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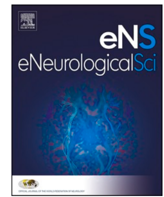

Next-generation sequencing approach to molecular diagnosis of Iranian patients with Duchenne/Becker muscular dystrophy: Several novel variants identified

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ABSTRACT

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) constitute the second most prevalent muscular dystrophy, with large deletions or duplications accounting for 66% of cases. No effective treatment exists for DMD/BMD. At present, genetic diagnosis serves as the foundation for gene therapy treatments. In this study, a comprehensive molecular investigation was conducted. The subjects diagnosed with DMD/BMD were initially examined using multiplex ligation-dependent probe amplification (MLPA) technology. The negative MLPA results were analyzed further using next-generation sequencing (NGS) technology. The MLPA detected 201 deletions (65.9%) and 20 duplications (6.6%) along the dystrophin gene among the 305 Iranian patients examined. The deletion of exon 52 in the amenable skipping subgroup was associated with an earlier onset age and a more severe phenotype. Twenty-one of the small mutations found in 58 MLPA-negative patients were novel. The most prevalent variants were nonsense variants (46.5%), frameshift variants (31%), splicing variants (6.9%), missense variants (10.4%), and synonymous mutations (5.1%). Our results demonstrate that MLPA and NGS can be effective diagnostic tools for very young patients with a single exon deletion.

1. Introduction

Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy (DMD) constitute the most prevalent types of muscular and inherited myopathy in live males, respectively [1]. As X-linked recessive genetic disorders, these severely debilitating conditions with an incidence rate of 1 in 3600–6000 are caused by variants in the Xp21.2-located dystrophin gene, resulting in the absence or malformation of the dystrophin cytoskeletal protein [2,3]. The most common symptoms of DMD/BMD are delayed walking, frequent falls, waddling gait, toe walking, calf hypertrophy, difficulty rising (Gower's sign), and difficulty climbing stairs [4].

The approximate age of diagnosis is five, which is when the first symptoms appear [5]. Clinical analysis of DMD reveals a severe phenotype, which is characterized by progressive muscle function loss beginning between the ages of two and five, loss of ambulation by the age of thirteen, and death around the age of twenty. Moreover, BMD is the less severe form, and patients lose their ability to walk after age 16 [6].

As one of the largest genes, dystrophin contains 2.4 million base pairs with 79 exons forming a 14-kb mRNA. The mutation spectrum of DMD/BMD consists of large deletions/duplications ($\geq$ 1 exon) occurring in approximately 70% of patients, while the remaining 25–30% of the patients carry point variants (<1 exon), including missense and nonsense variants as well as splicing defects and small insertions, deletions, and inversions [7,8]. Mutation variations in the *DMD* gene are responsible for the clinical differences between BMD and DMD. Patients exhibit the DMD phenotype when the mutation results in a frameshift (out-of-frame mutation) or a premature stop codon, whereby a nonfunctional dystrophin protein is produced. In contrast, BMD is diagnosed when the mutation occurs within the reading frame (in-frame mutation), and a partially functional dystrophin protein is generated [9]. Studies have demonstrated that dystrophin gene deletions cluster in two distinct hotspot regions, including the major mid-distal region exons 45–52 and the minor proximal region exons 3–19 [10].

Multiplex ligation-dependent probe amplification (MLPA) is a quick and cost-effective method that can be used as a first-pass assessment of DMD. Moreover, it can detect deletion and duplication variants in all 79

