Genetic Landscape of Dystrofin Gene Deletions and Duplications from Turkey: A single Center Experience

Distrofin Genindeki Delesyon ve Duplikasyonların Türkiye'deki Profili: Tek Merkez Deneyimi

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ABSTRACT

Objective: Dystrophinopathies are the most frequently researched neuromuscular disease group due to their characteristic and diverse clinical and genetic spectrum. This study aims to evaluate the deletion and duplication profile of the dystrophin gene in Turkey by investigating data from a tertiary center.

Material and Methods: Dystrophin MLPA and microarray results of 53 patients, 49 with a dystrophinopathy and 4 with a neurogenetic and syndromic disorder pre-diagnosis, who were referred to the Medical Genetics Clinic of Ankara City Hospital between February 2019-December 2020 were retrospectively evaluated.

Results: Of the 53 patients, 4 had various exon duplications and 49 had deletions. 33 of these mutations caused frame-shift (62.3%), while 20 caused in-frame (37.7%) changes. Fifty (94.3%) patients underwent maternal studies and 14 (26.4%) of these had de novo mutations. Mutations were observed most frequently in the central rod domain (69.7%) followed by the actin-binding domain (7.5%) of the dystrophin gene and 12 of 33 patients with frameshift mutation (36%) patients were found to be candidates for the exon skipping treatments that are still subject to clinical research.

Conclusion: This study has shed light on the incidence of dystrophin deletion/duplication mutations in our population and has revealed that a majority of patients are suitable candidates for treatments which are still not in routine use. Considering ever-growing number of dystrophin gene-based treatment options, data on population-specific mutation types is of great importance.

Key Words: Deletion/duplication, Duchenne muscular dystrophy, Exon skipping, MLPA

ÖZ

Amaç: Distrofinopatiler; kendilerine özgü ve oldukça geniş klinik ve genetik spektrumu ile nöromusküler hastalıklar içinde halen en sık araştırma konusu olan gruptur. Bu araştırmada bir merkezden elde edilen sonuçlar değerlendirilerek Türkiye'deki distrofin geni delesyon ve duplikasyon profilinin ortaya konulması amaçlanmıştır.

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Gereç ve Yöntemler: Ankara Şehir Hastanesi Tıbbi Genetik Polikliniği'ne Şubat 2019-Aralık 2020 tarihleri arasında 49'u distrofinopati, 4'ü nörogenetik-sendromik bozukluklar klinik ön tanısı ile yönlendirilen 53 hastaya ait distrofin MLPA ve mikrodizin sonuçları retrospektif olarak değerlendirildi.

Bulgular: Çalışmaya alınan 53 hastanın 4'ünde distrofin geninde çeşitli ekzon duplikasyonları saptanmış olup kalan 49 hastada delesyon olduğu görüldü. Bu mutasyonların 33'ü frame-shift (%62.3), 20'si in-frame (%37.7) değişikliğe neden olmaktadır. Maternal çalışma yapılan 50 hasta (%94.3) değerlendirildiğinde 14 hastada (%26.4) de novo mutasyon olduğu görüldü. Distrofin geninde en sık santral rod domain'de (%69.7), ikinci sıklıkla aktin bağlayıcı bölümde (%7.5) mutasyonlar izlenmiştir. Henüz klinik araştırmaları devam eden güncel ekzon atlatma tedavileri açısından çerçeve kayması tipi mutasyona sahip 33 hastanın 12'sinin (%36) aday olduğu saptandı.

Sonuç: Bu çalışma ile popülasyonumuz açısından distrofin delesyon/duplikasyon mutasyon sıklıklarına ışık tutulmuş ve henüz rutin kullanıma girmeyen tedaviler açısından dahi hastaların önemli bir kısmının aday olduğu tespit edilmiştir. Son yıllarda gelişen distrofin geni temelli tedavi olanakları da göz önünde tutulursa populasyonlara ait mutasyon tipi sıklıklarının bilinmesi büyük önem taşımaktadır.

