). Of the 9 other female relatives, mutations were found in 4. In the case of a mother who was confirmed as a carrier, deletion was detected in 2 of 3 foetuses. These results point to the importance and need to determine mutations in probands, as well as the status of the carrier of the mutation of women in families with Duchenne and Becker muscular dystrophies, which will allow individuals and other family members to receive adequate genetic advice.

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Table 1

Genetic analysis in DMD/BMD probands

phenotype	deletion	duplication	total
Duchenne	15	4	19
Becker	12	/	12

Table 2

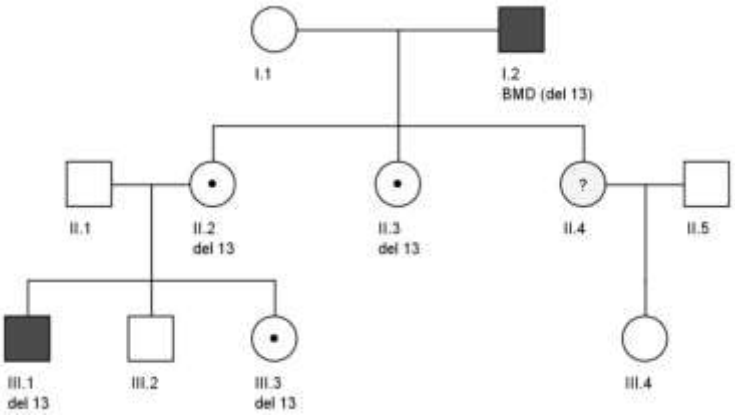
Mutations found in probands and their female relatives

Phenotype of proband	Age of proband	Mutation/proband	Mutation/mother	Mutation /other female relatives
1-DMD	19	del*1	no del/dupl	
2-DMD	8	dupl† 8-16	no del/dupl	
3-DMD	5	del 25-43	no del/dupl	
4-DMD	2	del 50	no del/dupl	
5-DMD	6	del 49,50	del 49,50	grandmother - del 49,50
6-DMD	9	del 45-52	del 45-52	
7-DMD	12	del 1, DP427c	del 1, DP427c	
8-DMD	/	del 59	del 59	
9-DMD	7	del 35-52	del 35-52	
10-DMD	21	dupl 2	dupl 2	sister 1, 2 - no del/dupl
11-DMD	16	del 45-50	no del/dupl	
12-DMD	6	del 46-50	no del/dupl	
13-DMD	6	del 44	no del/dupl	
14-DMD	2	del 33,34	del 33,34	
15-DMD	9	dupl 18-42 i 45-48	dupl 18-42 i 45-48	
16-DMD	7	dupl 52-62	dupl 52-62	
17-DMD	6	del 12-19	no del/dupl	
18-DMD	4	del 46-52	del 46-55	
19-DMD	9	del 3-15	del 3-15	sister - no del/dupl
20-BMD	34	del 44-48		niece – del 45-47
21-BMD	38	del 44-49	del 45-47	
22-BMD	34	del 48	del 48	
23-BMD	33	del 45-47	del 45-47	
24-BMD	24	del 45-49	no del/dupl	
25-BMD	22	del 45-49	no del/dupl	
26-BMD	30	del 45-48	del 45-48	
27-BMD	32	del 45-48	del 45-48	

28-BMD	14	del 9-12	no del/dupl	
29-BMD	24	del 12-43	no del/dupl	sister 1 i 2 - no del/dupl
30-BMD	/	del 45-48	del 45-48	
31-BMD	/	del 13		daughter 1 i 2 – del 13

*del – deletion, †dupl- duplication

Figure 1. Genealogy of case 31 – BMD. Daughter II.2 – male foetus III.1 has del 13 and female foetus III.3 has del 13; daughter II.4 has female foetus without homozygous deletion.



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