Research Article / Araştırma Makalesi

The Spectrum of Dystrophin Gene Deletions and Duplications in a Cohort of Patients with Duchenne/Becker Muscular Dystrophy in Türkiye

Türkiye'de Duchenne/Becker Musküler Distrofisi Kohortunda Distrofin Gen Delesyonları ve Duplikasyonlarının Dağılımı

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Abstract: Duchenne Muscular Dystrophy (DMD) is the most prevalent muscle disease in children, and unfortunately, currently there are no effective treatments for either DMD or Becker Muscular Dystrophy (BMD). Nevertheless, targeted gene therapy treatments have recently emerged, and genetic diagnoses is now the basis of treatment. In addition, genetic and prenatal diagnosis have significantly reduced the incidence rates of these diseases. The aim of this study was to identify the most common deletion and duplication regions in the Turkish population using the Multiplex Ligation-dependent Probe Amplification (MLPA) method, as well as to determine the suitability of patients for current treatments and identify new treatment target regions based on the findings. In clinical practice, data from 103 patients with Duchenne and Becker muscular dystrophy who have been identified with the deletion/duplication using the Multiplex Ligation-dependent Probe Amplification (MLPA) method, as well as 35 participants carrying the deletion/duplication for these diseases, were analyzed. The aim was to detect the most common deletion/duplication regions of the Dystrophin gene in the Turkish population. The majority of patients had deletions (89.9% in males and 75% in females), while a smaller percentage had duplications. The most common deletions occurred in exons 50 and 49, while the most common duplication was in exon 7. The deletions in exons 45-52 accounted for over half of all deletions, and most deletions involved 5 or less exons. The longest deletions involved 30 exons and were found in 2 patients. The findings of this research have provided valuable insights into the prevalence of deletions and duplications in the dystrophin gene among individuals in our population. The results indicate that a significant proportion of patients may be eligible for treatments that are not yet widely available. This study highlights the critical role of population-specific data in advancing the field of dystrophin gene-based therapies.
Keywords: Duchenne Muscular Dystrophy, Becker Muscular Dystrophy, dystrophin, deletion, duplication

Özet: Duchenne Musküler Distrofi(DMD) ve Becker Musküler Distrofi(BMD), çocuklarda en sık görülen kas hastalıklarıdır. DMD ve BMD için kesin bir tedavi bulunmamakla birlikte, son zamanlarda hedefe yönelik gen tedavileri ortaya çıkmış ve bu tedavilerin temeli genetik tanıya dayanmaktadır. Genetik testlerin yaygın olarak kullanımı ve özellikle prenatal dönemde yapılan genetik testler hastalıkların insidanslarını önemli ölçüde azaltmaktadır. Bu çalışmada uygun maliyetli ve verimli tanı yöntemi olan multiplex ligasyona bağımlı prob amplifikasyon teknolojisi (MLPA) kullanılarak Türk popülasyonunda en sık görülen delesyon ve duplikasyon bölgelerinin tespit edilmesinin yanı sıra hastaların mevcut tedavilere uygunluğunun belirlenmesi ve buna göre yeni tedavi hedef bölgelerinin belirlenmesi amaçlamıştır. Klinik olarak Duchenne ve Becker muskuler distrofisi olan ve Multipleks Ligaz bağımlı Prob Amplifikasyonu (MLPA) yöntemi ile delesyon/duplikasyon tespit edilmiş olan 103 hasta ve bu hastalıklar için delesyon/duplikasyon taşıyıcısı olan 35 katılımcının verileri analiz edildi. Türkiye populasyonunda en sık görülen Distrofin gen delesyon/duplikasyon bölgelerinin tespit edilmesi amaçlandı. Hastaların çoğunda delesyon (erkeklerde %89,9 ve kadınlarda %75), daha küçük bir oranda ise duplikasyon saptandı. En yaygın delesyonlar ekzon 50 ve 49'da olurken, en yaygın duplikasyon 7. ekzonda görüldü. Ekzon 45-52'yi içeren delesyonlar, tüm delesyonların yarısından fazlasını oluşturuyordu ve çoğu delesyon 5 veya daha az ekzon içeriyordu. En uzun delesyonlar 30 ekzon içeriyordu ve 2 hastada bulundu. Bu araştırma ile Türk popülasyonunda bireyler arasında distrofin genindeki delesyonların ve duplikasyonların yaygınlığına ilişkin değerli bilgiler elde edilmiştir. Sonuçlar, hastaların önemli bir bölümünün henüz yaygın olarak uygulanmayan tedaviler için uygun olabileceğini göstermektedir. Bu çalışmada, popülasyona özgü spesifik mutasyon tiplerini belirlemenin, distrofinopatiler için geliştirilen genetik temelli tedavilerin ilerlemesindeki kritik rolü vurgulanmaktadır.
Anahtar Kelimeler: Duchenne kas distrofisi, Becker kas distrofisi, distrofin, delesyon, duplikasyon