Anahtar Sözcükler: Delesyon/duplikasyon, Duchenne musküler distrofi, Ekzon atlama, MLPA

INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD and BMD, respectively), also known as X-linked dystrophinopathies, are the most common neuromuscular diseases of childhood. Dystrophin gene, which spans 2.2 Mb and is composed of 79 exons, codes the main skeletal frame protein dystrophin, located on the cytoplasmic surface of the skeletal and cardiac muscle cell membranes. Loss of function mutations of Dystrophin gene causes progressive and fatal muscle weakness (1,2). Clinical and laboratory findings are helpful in the diagnosis of this disease however, disease severity is related to mutational features (1-4). The fundamental point in defining the clinical features and subsequently the type of dystrophinopathy and its prognosis is the reading-frame principle which is observed in 90% of the patients (1-4). Out-of-frame mutations cause DMD, while in-frame mutations cause BMD (2). Approximately two-thirds of the considerably complex mutations in BMD/DMD are large deletions or duplications in one or more exons while the remainder is minor deletions, insertions, point mutations, and splicing mutations (1-9). Although the mutation frequency and spectrum depend on the country, DNA/RNA-based therapeutic approaches have rendered population-based genetic features more important in dystrophinopathies which still lack a curative treatment, especially in the last decade (5-9). Quantitative techniques such as microarray-based comparative genomic hybridization (array-CGH) and the more frequently utilized Multiple Ligation Probe Assay (MLPA) which detect the deletions and duplications are the first choices for the diagnosis of the disease (1-3). For the cases which can not be diagnosed using these techniques, also known as MLPA-negative cases, NGS and Sanger sequencing are recommended (1-3). Genetic counseling is also recommended for this disease where ¼ of the mutations are *de novo* and molecular genetic diagnosis is necessary (3). This study aims to define the mutation spectrum and features of the dystrophin gene in our country by evaluating the molecular genetic diagnostic tests performed in our center in a large patient population with a diagnosis of DMD or BMD to provide genetic counseling, a more accurate prognosis and define targeted gene therapies in addition to providing insight for national and international clinical studies.

MATERIAL and METHODS

A retrospective, descriptive study was planned after obtaining the approval of the Ankara City Hospital Ethics Committee, No.2 (E2-21-07). The study was conducted in concordance with the Helsinki declaration and written in accordance with the STROBE statement.

Patients and Samples

Fifty-three male children were referred for low effort capacity, Gower's sign, leg pain, a family history of DMD, proximal muscle weakness and elevated CK levels to the Medical Genetics Clinic of the Ankara City Hospital between February 2019- December 2020 were included in this study. Patients with deletion/duplications in MLPA analysis of the DMD gene and their mothers who provided written informed consent for a family study were included in this study. Patients with a negative DMD MLPA result or patients who did not undergo this testing were excluded from the study. Four of the patients were referred due to congenital hypotonia, epilepsy, atypical autism, and growth retardation, underwent microarray testing, and subsequent MLPA study because a deletion in Xp21.1 was detected. Only one patient from a family was included in the study after the evaluation of pedigree analysis and family history. Deletions and duplications were classified as disrupting or non-disrupting the reading frame according to the DMD gene reading frame principle. Mutations affecting the central rod domain, cysteine rich domain, C-terminal domain, and the actin-binding domain of the dystrophin gene were classified based on the affected domain.

Genetic Analyses

Two milliliters of peripheral blood samples were obtained from the patients after they provided written informed consent. Genomic DNA was extracted from peripheral blood lymphocytes with an automated QIA symphony DSP DNA Mini Kit (QIAGEN Inc., Germany).

