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Analysis of duplications versus deletions in the dystrophin gene in Serbian cohort with dystrophinopathies

Uporedna analiza duplikacija i delecija u genu za distrofin u grupi bolesnika sa distrofinopatijom iz Srbije

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Abstract

Background/Aim. Duchenne muscular dystrophy (DMD) and its allelic form Becker muscular dystrophy (BMD) are X-linked diseases that affect males, characterized by progressive muscle and cardiopulmonary weakness, especially in DMD as a severe form of the disease. They result from mutations in the dystrophin gene, and the most common changes are large intragenic deletions and duplications (80%). One third of patients have *de novo* mutation and 2/3 of the mothers are estimated as carriers. The aim of the study was to analyze the frequency of duplications versus deletions in the dystrophin gene in patients with dystrophinopathies, as well as to analyze the phenotypic effect of large mutations obtained and to determine the carrier status of female relatives in probands with duplications. **Methods.** We examined 22 DMD and 35 BMD unrelated patients and 6 female relatives of the probands where duplications were found. We used polymerase chain reaction (PCR) and multiplex ligation-dependent probe amplification (MLPA) methods, according to the protocol, to detect or confirm mutations in probands and female carriers. **Results**. In probands, there were 34 (59.6%) large deletions (mostly affected exons 44–60) and 6 (10.5%) large duplications in 4 DMD and 2 BMD patients. Also, duplications were found in 3 out of 4 (75%) tested mothers. The distribution of duplications was heterogeneous, affecting N-terminal and central rod domain, and included more exons, except for one DMD patient who had duplication of exon 2. An exception from the Monaco rule was present in 9.5% of DMD and 15.8% of BMD probands, i.e. in 12.5% of DMD/BMD cases. **Conclusion.** In 57 DMD/BMD probands, we found 59.6% of large deletions and 10.5% of large duplications. The most affected region of the DMD gene was the central rod domain. An exception to Monaco's rule was present in 12.5% of DMD/BMD cases. Three out of 4 examined proband's mothers were confirmed as carriers.

Key words:
gene deletion; gene duplication; genetics, medical; genetic diseases, inborn; muscular dystrophy, duchenne; women.

Apstrakt

Uvod/Cilj. Dišenova mišićna distrofija (DMD) i njegova alelna forma, Bekerova mišićna distrofija (BMD), su X-vezane nasledne bolesti od kojih obolevaju muškarci, a karakteriše ih progresivna mišićna i kardiopulmonalna slabost, posebno kod DMD kao težeg oblika bolesti. Ove bolesti nastaju kao posledica mutacija u genu za distrofin, a najčešće su prisutne intragenske delecije i duplikacije (80%). Novonastalu mutaciju ima1/3 bolesnika, a procenjeno je da su 2/3 majki nosioci. Cilj rada je bio da se analizira učestalost duplikacija u odnosu na delecije u genu za distrofin kod bolesnika sa distrofinopatijom, kao i da se ispita efekat dobijenih mutacija na fenotip kod probanda i utvrdimo status nosioca kod ženskih srodnika probanda sa duplikacijama. **Metode.** Studijom je bilo obuhvaćeno 22 DMD i 35 BMD nesrodnih bolesnika i šest ženskih srodnika probanda kod kojih su bile otkrivene duplikacije. Za otkrivanje ili potvrdu mutacije, kod probanda i ženskih nosioca, korišćene su metode: lančana reakcija polimerazom (PCR) i višestruko umnožavanje vezanih sondi (MLPA), prema datom protokolu. **Rezultati.** Kod probanda je nađeno 34 (59,6%) velikih delecija (najčešće su bili zahvaćeni egzoni 44–60) i 6 velikih duplikacija (10,5%) kod 4 DMD i 2 BMD bolesnika.