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1. Introduction

Duchenne and Becker muscular dystrophies (DMD/BMD) are neuromuscular diseases caused by mutations in the dystrophin (DMD) gene on the Xp21.2 region. DMD is the more common form, with a prevalence of 1 in 3,500-5,000 live male births (1). BMD is a milder form of DMD, and its incidence is 1 in 18,518 male births. The mean age at diagnosis of DMD is around 4.5-5 years, and it takes about 2 years to receive a diagnosis after the presentation of clinical findings (2). DMD manifests as a balance disorder, difficulty in standing up, difficulty walking, and eventually, loss of walking ability. DMD patients typically lose the ability to walk at an average age of 10 years (3). BMD generally occurs at a later age compared to DMD, typically between 5 and 15 years old, and the severity or progression of the disease varies among patients (4). In addition to the limitation of movement, DMD patients experience weakened respiratory and cardiac muscles, significantly shortening their life expectancy and reducing their quality of life (5). Variability in the amount of fibro-fatty replacement in dystrophic muscle can be detected in biopsies, and different mutations in the dystrophin gene can lead to this situation (5,6). The dystrophin gene, located at Xp21.2, is the largest known gene and contains 79 exons spanning over 2500 kilo bases of genomic DNA (7). The mutations in this gene lead to two types of muscular dystrophy depending on the preservation or disruption of the translational reading frame: severe DMD due to out-of-frame mutations causing loss of protein function, or a milder form of muscular dystrophy known as Becker muscular dystrophy, which occurs due to mutations that preserve the frame, resulting in a decrease in the quantity and/or size of the dystrophin protein(3).

The multiplex ligation-dependent probe amplification (MLPA) method, widely used in the molecular diagnosis of DMD, detects quantitative changes (8). These technologies have facilitated the detection of deletions and repeats in various diseases, including DMD/BMD (8). By individually testing all 79 exons of the DMD gene, the application of these techniques confirmed the presence of previously detected deletions and a significant number of new deletions (up to 5%) and repeats (5-8%) (5). The most common molecular defect in the DMD gene is the deletion of one or more exons (65%), although duplications (6-10%), small mutations (10%), and other smaller rearrangements are also observed (4). There is a wide range of mutations in DMD, with nearly all patients having unique mutations, and approximately one in three mutations being de novo (9).

The aim of this study is to analyze the most common deletions/duplications in the dystrophin gene in the Turkish population and contribute to the development of treatments.

2. Materials and Methods

In this study, we analyzed the results of 138 participants in whom deletions or duplications had been identified between January 1, 2017, and November 31, 2021. The study group consisted of 103 unrelated patients with DMD/BMD and 35 carriers. Patients were referred for elevated CK levels, low effort capacity, Gower's sign and, muscle weakness to Medical Genetics Clinic of Haseki Training and Research Hospital were included in this study. Four of the patients were female, and they had elevated CK levels. Carriers were mothers or sisters of DMD/BMD patients analyzed for MLPA in another laboratory, so the mutations

observed in carriers were also included in the mutation frequency ratio. Although the age distribution varies, the average age of patients was calculated as 6.1. We performed mutation analysis in the dystrophin gene using the Multiplex Ligation-dependent Probe Amplification (MLPA) method. Written informed consent was obtained from each participant. The study was approved by the ethical committee under protocol no: 32-2022 (dated April 20, 2022).