Multiplex ligation-dependent probe amplification analysis

Multiplex ligation-dependent probe amplification (MLPA) was performed as recommended by the manufacturer (MRC-

Holland®, Netherlands). The SALSA® MLPA® probemix P034 and P035 were used for detection of copy numbers of the DMD gene. The MLPA data were analyzed using the Coffalyser software package (MRC- Holland®). The standard deviation for all probes in the reference samples was <0.10, and the relative probe intensity or dosage quotient (DQ) of the reference probes in the patients' samples was between 0.80 and 1.20 for healthy/normal individuals. For samples with heterozygous deletions, the DQ of the probes was between 0.40 and 0.65, whereas for samples with heterozygous duplications, the DQ of the probes was between 1.30 and 1.65.

Chromosomal Microarray Analysis (CMA)

The Infinium CytoSNP-850K v1.2 BeadChip (Illumina, Inc., San Diego, CA, USA) were used to perform CMAs. A data analysis was carried out using the BlueFuse Multi Software. Variants were evaluated based on the phenotype using standard in silico tools. The obtained results were analyzed and interpreted using public genomic databases such as UCSC (https://genome.ucsc.edu/cgi-bin/hgTracks), OMIM (https://omim.org/), DGV (http://dgv.tcag.ca/dgv/app/home), DECIPHER (https://decipher.sanger.ac.uk/), and CLINGEN (https://clinicalgenome.org/).

RESULTS

Dystrophin gene MLPA test results revealed 4 (7.5%) patients with duplications in various exons while the remaining (92.4%) patients had deletions. Thirty-three (62.3%) of these mutations caused a frame-shift while 20 (37.7%) caused in-frame changes. Maternal studies were performed for 50 (94.3%) patients and 14 (26.4%) patients had *de novo* mutations while 36 had a maternal inheritance.

The mutation spectrum based on dystrophin gene domains is presented in Figure 1. Mutations were found most frequently in the central rod domain of the dystrophin gene. Three patients had 44th exon deletion and 44-47th exon deletion in the central rod domain in common. Additionally, two patients had 48th exon, 48-50th exon, 49-52nd exon, 50-52nd exon, and 51st exon deletion in the central rod domain, 2nd exon duplication in the actin-binding domain, and 8-12nd exon duplication in both domains in common (Figure 1).

The main clinical findings of patients who underwent microarray testing for various reasons and their results are presented in Table I. Two of the mutations detected were maternal mutations and one of these was a *de novo* mutation. A family study could not be performed for one patient. All changes were deletions and none of them disrupted the reading frame.

12 patient variations among 33 frameshift variants were detected to be amenable for developed exon skipping therapies (%36). Deletions amenable for exon 51 skipping (the orphan drug being eteplirsen) were detected in 12% (n:4), for exon 45 skipping in 9% (n:3) patients, and for exon 53 skipping in 15% (n:5) of the patients (Figure 2).

