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The importance of direct genetic testing for determining female carriers of the mutation in dystrophinopathies

Značaj direktnog genetičkog testiranja za otkrivanje žena prenosioca mutacije kod distrofinopatija

Jasmina Maksić*, Nela Maksimović[†], Lukas Rasulić[†‡], Olgica Milankov[§||], Ana Marjanović[¶], Dragana Cvetković, Vidosava Rakočević Stojanović[†¶], Ivana Novaković[†]**

University of Belgrade, *Faculty for Special Education and Rehabilitation, [†]Faculty of Medicine, **Faculty of Biology, Belgrade, Serbia; University Clinical Center of Serbia, [‡]Clinic for Neurosurgery, [¶]Clinic for Neurology, Belgrade, Serbia; [§]Institute for Health Care of Children and Youth of Vojvodina, Novi Sad, Serbia; [||]University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia

Abstract

Background/Aim. Duchenne muscular dystrophy (MD) and Becker MD are caused by mutations in the gene for dystrophin (*DMD*). They are X chromosome-linked recessive diseases where males are affected, and females are healthy carriers of the mutation in most cases. It is estimated that 2/3 of mothers of Duchenne MD probands are carriers, while 1/3 of probands have *de novo* mutations. The aim of the study was to confirm the carrier status of female members of the families of Duchenne MD/Becker MD probands using direct genetic testing methods. **Methods.** The study included 38 females from 31 families of Duchenne MD/Becker MD probands with deletion/duplication in the *DMD* gene. Moreover, 4 cases of prenatal diagnosis of Duchenne MD/Becker MD were included. The methods of polymerase chain reaction - PCR and the multiplex ligation-dependent probe amplification - MLPA were applied for detecting deletions, i.e., deletion/duplication mutations in the *DMD* gene. **Results.** In the total of 31 Duchenne MD/Becker MD probands, 87.1% of deletions and 12.9% of duplications of one or more exons in the *DMD* gene were detected. Of the 29 tested mothers, mutations were found in 17 of them (14 deletions and 3 duplications). Mutations were detected in 11 (57.9%) out of 19 mothers of probands with the Duchenne MD phenotype and 6 (60%) out of 10 mothers of Becker MD probands. Furthermore, 14 (56%) out of 25 mothers were carriers in probands with deletions, and 3 (75%) out of 4 mothers were carriers in probands with duplications. In the remaining 9 other female relatives of the patients, mutations were found in 4. In prenatal diagnosis, we identified a deletion in one male and one female fetus of one single mother who was confirmed as a carrier. **Conclusion.** The study showed that mothers were carriers in almost 60% of sporadic cases of Duchenne MD/Becker MD with deletions and duplications. In addition, the carrier frequency tended to be higher in mothers of the probands with duplications (75%) compared to mothers of probands with deletions (56%).

Key words:
genes; genetic testing; muscular dystrophies; mutation; prenatal diagnosis; women.

Apstrakt

Uvod/Cilj. Dišenova mišićna distrofija (MD) i Bekerova MD su uzrokovane mutacijama u genu za distrofin (*DMD*). To su recesivne bolesti vezane za X hromozom, od kojih obolevaju muškarci, a žene su uglavnom zdravi nosioci mutacije. Procenjeno je da su kod probanada obolelih od Dišenove MD 2/3 majki nosioci mutacije, dok 1/3 probanada ima *de novo* mutaciju. Cilj rada bio je da se potvrdi status nosioca mutacije kod ženskih članova porodica probanada obolelih od Dišenove MD/Bekerove MD primenom metoda direktnog genetičkog testiranja. **Metode.** Studija je obuhvatila ukupno 38 žena iz 31 porodice probanada obolelih od Dišenove MD/Bekerove MD sa delecijom/duplikacijom u *DMD* genu. Takođe, u studiju su bila uključena i 4 slučaja Dišenove MD/Bekerove MD otkrivena prenatalnom dijagnostikom. Metoda lančane reakcije polimeraze (*polymerase chain reaction* – PCR) i metoda višestrukog umnožavanja vezanih proba (*multiplex ligation-dependent probe amplification* -MLPA) su korišćene za detekciju delecija, odnosno delecija/duplikacija mutacija u *DMD* genu. **Rezultati.** Kod ukupno 31 probanada obolelih od Dišenove