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Takođe, duplikacije su nađene kod 3 od 4 (75%) testirane majke. Distribucija duplikacija je bila heterogena, obuhvatala je N-terminalni i štapićasti region i uključivala je veći broj egzona, osim kod jednog DMD bolesnika koji je imao duplikaciju egzona 2. Odstupanje od Monakovog pravila je bilo prisutno kod 9,5% DMD probanda, odnosno kod 15,8% BMD probanda, to jest kod 12,5% slučajeva. **Zaključak.** Kod 57 DMD/BMD probanda nađeno je 59,6% velikih delecija i 10,5% velikih duplikacija. Najčešće je bio zahvaćen štapićasti domen u DMD genu. Odstupanje od Monakovog pravila je bilo prisutno u 12,5% DMD/BMD slučajeva. Tri od četiri ispitane majke probanda su bile potvrđene kao nosioci.

Ključne reči:
geni, delecija; geni, duplikacija; genetika, medicinska; genetičke bolesti, urođene; distrofija, mišićna, dišen; žene.

Introduction

Duchene muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are dystrophinopathies that result from mutations in the gene encoding the dystrophin protein. They have X-linked recessive inherited pattern, where male family members are affected, while women are mostly the healthy carriers of the disease. DMD is the most severe form of dystrophinopathy, with an incidence of 1: 3,500 live-born males. Symptoms of the illness occur around the third year of life, progressing over time with increasingly pronounced weakness of the skeletal and cardiac muscles (cardio/pulmonary weakness) in the twentieth year of life. Its allele form, BMD, is characterized by a lower incidence, a later onset of the disease, a slower progression of symptoms and sometimes by a very mild clinical picture[1]. The gene for dystrophin protein (DMD gene) is located on the short arm of the X chromosome (Xp21.2–p21.1). With a size of 2.4 million base pairs, 79 exons and a main transcript length of 14 kb, it provides the full length of the protein (427 kDa) [2]. Four functional domains can be distinguished in the structure of dystrophin. Through the N-terminal domain, dystrophin binds to the f-actin of the cytoskeleton, and through the C-terminal domain it binds with proteins and glycoproteins of the sarcolemma, so called dystrophin associated-proteins which produce a dystrophin-glycoprotein complex [3, 4]. This complex stabilizes the sarcolemma and protects muscle fibers from damage caused by their long-term contraction [3, 5].

Due to its extreme size, the DMD gene is often subject to change. Of all the mutations in the DMD gene, in 65%–70% of cases there are intragenic deletions of one or more exons. The disposition of the detected deletions in the gene is specific, and the most commonly affected are exons 45–55 (the distal part of the gene), and exons 2–20 (the proximal part), the so-called "hot spots". Duplications are present in 5%–15% of cases, while the remaining cases are due to small mutations (less than one exon) – point mutations, small deletions, small insertions, splice sites changes [6, 7]. One-third of the mutations in the DMD gene are *de novo* mutations. It has been shown that the size and localization of the mutations are not correlated with the severity of the clinical presentation, bearing in mind that small lead to a more severe DMD phenotype, i.e. mutations of a large number of exons can result in a milder BMD phenotype. According to the Monaco rule, the effect of mutations on the phenotype depends on whether the mutation changes the reading frame of the genetic code or not [8]. The severe clinical presentation of DMD patients is the result of frameshift mutations in the dystrophin gene. These mutations (deletions or duplications) change the reading frame (out-of-frame), leading to the creation of practically undetectable amounts of shortened, nonfunctional protein. Deletions that do not change the reading frame (in-frame), result in the creation of shortened, partially functional protein, which is associated with a milder clinical presentation of BMD patients. However, about 10% of DMD/BMD patients deviate from this rule [9].

Grouping deletions in predilected areas in the DMD gene facilitates their detection. Multiplex polymerase chain reaction (PCR) is a technique that identifies 98% of all deletions in the DMD gene by analyzing 19 exons [10, 11]. The disadvantage of this method is that it does not detect duplications in the DMD gene of the proband, nor mutations in female carriers. For this purpose, Southern blotting, quantitative PCR (qPCR) and multiplex amplification and probe hybridization (MAPH) methods were used, but have proved complicated for routine application in practice [12]. In recent years, the principal method for detecting deletions and duplications in the DMD gene is multiplex ligation-dependent probe amplification (MLPA). The MLPA method enables the analysis of all 79 exons in the dystrophin gene and the detection of deletions which are not in predilected areas as well as duplications in the DMD gene; it is particularly important in determining the carrier status of female members [13–15]. However, point mutations cannot be detected by these methods.