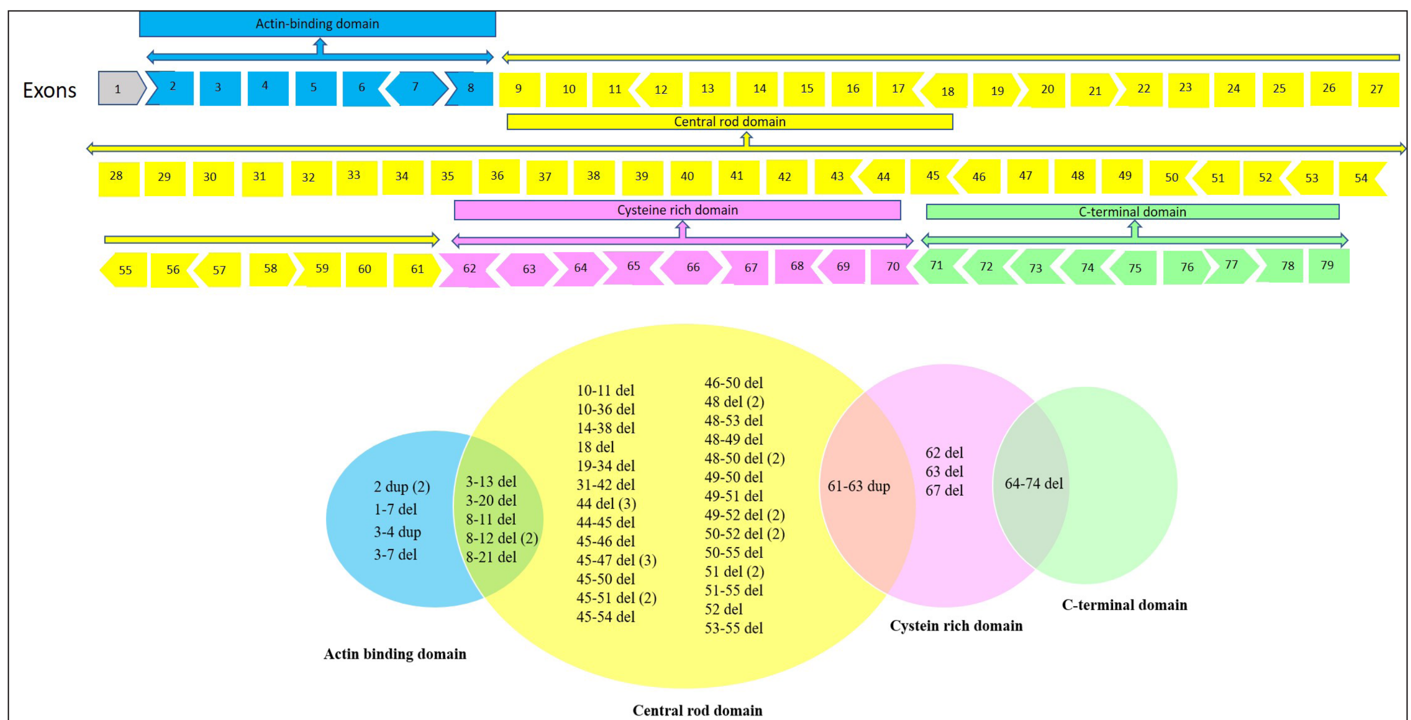

Figure 1: Mutations detected according to the dystrophin gene exon and domain characteristics.