Correspondence to: Lukas Rasulić, University Clinical Center of Serbia, Clinic for Neurosurgery, Dr. Koste Todorovića 4, 11 000 Belgrade, Serbia. E-mail: lukas.rasulic@gmail.com

MD/Bekerove MD, utvrđeno je 87,1% mutacija tipa delecije i 12,9% mutacija tipa duplikacija jednog ili više egzona u *DMD* genu. Od 29 testiranih majki probanada, mutacije su nađene kod njih 17 (14 delecija i 3 duplikacije). Mutacije su detektovane kod 11 (57,9%) od 19 majki probanada sa fenotipom Dišenove MD i kod 6 (60%) od 10 majki probanada obolelih od Bekerove MD. Takođe, kod probanada sa delecijom, kod 14 (56%) od 25 majki je potvrđeno da su nosioci mutacije, a kod probanada sa duplikacijom, 3 (75%) od 4 majke su bile nosioci mutacije. Od ostalih 9 ženskih srodnika probanada obolelih od Dišenove MD/Bekerove MD, mutacije su nađene kod nijh 4. Prenatalnom dijagnostikom utvrđene su delecije kod jednog muškog i jednog ženskog fetusa iste majke koja je bila potvrđena kao nosilac mutacije. **Zaključak.** Istraživanje je pokazalo da su majke bile nosioci mutacija u skoro 60% izolovanih slučajeva obolelih od Dišenove MD/Bekerove MD sa delecijama i duplikacijama. Takođe, učestalost majki nosioca mutacije kod probanada sa duplikaciom (75%) se pokazala višom nego kod majki probanada sa delecijom (56%).

Ključne reči:
geni; genetičko testiranje; mišići, distrofija; mutacija; prenatalna dijagnoza; žene.

Introduction

Duchenne muscular dystrophy (MD) and Becker MD are diseases that result from mutations in the dystrophin gene (*DMD)*. The gene is located on the short arm of the X chromosome (Xp21.1). Duchenne MD is the most severe form of dystrophinopathies because of the missing protein dystrophin. The incidence of Duchenne MD is 1 : 3,500 in liveborn males, and it is characterized by early onset (approximately the second year of life), progressive weakness of skeletal and cardiac muscles, and a fatal outcome in the early twenties [1]. Becker MD occurs 10 times less often and represents a milder form of the disease that starts to manifest mostly around the age of 10, with variability in the clinical features – from practically asymptomatic forms to severe forms such as Duchenne MD.

The *DMD* gene is the largest human gene – it is 2.4 megabases in size and shows a high rate of spontaneous mutations [2]. The changes occurring 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 the specific distribution in the gene, appearing in the so-called “hot spots” of the 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 Duchenne MD are familial cases, while one-third of the patients have *de novo* mutations [6]. Likewise, duplications and point mutations are more likely to be family cases [7].

Duchenne MD and Becker MD are monogenic diseases, where males as hemizygous are affected, while females 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 the mutation, has a 50% chance that her daughter will inherit a risky X-chromosome and become a carrier or a 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 probe amplification (MLPA) is a direct molecular genetic method that allows a quantitative analysis of all 79 exons of the *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. The indirect analysis is based on 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. However, indirect detection of mutations has its own limitations, which relate to the possibility of recombination within the gene, i.e., within the analyzed region, as well as on the limited information markers [13–16].

The aim of this study was to confirm the carrier status of females in the families of Duchenne MD/Becker MD probands using direct genetic methods.

Methods

Study groups

The study group consisted of 38 female members (29 mothers and 9 other female relatives) from families of 19 Duchenne MD and 12 Becker MD probands with confirmed deletion/duplication in the *DMD* gene. All the cases of those affected were sporadic, except for one family with two affected sons (only one was analyzed in the study). In 25 cases, only the mothers of the patients were tested, and in 6 cases, the mothers and/or other female relatives of the patients were tested. In addition, 4 cases of prenatal diagnosis of Duchenne MD/Becker MD were included in the study.