Blood samples (4 ml) were collected from the participants, and genomic DNA was extracted from EDTA-treated peripheral blood samples using a spin column method with the DNA isolation kit (PureLink® Genomic DNA Kits) following the manufacturer's instructions. The DNA samples were stored at -20°C until further use. MLPA was performed using SALSA MLPA Probemixes P034-B2 DMD-1 & P035-B1 DMD-2 (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's protocol. Amplification products were analyzed by capillary electrophoresis on an ABI 3130 genetic analyzer (Thermo Fisher Scientific, MA, USA). The data were analyzed using Genemapper 4.0 and Coffalyser.net software. Dystrophin gene sequence analysis was performed using an APPLIED 3730 DNA Analyzer (48-capillary) automated DNA sequencer following the manufacturer's protocol. The raw sequence data were analyzed using the CHROMAS analysis program.

Statistical analysis: Statistical data analysis was performed using Microsoft Excel and general percentage calculation tools were used, apart from these, statistical analysis program was not used.

3. Results

In this study, we analyzed the MLPA results of 103 patients with DMD/BMD and 35 carriers for DMD/BMD. Among the patients, four were females who exhibited elevated creatine kinase (CK) levels and mild clinical findings. The carriers did not show any clinical findings. The average age of diagnosis for the patients was 6.1 years. These patients exhibited muscle weakness, developmental milestones retardation, elevated CK levels, Gowers' sign, and hypertrophic gastrocnemius.

Deletion was detected in 89 out of 99 male patients (89.9%), while duplication was detected in 10 out of 99 male patients (10.1%). Among the female patients, three out of four mutations were deletions (75%), and one of them was a duplication (25%). Deletions were detected in 31 out of 35 carriers (89%), while duplications were detected in four out of 35 carriers (11%). The distribution of deletions and duplications in the dystrophin gene is presented in Figure 1 and Figure 2. The most common deletions were observed in exon 50 (42/514), followed by exon 49 (28/514). Deletions of exons 45-52 accounted for 52% of all deletions and were the most common type. The most common duplication observed in the patients was a duplication of exon 7. When examining the exon contents of the deletions and duplications in the patients, it was found that 64% (58/90) of the deletions involved the deletion of 5 or fewer exons. The longest deletions encompassed 30 exons in 2 patients and the deleted exons were between exons 13-42 and 10-39. 57% (8/14) of all duplications consisted of copies of 5 or fewer exons. The largest duplication involved exons 45-67 and encompassed 23 exons. In this study, deletions or duplications associated with all exons were identified in patients and carriers. The exons affected by deletions and duplications are summarized in Table 1. In one male patient with high CK levels, the copy number ratio in exon 64 was found to be 0.5 in the MLPA test, the

other exons' copy number ratios were normal. This result was not a classic deletion image in MLPA. The test was repeated twice and the results were consistent, so the DMD gene was sequenced using the Sanger method. A hemizygous missense mutation, c.9338G>A (p.Arg3113Gln) (NM_004006.3), was identified in the exon 64 of the gene (Figure 3). Segregation analysis revealed that the mother was heterozygous for this mutation and did not exhibit any clinical findings. Consequently, it was hypothesized that because this region was within the MLPA probe binding site, the probe could not bind, thus appearing as heterozygous.