The aim of study was to detect or confirm mutations in DMD/BMD probands, analyze the mutations obtained, and in patients with proven duplications, to determine the carrier status of the female members in the family.

Methods

The study group consisted of a total of 63 respondents, 57 unrelated DMD/BMD patients (22 DMD and 35 BMD) and 6 female relatives. The study was conducted at the Neurology Clinic, Clinical Center of Serbia, Belgrade and at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade. Patients were selected according to the clinical parameters for DMD/BMD [the onset of the disease, the clinical presentation, electromyography (EMG) findings, hyper creatine phosphokinase (CPK)]. The genomic DNA was extracted from the peripheral blood samples of the subjects according to standard salting-out procedure [16]. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade.

The multiplex PCR method was applied to one group of patients. For the analysis of 26 exons of the DMD gene, three sets of primers were used – A, B, and C. Set A covered exons: 4, 8, 12, 17, 19, 44, 45, 46, 48, 51; Set B covered exons: Pm, 3, 6, 13, 43, 47, 50, 52, 60; and Set C covered exons: Pb, 16, 32, 34, 41, 42, 49 [17]. The analysis was performed according to the DMD/BMD multiplex PCR protocol [18].

The MLPA method was applied to newly diagnosed patients who did not previously have PCR, as well as to those later diagnosed to have. In patients with no deletions in the DMD gene detected by the PCR method, the MLPA was used to detect either deletions in other areas of the gene, or gene duplication. In patients where a deletion was detected using the PCR method, the MLPA was applied in order to more precisely define the mutation rates. In the probands with duplications found, the MLPA method was also used to examine the female members of the families. Two complementary SALSA MLPA kits, P034 and P035 (MRC Holland, Netherlands) were used to detect duplications and deletions in the DMD gene, according to the given protocol [13]. The analysis was carried out using ABI Thermal CyclerVerity and ABI 3500 Genetic Analyzer apparatus, and soft data processing was carried out using Coffalyser. Net. To predict the effect of duplication on the phenotype, we used the Reading-frame checker, version 1.9, which is available at www.dmd.nl [19].

For statistical analysis, frequencies, percentages, means and standard deviations (SD) were used as descriptive statistics and the χ^2 test for interrelation between variables. Analyses were performed in SPSS Statistics, version 20.

Results

The total sample consisted of 63 respondents, 57 unrelated DMD/BMD patients and 6 female relatives of probands where duplications were found. First, 57 unrelated patients were examined, 22 (38.6%) DMD and 35 (61.4%) BMD. In 9 patients, only the PCR method was applied, in 28 only the MLPA method, and in 20 patients, both methods were applied.

At the time of first neurological evaluation and the first genetic analysis, the average age of DMD/BMD probands was 17.24 ± 12.39 years, where the youngest patient was 1 year, and the oldest one 49 years old. Among DMD probands, the average age was 7.71 ± 5.29 years, whereby the youngest patient was 1 year, and the oldest one 21 years old. In BMD patients the average age was 23.90 ± 11.57 years, the youngest patient was 3 years, and the oldest one 49 years old.

The PCR method was used in a total of 29 DMD/BMD patients. In 18 (62.1%) patients, deletions of one or more exons were found, while in 11 (37.9%) patients, no deletions were found in the dystrophin gene.

The MLPA method was used in a total of 48 patients. In 25 (52.1%) patients, large deletions were found, in 6 (12.5%), large duplications were found, while in 17 (35.4%) patients, no deletions and duplications were found.

Analysis of the overall sample of patients, regardless of the method applied, showed that in 34 (59.6%) respondents deletions were found, in 6 (10.5%) duplications were found, while in 17 (29.8%) respondents no deletions or duplications were found.