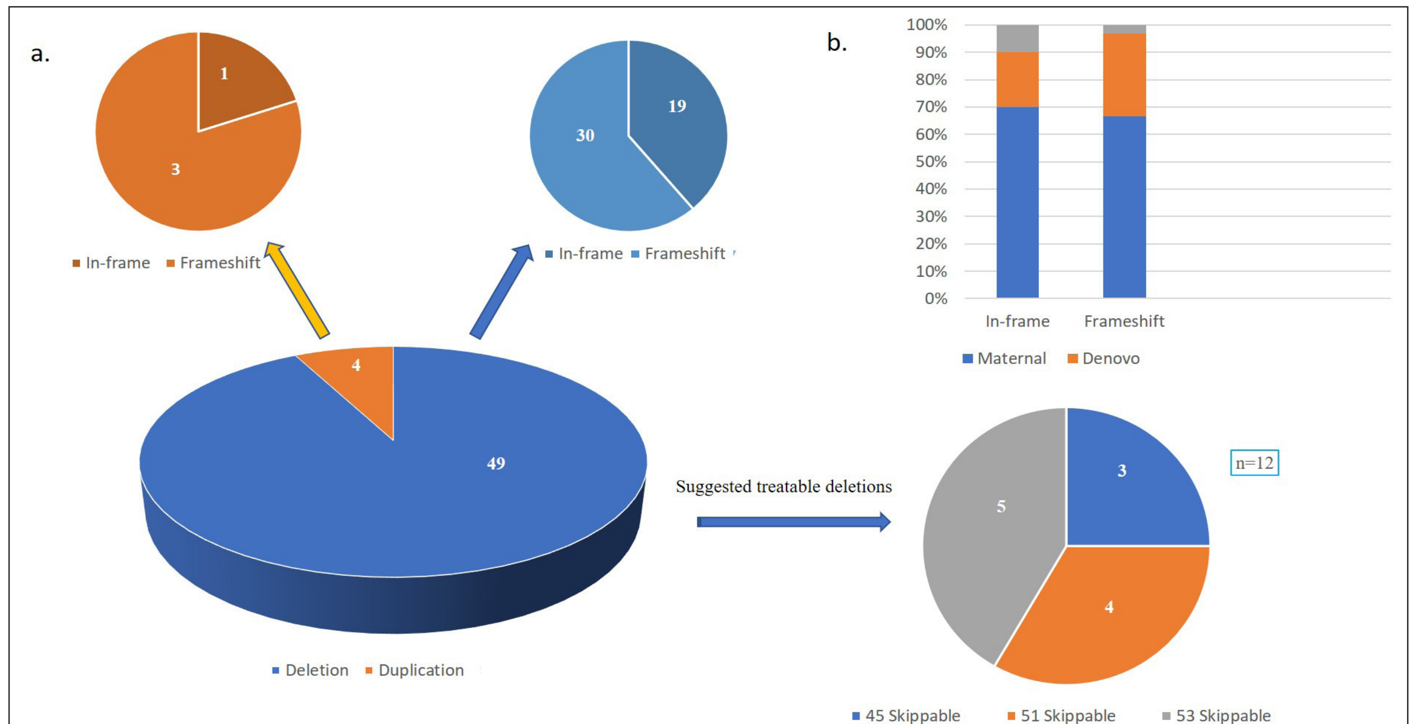

Figure 2: Distribution of patient numbers according to mutation type and amenability with evolving treatment options.

Table I: Clinical and genetic characteristics of the patients have been firstly evaluated by Microarray.

Patient	Exonic Deletion / Duplication	Inheritance	Open-Reading Frame	Age	Main clinical Symptoms	Microarray Results
P9	45-51 Deletion	Denovo	in-frame	7 year, 6 month	Congenital hypotonia, epilepsia, developmental delay	arr[GRCh38] Xp21.1(301748528_32018674) x0
P18	48 Deletion	Maternal	in-frame	7 year, 4 month	Atipic Autism, Speech delay	arr[GRCh38] Xp21.1(31844495_31893967)x0
P20	14-38 Deletion	Maternal	in-frame	3 years, 11 month	Epilepsy, developmental delay	arr[GRCh38] Xp21.1(32346780_32589606)x0
P25	48-49 Deletion	N/A	in-frame	8 years 9 month	Developmental delay, absent speech	arr[GRCh38] Xp21.1(31844475_31975958)x0

DISCUSSION

DMD and BMD are the most common hereditary neuromuscular disease group of the childhood caused by the mutations which cause a defect in the synthesis of the fully-functional 427-kDa dystrophin protein (1-9). They make up approximately 50% of the neuromuscular diseases in our country (10). Large rearrangements secondary to deletions or duplications in one or more exons are the most frequent cause of the defects in the dystrophin protein synthesis (1,3). The MLPA results for the dystrophin gene were evaluated in our study which revealed a frequency of 92.4% for deletions and 7.5% for duplications. In a study that evaluated the frequency of mutations in the dystrophin gene in our country, the frequency of gross