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. The study was carried out at the Clinic for Neurology, University Clinical Center of Serbia, Belgrade, and at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade. The second study group consisted of patients (probands) selected based on the following clinical parameters: the onset of the

disease, the clinical features, electromyography findings, and elevated levels of creatine phosphokinase.

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 villus samples using a commercial kit (Qiagen DNA mini kit).

Genetic analysis of the patients and their female relatives

In the probands, we previously applied the polymerase chain reaction (PCR) and/or the multiplex ligation-dependent probe amplification (MLPA) for detecting deletions/duplications in the *DMD* gene [18, 11]. Likewise, the MLPA method was applied to detect deletions/duplications in female carriers. For prenatal diagnosis of Duchenne MD/Becker MD mutations, after determining the sex, the PCR method was used on one female and two male fetuses, while the MLPA method was used on one female fetus.

MLPA method

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

PCR analysis

For the PCR analysis of 26 exons of the *DMD* gene, three sets of primers, A, B, and C, were used [18]. The PCR products were analyzed 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 of Duchenne MD/Becker MD probands

In 19 Duchenne MD and 12 Becker MD probands (total n = 31) with major changes in the *DMD* gene, deletions were identified in 27 (87.1%) and duplications in 4 (12.9%) probands. 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 Duchenne MD phenotype, 15 deletions and 4 duplications were identified, while deletions were found in all patients with the Becker MD phenotype (Table 1).

Results of genetic analysis of mothers of Duchenne MD/Becker MD probands

Of the 29 tested mothers of 19 Duchenne MD and 10 Becker MD probands, mutations in the *DMD* gene were found in 17 (58.6%) out of 29 mothers. Deletions were detected in 14 (82.4%) out of 17 mothers, and duplications were detected in 3 (17.6%) out of 17 mothers. 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 bigger and the other smaller (Table 2).

Concerning the phenotype of the probands, mutations were found in 11 (57.9%) out of 19 mothers whose sons had a Duchenne MD phenotype and 6 (60%) out of 10 mothers whose sons had a Becker MD phenotype.

Concerning the type of mutation, in probands with deletions, mothers were carriers in 14 (56%) out of 25 cases, and in probands with duplications, mothers were carriers in 3 (75%) out of 4 cases.

Results of genetic analysis in other female relatives

Out of the remaining 9 female relatives of the Duchenne MD/Becker MD probands (5 sisters of 2 Duchenne MD and 1 Becker MD probands, 1 grandmother of a Duchenne MD proband, 1 niece whose uncle was a Becker MD proband, and 2 daughters whose father was a Becker MD proband), mutations were found in 4 [Table 2, case No. 5 (Duchenne MD) – grandson whose grandmother (mother’s line) had deletions in exons 49, 50; case No. 20 (Becker MD) – uncle had deletions in exons 45–47, his niece was a carrier of mutation; case No. 31 (Becker MD) – father had deletions in exon 13, 2 daughters were tested, and both were confirmed as carriers. The affected father had a third daughter who was not tested for carrier status].

Out of all the tested sisters of the probands, not one was confirmed as a carrier.

Results of prenatal testing

A prenatal diagnosis was performed on two of the three daughters whose father suffered from Becker MD (deletion of exon 13). A prenatal diagnosis was performed

Table 1

Genetic analysis of Duchenne MD and Becker MD probands

Phenotype	Deletion	Duplication	Total
Duchenne MD	15	4	19
Becker MD	12	/	12

MD – muscular dystrophy.
Results are given as number of Duchenne MD or Becker MD probands.

for the pregnancies of one daughter, previously tested and confirmed as a carrier (case No. 31, Table 2), and for the other daughter not previously tested on the carrier status. For the first daughter, in three pregnancies, the PCR method confirmed the deletion of exon 13 in one male fetus, while there was no deletion in the other male fetus. In the third – female fetus, the MLPA method confirmed heterozygous deletion in exon 13 (carrier) (Figure 1).

As for the second daughter, the PCR method did not confirm homozygous deletion in her female fetus, but it is still possible that the female fetus is a heterozygous carrier of the deletion.