Both methods, the PCR and MLPA, were applied to a total of 20 patients. Among nine patients, deletions were found using the PCR method, while with the MLPA method, the same deletion rate was found in 5 (55.6%) patients, and a higher deletion rate was found in 4 (44.4%) patients. Among the remaining 11 patients, no deletions were found using the PCR method, while using the MLPA method, 5 (45.5%) patients were found to have deletions in another area, duplications were found in 4 (36.4%) patients, and in 2, no deletions or duplications were found (Table 1).

We analyzed the age in which the first genetic analysis was done in DMD/BMD probands with deletion, duplication, and with no deletion or duplication, respectively. In the group with deletions, the average age was 16.31 ± 12.16 years, in the group with duplications 11.00 ± 5.18 years, and in probands with no deletion or duplication the average age was 22.38 ± 3.88 years.

We also analyzed the correlation between the mutations found and the phenotypes (Table 2). In patients with a diagnosis of DMD, the mutations found were significantly higher (77.3% deletion and 18.2% duplication) than in patients with BMD. In 45.7% of the patients with BMD, no mutations were found (neither deletions nor duplications), while in patients with DMD this percentage was 4.5%.

The results of the chi-square test showed that there was a statistically significant difference [χ^2 (1) = 11.54, p = 0.003] in the frequency of the mutations regarding established phenotypes.

Table 1
Findings in probands using the polymerase chain reaction (PCR) and multiplex ligation-dependent probe ampiflication (MLPA) methods

PCR	MLPA					Total
	confirmed deletion	confirmed deletion and found larger	deletion at another location	duplication	no deletion or duplication	
Patients with deletions, n (%)	5 (55.6)	4 (44.4)	0 (0.0)	0 (0.0)	0 (0.0)	9 (100.0)
Patients with no deletions, n (%)	0 (0.0)	0 (0.0)	5 (45.5)	4 (36.4)	2 (18.2)	11 (100.0)
Total patients, n (%)	5 (25.0)	4 (20.0)	5 (25.0)	4 (20.0)	2 (10.0)	20 (100.0)

Table 2

Frequency of the mutations in relation to the proband's phenotype

Proband's phenotype	Mutations			Total	*p*
	deletion	duplication	no deletion or duplication		
DMD, n (%)	17 (77.3)	4 (18.2)	1 (4.5)	22 (100.0)	
BMD, n (%)	17 (48.6)	2 (5.7)	16 (45.7)	35 (100.0)	< 0.05
Total, n (%)	34 (59.6)	6 (10.5)	17 (29.8)	57 (100.0)	

DMD – Duchenne muscular dystrophy; BMD – Becker muscular dystrophy.

Table 3

Phenotype by Reading frame checker crosstabulation

Proband's phenotype	Mutations		Total
	in-frame	out of-frame	
DMD, n (%)	2 (9.5)	(90.5)	21 (100)
BMD, n (%)	16 (84.2)	3 (15.8)	19 (100)
Total, n (%)	18 (45.0)	22 (55.0)	40 (100)

DMD – Duchenne muscular dystrophy; BMD – Becker muscular dystrophy.

Using the Reading-frame checker (version 1.9) we analyzed the large mutations obtained as well as their correlation to the probands' phenotype (Table 3). Among DMD probands, 2 (9.5%) of them had in-frame mutations (del 3–15; del 33.34), and 19 (90.5%) had out-of-frame mutations. In BMD probands, 16 (84.2%) had in-frame mutations, and 3 (15.8%) had out-of-frame mutations (del 44–48; del 44–49; dupl. 18–27).

Deletions were found in 17 DMD and 17 BMD probands. In 27 (79.4%) DMD/BMD probands more exons were affected and were mostly localized in the distal part of DMD gene (exons 44–60), in 6 DMD and 12 BMD patients (66.7%). Among BMD probands, the most common was deletion of exons 45–48 (5 times) and deletions of exons 45–47 and 45–49 (3 times each), while in DMD patients affected exons were more heterogeneous. The largest deletions covered 31 exons in one BMD patient (exons 12–43). Deletions of one exon were present in 7 (20.6%) patients – exons 1, 44, 48, 50 and 59, of which 5 patients (71.4%) had the DMD phenotype and both patients with the BMD phenotype had single deletion of exon 48. In 13 DMD and all BMD probands affected exons covered the central rod domain of the DMD gene, while in 2 DMD probands deletion included only N-terminal domain, in one patient both of those domains, and in one DMD patient the central rod domain and C-terminus were included. An exception to Monaco's rule in patients with deletions was present in 2 DMD and 2 BMD probands.