deletions was 48.8% and 9.2% for duplications (11). Another study has reported 67% deletions, and 18% duplications (12). In a recently published study where 1660 patients were evaluated, 65.7% of the patients had deletions while 5.9% had duplications (13). The same study reported that deletions were most frequently detected on the 45th exon while duplications were most frequently detected on the 2nd exon (13). There were no patients with a deletion on the 45th exon in our study while half of the duplications detected in 4 patients were located on the 2nd exon. Detection of the mutations has become more important in the last decade and skipping treatments for 45th, 51st, and 53rd exons are expected to become a part of routine treatments shortly soon (14). At this point, we would like to state that 36% (12 of 33 frameshift variation) of our patients are suitable candidates for skipping treatments targeting these 3 different exons. Along with these 3 exons, there are 9 more patients suitable for exon skipping that can be developed (exons 7,12,17,52 (for 2 patients), 55, 56 (for 2 patients) and 62 skippable variations). As a result, 21 (63%) of 33 patients with frameshift mutation among the patients included in our study are suitable for exon skipping treatment and this finding is compatible with the literature knowledge about the frequency of DMD patients eligible for exon skipping treatments (15).

The gold standard method for DMD diagnosis is the MLPA technique, because deletions and duplications are frequently observed in DMD (16). Microarray technology is capable of detecting minor deletions and duplications in single-gene disorders due to high single nucleotide polymorphisms (SNP) and small copy number variations (CNV) (17). Deletions in the dystrophin gene were incidentally detected in four patients who were initially scheduled to undergo microarray testing due to congenital hypotonia, epilepsy, growth retardation, and atypical autism. The fact that all these mutations were in-frame can explain the lack of prominent dystrophinopathy-related symptoms.

The scope of DMD gene mutations is determined by whether the variants disrupt the reading frame or not. Frameshift mutations disrupt the open reading frame of the DMD gene which causes dystrophin deficiency and serious DMD phenotype. On the other hand, in-frame mutations retain the reading frame, result in a partially functional dystrophin protein, and cause the less severe Becker muscular dystrophy (BMD) disease. The BMD phenotype can vary between asymptomatic and borderline DMD depending on the different mutation types. The phenotype can be foreseen with 90% accuracy by using the mentioned reading frame rule, which is also known as the Monaco rule (2,18). Thus, we believe that 62.3% of our patients had DMD features due to a frameshift mutation while 37.7% presented with BMD features due to in-frame mutations. However, a definitive diagnosis can be confirmed with family history, clinical examination, and muscle biopsy when becomes necessary.

The most frequent mutation in our patient population was found in the central rod domain of the dystrophin gene while the second most frequent mutation was found in the actin binding domain. The actin-binding domain (N-terminal area) contains exon 2-8, the central rod domain contains exon 9-61, the cysteine-rich domain (CR) contains exon 64-70 and the C-terminal domain (CT) contains exon 71-79. In the skeletal muscle, the central rod domain 1-3, and 10-12, CR and the CT domains are called membrane-binding domains (MBDs) and are bound to the sarcolemma. In the heart muscle, the central rod domain 10-12 is not bound to the sarcolemma. The N-terminal domain contains the primary actin-binding domain which binds F-actin. The first part of CR and CT binds to transmembrane β-dystroglycan. CT contains the dystrobrevin and syntrophy binding domains which bind to two transmembrane proteins in the sarcolemma. Since NT, CR, and CT are very important for dystrophin function, frameshift mutations in these domains cause more severe clinical features (19,20).

This study has evaluated patients referred from various parts of Turkey who received a molecular genetic diagnosis as a result of deletions or duplications detected in dystrophin exons using the MLPA method.

The frequencies of mutations other than deletions and duplications could not be evaluated in this study since there were no patients who were diagnosed using dystrophin gene sequencing (NGS) in the study population. However, the fact that several of our patients are suitable candidates for emerging genetic-based treatments is promising. It should be noted that new studies with larger populations where patients diagnosed with techniques other than MLPA are necessary to determine the genotype distribution of dystrophinopathies in our country.

As a result the DMD gene mutations can be identified incidentally or causally in many patients with a neurodevelopmental disorder since it is prone to mutations. In our study, we determined 36% of patients with frameshift mutation are suitable for recent exon-skipping treatments, and 63% of them may be suitable for exon-skipping that can be developed in near future. Elucidation of major deletions and duplications in the DMD gene based on ethnicity will aid in population-specific studies on new genetic and molecular therapies.

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