Table 2

Mutations found in Duchenne MD and Becker MD probands and their female relatives

Proband phenotype (case number)	Proband age (years)	Proband mutation type	Mother mutation type	Other female relatives mutation type
1- Duchenne MD	19	del 1	no del/dupl	
2- Duchenne MD	8	dupl 8-16	no del/dupl	
3- Duchenne MD	5	del 25-43	no del/dupl	
4- Duchenne MD	2	del 50	no del/dupl	
5- Duchenne MD	6	del 49,50	del 49,50	grandmother (del 49,50)
6- Duchenne MD	9	del 45-52	del 45-52	
7- Duchenne MD	12	del 1, DP427c	del 1, DP427c	
8- Duchenne MD	/	del 59	del 59	
9- Duchenne MD	7	del 35-52	del 35-52	
10- Duchenne MD	21	dupl 2	dupl 2	sister 1, 2 (no del/dupl)
11- Duchenne MD	16	del 45-50	no del/dupl	
12- Duchenne MD	6	del 46-50	no del/dupl	
13- Duchenne MD	6	del 44	no del/dupl	
14- Duchenne MD	2	del 33,34	del 33,34	
15- Duchenne MD	9	dupl 18-42 and 45-48	dupl 18-42 and 45-48	
16- Duchenne MD	7	dupl 52-62	dupl 52-62	
17- Duchenne MD	6	del 12-19	no del/dupl	
18- Duchenne MD	4	del 46-52	del 46-55	
19- Duchenne MD	9	del 3-15	del 3-15	sister (no del/dupl)
20- Becker MD	34	del 44-48	/	niece (del 45-47)
21- Becker MD	38	del 44-49	del 45-47	
22- Becker MD	34	del 48	del 48	
23- Becker MD	33	del 45-47	del 45-47	
24- Becker MD	24	del 45-49	no del/dupl	
25- Becker MD	22	del 45-49	no del/dupl	
26- Becker MD	30	del 45-48	del 45-48	
27- Becker MD	32	del 45-48	del 45-48	
28- Becker MD	14	del 9-12	no del/dupl	
29- Becker MD	24	del 12-43	no del/dupl	sister 1 and 2 (no del/dupl)
30- Becker MD	/	del 45-48	del 45-48	
31- Becker MD	/	del 13	/	daughter 1 and 2 (del 13)

MD –muscular dystrophy; del – deletion; dupl – duplication.

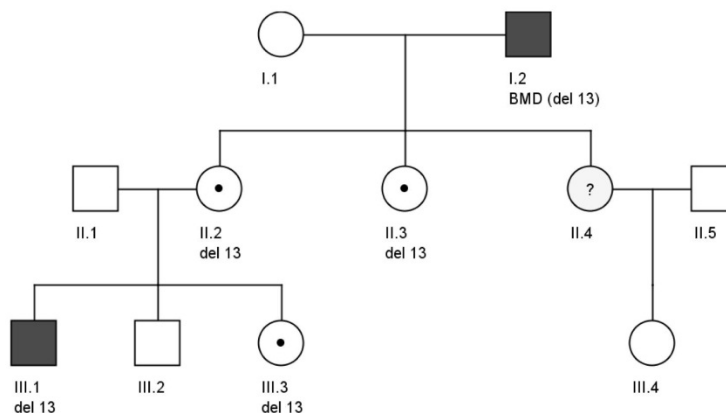

Fig. 1 – Genealogy of case 31 – Becker muscular dystrophy (BMD) proband. Daughter II.2 is a confirmed carrier; she had a male fetus III.1 with del 13 and a female fetus III.3 with del 13. Daughter II.4 has not been tested for being a potential carrier; she had a female fetus without homozygous deletion. del – deletion.

Discussion

Our study included 19 Duchenne MD probands (mean age 8.5 years) and 12 Becker MD probands (mean age 28.5 years), where deletions or duplications were found in the *DMD* gene, using PCR and/or MLPA methods. The incidence of 87.1% of deletions and 12.9% of duplications, established in our group of probands, correspond to the data in literature [3]. 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 *DMD* gene [11, 20–22]. The largest number of mutations was localized in the distal part of the gene (exons 45–55) in 19 out of 31 (61.3%) cases, which is the most common localization of deletions/duplications in sporadic Duchenne MD/Becker MD cases [23].