Duplications were found in 4 DMD and 2 BMD patients. In 16.7% and 83.3% of probands, duplications of one exon and more exons were found, respectively. The distribution of duplications was heterogeneous, affecting N-terminal and central rod domain. Among the probands with the Duchenne phenotype, 1 (25%) had a duplication of exon 2, and in 3 (75%) of them, the duplication affected more exons, as well as in both BMD probands. Duplications of more exons are shown in Table 4. In 4 probands with the Duchenne phenotype, all of them had frameshift mutations, while in probands with the Becker phenotype, one of them had in-frame mutations and one out-of-frame mutations.

Also, for each DMD proband with duplication, the carrier status of the mother, and in one trial, of two sisters, was analyzed using the MLPA method. In two BMD probands, female relatives were not examined. According to the data, in half of the female relatives, duplications were found. Duplications were found in 3 (75%) mothers, while in one (25%) mother, no duplications were found. In addition, duplications found in 3 mothers were the same as in sons of the probands. In the family where three female members were examined – the mother and two sisters, duplication was found in the mother, but not in sisters (Table 4).

Table 4

Duplications in probands and female relatives

Respondents	Affected exons						Total
	2	8–16	18–27	21–42 / 45–48	31–44	52–62	
Probands							
DMD	1	1		1		1	4
BMD			1		1		2
Female relatives							
mother	1	0		1		1	3
sisters	0						0

DMD – Duchenne muscular dystrophy; BMD – Becker muscular dystrophy.
1 – duplication found; 0 – duplication not found.

Discussion

As the largest detected gene in the human genome, the DMD gene is susceptible to changes. Diagnostic genetic testing is performed on symptomatic patients and in making a prenatal genetic diagnosis, as well as in order to determinate the carrier status of female. In our sample of 57 probands (22 DMD and 35 BMD), the average age at the time of first neurological evaluation and the first genetic analysis was 17.24 years. Among DMD probands, the youngest patient was 1 year, and the oldest one 21 years old. In BMD probands, the youngest patient was 3 years and the oldest one 49 years old. The data obtained are in line with the fact that the symptoms of DMD begin around third year of life (sometimes earlier), and the loss of mobility is present up to 12 years of age [1]. On the other hand symptoms of BMD begin around 10 years of age and in most patients the symptoms are present up to 20 years, the course of the disease is slower, and even in severe forms of the disease the loss of mobility is not present before the age of 16. We also found that in DMD/BMD patients with duplication, the mean age was lower than in the group with deletion and in the group with no mutations detected.

The multiplex PCR is a method that detects deletions in predilected areas in the dystrophin gene. In our sample, using this method, deletions were detected in 62.1% of the patients. With the use of the MLPA method, all deletions found using the PCR method were confirmed. Also, in 20% of the cases, extended deletions were found, in 25% of the cases deletions were detected at another location (outside of the "hot spots"), and in 4 (36.4%) respondents duplications were detected. So far, these findings confirm the conclusions of a large number of authors on the effectiveness of the MLPA method [13–15, 20, 21].