Table 1. Deletions and duplications according to the number of exons they contain

No. of exons involved	**Exons deleted**
1(n=21)	Ex5 (n = 1), Ex16 (n = 1), Ex17 (n = 1), Ex19 (n = 1), Ex40 (n = 1), Ex43 (n = 2), Ex44 (n = 4), Ex45 (n = 4), Ex48 (n = 1), Ex50 (n = 1), Ex51 (n = 3), Ex53 (n = 1), Ex56 (n = 1), Ex64(n = 1)
2(n=8)	Ex7-8(n = 1), Ex8-9 (n = 1), Ex46-47 (n = 1), Ex49-50 (n = 4), Ex51-52(n = 1)
3(n=16)	Ex41-43(n = 1), Ex45-47 (n = 7), Ex46-48 (n = 1), Ex48-50 (n = 4), Ex50-52(n = 4)
4(n=8)	Ex27-30(n = 1), Ex45-48 (n = 3), Ex48-51 (n = 1) , Ex52-55(n = 1)
5(n=5)	Ex3-7(n = 1), Ex45-49 (n = 1), Ex46-50(n = 2), Ex48-52 (n = 1)
6(n=9)	Ex21-26(n = 2), Ex45-50 (n =5), Ex46-51 (n = 1), Ex49-54(n = 1)
7(n=4)	Ex45-51(n = 1), Ex47-53 (n =2), Ex48-54 (n = 1)
8(n=5)	Ex44-51(n = 1), Ex45-52 (n =3), Ex46-53 (n = 1)
9(n=4)	Ex45-55 (n =4)
10(n=4)	Ex3-12 (n =1), Ex42-51(n =1), Ex46-55 (n =2)
11(n=2)	Ex45-55 (n =2)
14(n=1)	Ex2-15 (n =1)
18(n=1)	Ex4-21 (n =1)
25(n=1)	Ex18-43 (n =1)
30(n=1)	Ex13-42 (n =1), Ex10-39 (n =1)
No.of exons involved	**Exons duplicated**
1(n=3)	Ex25 (n =1), Ex45(n =1), Ex51 (n =1)
2(n=1)	Ex18-19 (n = 1)
3(n=1)	Ex7-9 (n = 1)
5(n=3)	Ex3-7(n = 2),Ex12-16 (n = 1)
7(n=2)	Ex1-7(n = 1),Ex11-17 (n = 1)
12(n=1)	Ex8-19 (n = 1)
14(n=1)	Ex61-74 (n = 1)
17 (n=1)	Ex63-79 (n = 1)
23(n=1)	Ex45-67 (n = 1)

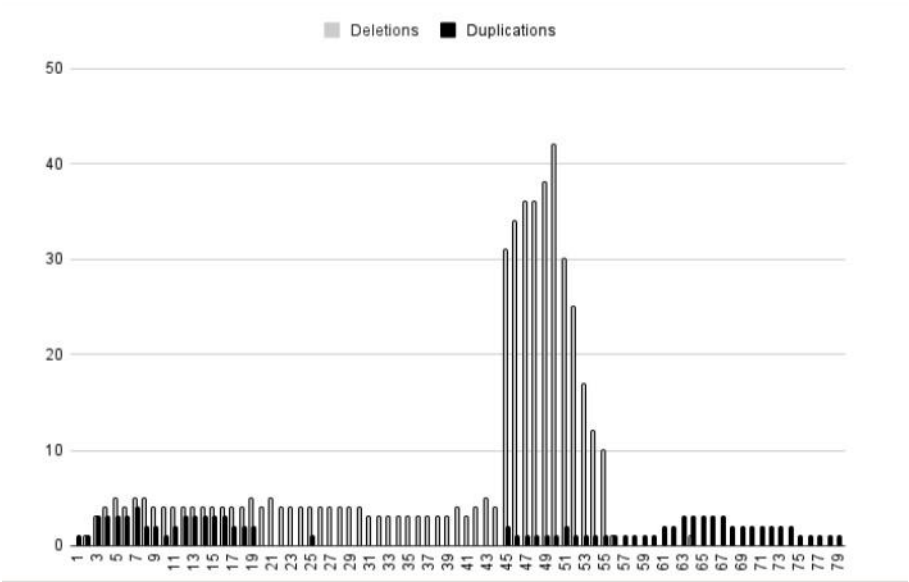

Figure 1. *Distrubition of deletions and duplications in dystrophin gene in probands (duplications:filled bars, deletions: unfilled bars)*