In order to analyze the status of the carriers, a total of 38 female members from the proband families were tested. Of the 29 Duchenne MD/Becker MD proband mothers tested, a mutation of the *DMD* gene was found in 17 (58.6%) out of 29 mothers, similar to the results of other authors [24]. In 14 (82.4%) out of 17 mothers, we discovered deletion, and in 3 (17.6%) out of 17 mothers, we detected a duplication. Mutations found in mothers were the same as in their affected sons, except in two cases.

In sporadic Duchenne MD cases, it was estimated that 2/3 of the mothers were carriers of the mutation, 5–10% had gonadal mosaicism, while 25–30% had no mutation. In our study, in sporadic Duchenne MD cases, the mother was confirmed as a carrier in 11 (57.9%) out of 19 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 [7, 25]. In the sporadic Becker MD cases of our sample, 6 (60%) out of 10 mothers were carriers, which is lower than expected since the milder form of the disease in Becker MD patients allows them to have offspring and transfer the risky X chromosome to their daughters, who transfer it to the next generation. For instance, Lee et al. [25] found that the mothers of Becker MD probands were carriers in 89.5% of cases. In relation to the type of mutation in the probands, we established that mothers were carriers in 14 (56%) out of 25 cases for deletions and 3 (75%) out of 4 cases for duplications. This finding is in accordance with the abovementioned, i.e., the risk of a mother being a carrier is greater for certain types of mutations [7, 26].

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, we could conclude whether they were carriers based on the MLPA findings in female relatives. However, in familial cases when the mutation cannot be identified, a mother who is an obligatory carrier has a 50% risk that her daughter will also be 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% [27].

Furthermore, in our study, a prenatal diagnosis was performed on two of the three daughters whose father had Becker MD (Table 2, case No. 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 fetus and one female fetus (carrier), while one male fetus was healthy. In the case of the daughter who was not tested but also considered an obligatory carrier, homozygous deletion was not confirmed in the female fetus (Figure 1), which does not exclude the possibility of the fetus 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 instance, 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 [28], while 1/3 of potential carriers are not tested at all [29]. It has been found that only 52.7% of women at risk of being a carrier of Duchenne MD/Becker MD gene mutations were tested for carrier status before conception [30]. It should be kept in mind that, in addition to the risk of bearing affected offspring, 10% of female carriers develop cardiomyopathy [31, 32], so early detection of carrier status would allow adequate cardiac monitoring of these women, which is recommended by the age of 16 [33]. In our environment, the practice is to let parents decide on testing the carriers, even prenatally. It is, therefore, of great importance to inform members of families affected by Duchenne MD/Becker MD about the nature of the disease, its inheritance, and possible risks, as well as about the method of testing and possible prevention.

In our study group, 12 (41.4%) out of 29 mothers were not confirmed as carriers, suggesting that mutation in the probands was new. It was mentioned earlier that 1/3 of mutations in the Duchenne MD probands were *de novo*. This occurrence is explained by an early fatal outcome in Duchenne MD patients, which leads to the elimination of 1/3 of 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 mainly occur due to events during spermatogenesis [26]. 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 Duchenne MD, later at Becker MD) and at a molecular level, sometimes significantly later – as shown in our sample. It is particularly difficult when a second male child is born before the diagnosis of the older brother is confirmed.

Modern molecular genetic tests have enabled the reliable detection of carrier status. In familial cases of Duchenne MD/Becker MD, 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. If molecular analysis of the mother does not determine the presence of a 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% [34]. 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 male fetuses, but there is still no consensus in the literature for this.

Conclusion

In 31 Duchenne MD/Becker MD probands, we identified 87.1% of deletions and 12.9% of duplications of one or more exons. Mothers were confirmed as carriers in almost 60% of sporadic Duchenne MD/Becker MD cases with deletions and duplications (mutations were found in 57.9% of mothers of Duchenne MD probands and 60% of mothers of Becker MD probands). In addition, the carrier frequency tended to be higher in mothers of the probands with duplications (75%) than 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 confirmed as a carrier, deletion was detected in 2 out of 3 of her fetuses. 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 female members in families with Duchenne MD and Becker MD, which will allow individuals and other family members to receive adequate genetic advice.

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