To date, the largest study including 7,149 respondents carried out by Bladen et al. [7], found major mutations in 80% of cases, out of which 86% were deletions (one exon or more) and 14% duplications (one exon or more). The remaining 20% constitute small mutations (less than one exon). In our sample of 57 unrelated patients, 22 DMD and 35 BMD, analysis of the results obtained using both methods (PCR and MLPA) showed that major mutations, deletions and duplications, were detected in 40 (70.2%) respondents (21 DMD and 19 BMD), while no mutations were detected in 17 (29.8%) respondents. The large deletions found in 25 of the patients were most represented and located in the distal part of the gene, exons 44–60, out of which 14 (63.3%) had the BMD phenotype. Deletions in BMD patients included exons 44–49, the typical localization for BMD of the moderate course, often with variability in the clinical picture [22–25]. The largest deletion found covered 31 exons in one BMD patient (exons 12–43). It is known that large deletions, which are limited to the rod-domain, predominantly result in the BMD phenotype [26]. Deletions of one exon were present in 7 (20.6%) patients – exons 1, 44, 48, 50, and 59, out of which 5 (71.4%) patients had the DMD phenotype. Both patients with the BMD phenotype had single deletions of exon 48, that generally causes a very mild form of the disease [27].

Out of 17 (29.8%) respondents with no deletions or duplications, the Becker phenotype was detected in 16 of them. Possible reasons for this are either point mutations, which could not be detected using the applied methods, or another kind of myopathy. As BMD shows a wide spectrum in the clinical presentation, from borderline DMD to very mild myopathy, this phenotype can have similarities with other types of muscular dystrophy or metabolic myopathies. Further examination of three patients using the Next Generation Sequencing method, showed that mutations of the CAPN3 gene (complex heterozygous) were present in two patients, while in one patient the findings in the muscle dystrophy gene panel were negative.

Comparing to deletions, duplications are much less common in the DMD gene, and they are present in 5%–10% of DMD patients and in 5%–19% of patients with BMD [28]. However, most authors using the MLPA method find no duplication in more than 10–14% of DMD/BMD patients [7, 29]. According to molecular analyses, while deletions are mainly due to unequal crossing-over during oogenesis, duplications are more often due to an event during spermatogenesis (grandpaternal germline). Basically, duplication can be caused by the same mechanism as deletion, during homologous or non-homologous recombination, or by insertion, although analysis of breakpoints has shown that it is more likely that they occurr due to the synthesis-dependent linking of nonhomologous areas [30]. Also, duplications are more often represented in families with increased risk of recurrence. Because it is an X-linked recessive disease, female family members are mainly heterozygous carriers of the disease. In sporadic cases of DMD, it is estimated that 2/3 mothers are carriers of the mutation, in 5%–10% there is gonadal mosaicism, while in 25%–30% of cases there is a new mutation [31]. In 5%–8% of cases, women can be manifesting carriers of the mutations. Also, the mother's risk of being a carrier is greater for duplication than for deletion.

In our total sample, there were 63 respondents, 57 DMD/BMD unrelated patients and 6 female relatives from 4 DMD probands families with duplications (4 mothers and 2 sisters of the probands). A total of 9 (14.3%) duplications were found, out of which 6 (10.5%) duplications were found in 4 DMD and 2 BMD patients, and 3 in DMD mothers. Thus, in 75% of the cases, mothers were confirmed as carriers of the mutation, while in one (25%) mother there was no mutation. Also, in the mothers of carriers, the same mutations were found in the affected sons. No duplications were found in the two sisters. Apart from being less frequent, the distribution of duplications in the DMD gene itself is very different, and most often localized in the vicinity of the 5'end of the DMD gene. The most frequent duplication of an exon is the duplication of exon 2 [30]. When it comes to the duplication of a greater number of exons, according to the TREAT-NMD DMD Global database, the most described duplications in literature are those of exons 3–7, 8–9, 8–11, 8–12, 5–7, 56–62 [7]. According to Takeshima et al. [29], the most present large duplications are those of exons 3–7, and the largest, of exons 3–43.

In our sample, in one DMD patient and his mother, a duplication of single exon 2 was found, while no mutations were found in his sisters. Exon 2 is part of the gene region encompassing exons 1-8 that encode the N-terminal Actin Binding Domain-1 containing three actin-binding sites through which the protein dystrophin, binds to the cytoskeletal actin [32]. In-frame mutations in this part essentially disturb the stability of dystrophic coupling and decrease affinity for binding with F-actin, resulting in a more severe clinical picture [33]. It is estimated that about 7% of DMD patients have mutations in this domain, and the most commonly affected are exons 2–7, with the most common being exon 2 [34, 35]. In BMD patients, the presence of mutations in this domain leads to a lower level of dystrophin and also results in a more severe clinical picture. In our patients, out-of frame duplication of exon 2 was associated with the DMD phenotype.