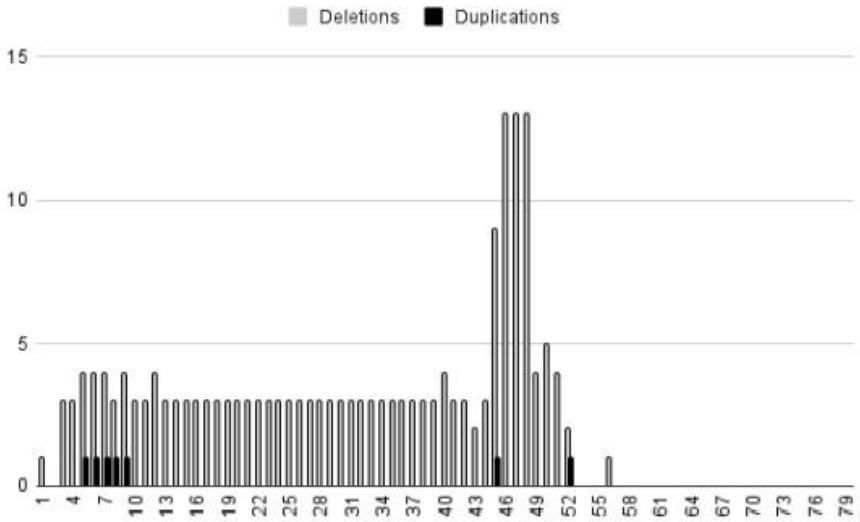

Figure 2. *Distrubition of deletions and duplications in dystrophin gene in carriers (duplications:filled bars, deletions: unfilled bars)*

Figure 3. *Image of MLPA analysis of the male patient with high CK levels. Sanger sequencing revealed the c.9338G>A (p.Arg3113Gln) (in exon 64) variant in this patient.*

4. Discussion

Numerous studies have demonstrated the usefulness of MLPA in quantitatively detecting mutations in the DMD gene, including deletions, duplications, and female carriers (10). In line with previous studies, our analysis revealed that 88.9% (88/99) of male patients had deletions, while 11.1% (11/99) had duplications, and 100% (4/4) of female patients had deletions (4). The most frequent deletions occurred in exons 45-55, in accordance with the literature. Hotspot regions in the DMD gene, such as exons 2-20 and 44-53, have been identified in previous studies in Turkish as well as other populations (11). Higher numbers of repetitive sequences in introns have been associated with an increased frequency of breakpoints and mutations, as reported in previous studies (10). Exon 45 was the most commonly affected exon in single-exon mutations, while exons 44 and 45 were the most common in single-exon deletions. Single-exon deletions were detected in 22.4% (24/107) of patients, which is lower than the findings of previous studies (12, 13). The most frequently deleted exon within the regions of multiple exon deletions was exon 51, consistent with previous studies (10). No deletions were detected in exons 1 and exons 57-79. The most common duplication involved exon 7, whereas a previous study conducted in Türkiye reported that the most common duplication occurred in exons 5-6 (14). Exon 2 duplication was frequently observed in previous studies (14,15). The location of duplications was predominantly proximal, with less frequent distal duplications, which is consistent with the existing literature.

Among the 90 patients with deletions or duplications, 64% (58/90) had deletions or duplications involving 5 or fewer exons. The longest deletions occurred in 2 patients and encompassed 30 exons, located between exons 10-39 and 13-42. Out of all the duplications, 57% (8/14) consisted of duplications involving 5 or fewer exons. The largest duplication involved exons 45-67, spanning a total of 23 exons. Deletions or duplications were detected in all exons of the patients and carriers included in the study. The distribution of deletions and duplications in introns varied, with intron 44 having the most initial breakpoints and intron 50 having the most frequent end breakpoints. However, no start or end breakpoints were found in introns 13, 14, 22, 23, 27-29, 31-38, 40, and 57-59, as well as 68-73 and 75-78, regardless of deletion or duplication. These findings may differ from the data reported in the literature. The clustering of breakpoints has been

associated with the formation of hotspot deletion points, and previous studies have suggested a relationship between repetitive sequences in introns and breakpoints (16). An increased number of repetitive sequences has been reported to result in more breakpoints and a higher incidence of mutations. Understanding the frequencies of breakpoints is crucial for treatment development.