In 3 DMD probands and two mothers, as in both BMD patients, there were duplications of a greater number of exons (77.8%). The distribution of these duplications was very different, and they all were localized in the central rod domain. In two BMD patients, there were duplications of exons 18–27 and 31–44, respectively. In one DMD patient and in another DMD patient and his mother carrier, there were duplications of exons 8–16 and 52–62, respectively; in one DMD patient and his mother, the largest duplications encompassing exons 21–42 and 45–48 were found.

The central rod domain (coded by exons 9–65) contains 24 spectrin-like repeats and four proline-rich hinges providing flexibility to the protein. Near the central part of the domain, there is the second actin-binding domain (ABD)-2, which together with ABD-1 builds a strong lateral connection with actin filaments on the one hand, while on the other hand, through a link with the cysteine-rich domain is connected to the C-terminus region [36]. It is believed that this domain contains entities that are of different functional significance, so the localization of the in-frame change has a different effect on the phenotype. In addition, it is known that major mutations in this domain, if they do not disturb the reading frame and have preserved the N-terminal and C-terminal regions, generally result in the BMD phenotype. In our patients, two duplications were associated with the BMD phenotype, and included the proximal and central part of the rod-domain. According to Beggs et al. [37], this localization results in the BMD phenotype of mild progression. In 3 patients, duplications were associated with the DMD phenotype, each with a different localization in the domain, and all were out of the scope of reading the genetic code, which led to the creation of very small amounts of shortened, non-functional protein.

According to Monaco et al. [8], out-of-frame mutation correlates with a severe clinical presentation in DMD patients, while the in-frame mutation results in a milder form of the disease, i.e. the BMD phenotype. Recent studies suggest that duplications, which are more commonly present in BMD, result in exceptions from the Monaco rule in over 30% of cases [28, 29]. By analyzing the detected mutations using the Reading-frame checker (version 1.9) [19], we found that there were exceptions from the Monaco rule in 9.5% of DMD probands, and in 15.8% of BMD probands. In DMD probands, 2 of them had in-frame mutations. One DMD proband had in-frame deletion of exons 3–15, that disturbs the 5' binding site in the gene which causes DMD, with a typical onset in 3rd or 4th exon, and extending into the rod domain [38, 39]. The other DMD proband had deletion of exons 33 and 34. Deletion of these exons, as an in-frame mutation, is mainly described in BMD patients, but smaller deletions, while only deletions of exon 33 or exon 34 lead to DMD [40, 41]. In our case del 33,34 led to DMD phenotypes in a boy of 2 years of age. Baumbach et al. [42] reported that deletion of exons containing HindIII fragments could result in either the DMD or the intermediate DMD/BMD phenotype.

Among BMD probands, 3 of them had out-of frame mutations (del 44–48; del 44–49; dupl 18–28). According to literature, the reason for this contradiction, is the appearance of an alternative splicing which, by the exon-skipping mechanism, leads to the reestablishment of the reading frame and the creation of shortened, but functional dystrophin, and the BMD phenotype [28, 43]. Apparently, the association of genotype with phenotype, apart from the size, location and state of the reading frame, has other complex impacts that can alter the phenotype of patients.

Conclusion

In 57 unrelated probands, 34 (59.6%) deletions of one or more exons (the most commonly affected exons 44–60) covering the central rod domain, and 6 (10.5%) duplications affecting N-terminal and central rod domain were found. Distribution of duplications was heterogeneous and included more exons, except for one DMD patient who had duplication of exon 2. In DMD probands, the mean age at the time of the first genetic analysis was 7.71 years, and in BMD probands it was 23.90 years. An exception from the Monaco rule was present in 9.5% of DMD probands, and in 15.8% of BMD probands. Also, duplications were found in 3 out of 4 (75%) tested mothers who were confirmed as carriers.

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