The number of studies on DMD treatment is increasing rapidly. Exon skipping therapy, which has gained prominence recently, aims to partially restore dystrophin production to create a milder phenotype in patients with impaired synthesis. Restoring the disrupted reading frame in the DMD gene can induce functional dystrophin production. Exon skipping therapy is mutation-specific, and each patient carries different mutations. However, approximately 70% of all patients, including two-thirds of our patients, have one or more exon deletions clustered between exon 45 and exon 55 (while each mutation has unique intronic breakpoints) (9). Therefore, skipping certain exons could be applicable to a large proportion of patients (17). Several antisense phosphodiamidate morpholino oligomers, such as Eteplirsen (Exon 51, Sarepta Therapeutics), Golodirsen (Exon 53, Sarepta Therapeutics), Casimersen (Exon 45, Sarepta Therapeutics), and Viltolarsen (Exon 53, NS Pharma), have been approved by the FDA as exon skipping therapeutics for the treatment of DMD. Confirmatory clinical trials for these therapies are currently underway(18). In addition to these molecules, many more are under development, targeting various DMD exons (https://www.ncbi.nlm.nih).gov/NBK584230/)(19). Eteplirsen has shown efficacy in 14% of patients, golodirsen in 10% of patients, and casimersen in 9% of patients (https://www.ncbi.nlm.nih).gov/NBK584230/)(19). Consequently, 33% (35) of our patients were deemed suitable for exon skipping treatment.

In a male patient with a copy number of 0.5 in exon 64 of the DMD gene, as detected by MLPA, Sanger sequencing revealed the c.9338G>A (p.Arg3113Gln) variant. This missense mutation had previously been associated with elevated creatine kinase levels (20). While the MLPA method cannot detect point mutations and small deletions, it may fail to detect certain mutations if the MLPA probe does not bind to the splice region, as observed in this patient. MLPA can diagnose DMD in approximately 75% of cases, while other molecular diagnostic methods are used in the remaining 25% (21). If MLPA fails to provide a diagnosis in patients with clinically suspected DMD/BMD, additional methods such as microarray, Sanger sequencing, and next-generation sequencing should be employed (4,21).

5. Conclusions

Advancements in technology have led to significant progress in the diagnosis of DMD through comprehensive molecular tests capable of identifying different types of mutations (deletions/duplications, point mutations, and deep intronic events). The utilization of comparative genomic hybridization (CGH) and next-generation sequencing (NGS) enables earlier and more effective intervention for patients. Discovering safe and effective molecules for the treatment of DMD remains challenging. Currently, corticosteroids are the only available treatment, although they do not provide a definitive cure. Understanding the pathophysiological mechanisms of dystrophin, developing new molecules, and utilizing nanomaterials for their delivery hold promise for DMD treatment. Knowledge

of the mutation distribution frequencies in the patient population aids in guiding research. In this study, we determined the distribution of the most frequently detected mutations in Turkish patients with DMD/BMD and identified the number of patients suitable for treatment.

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Ethics

Ethics Committee Approval: The study was approved by Haseki Training and Research Hospital Clinical Research Ethical Committee (Decision no:32/2022, Date: 20.04.2022).
Informed Consent: The authors declared that it was not considered necessary to get consent from the patients because the study was a retrospective data analysis.
Authorship Contributions: "Concept: FNÖ. Design: FNÖ, PÖÇ. Data Collection or Processing: FNÖ, PÖÇ, TAD. Analysis or Interpretation: FNÖ, TAD. Literature Search: FNÖ. Writing:FNÖ."
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