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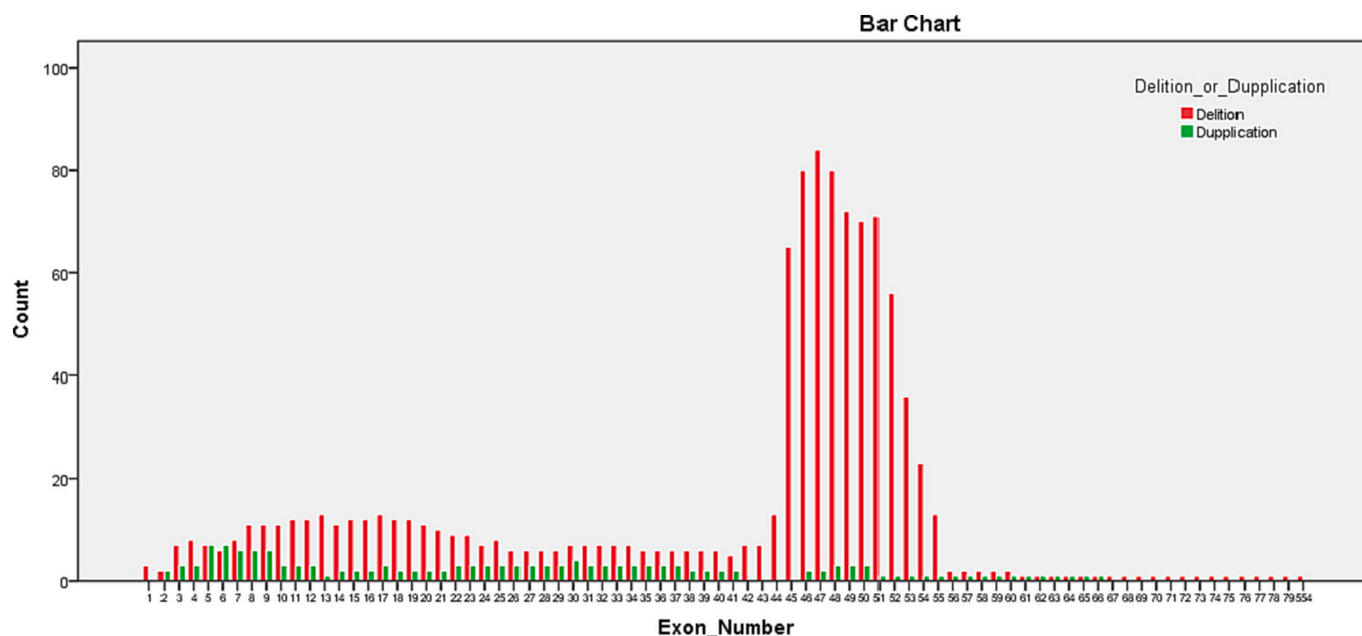

Fig. 1. Distribution of deleted or duplicated exons of the DMD gene as determined by MLPA, with red indicating deletion and green indicating duplication. The results demonstrated that deletion in exon 48 is the most affected region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

exons of the dystrophin gene [11].

NGS is a sequencing technique with a greater sequencing range than Sanger sequencing. It is a well-known testing method for DBMD, used in conjunction with other strategies, such as MLPA, for genetic diagnosis. Targeted NGS can provide a more precise depiction of unclear and undefined variants, whereas MLPA is incapable of distinguishing certain dystrophin variants [4,12,13].

In this study, we identified novel mutations in the DMD gene using both MLPA and NGS as the most cost-effective and efficient diagnostic methods. Moreover, the phenotypic and genotypic characteristics of dystrophinopathy patients in Iran were evaluated.

2. Materials and methods

2.1. Patient reports

This study enrolled all male patients with a suspected DMD/BMD who were referred to the Neuromuscular Clinic at Tehran University Hospital between January 2015 and April 2021.

Male sex, proximal muscle weakness (Gower's sign), calf hypertrophy, and a creatine phosphokinase (CPK) level of >1000 (U/L) constituted the inclusion criteria as determined by clinical assessments.

Before analysis, written informed consent was obtained from all patients or their parents/legal guardians. Moreover, the study was approved by the ethics committee of Tehran University of Medical Sciences (Identifier: IR.TUMS.REC.1394.2076).

2.2. MLPA technology

All patients recruited for the MLPA examination were referred to a university laboratory. Briefly, DNA was extracted from the peripheral blood samples using standard procedures. MLPA analysis was performed on the dystrophin gene's 79 exons using DMD kits (P034 and P035, MRC Holland, Amsterdam, Netherlands) according to the standard manufacturer's protocol. MLPA was utilized to determine the carrier status of the mothers of 172 patients.

2.3. NGS and data analysis

Subsequently, NGS was used to detect small variants in the 58 MLPA-negative patients. The remaining 26 cases did not participate in the project. Next Generation Sequencing (NGS) was followed by Nimblegen chip-based target region capture in the genes of interest. The mutation was confirmed via Sanger sequencing. In addition, three patients with a single exon deletion were sequenced to verify the identified variants, while the remaining patients did not participate in the verification process.

2.4. Statistical analysis

In this study, descriptive statistical measures were employed. The mean was calculated for quantitative data. Both absolute numbers and percentages are reported for qualitative variables. We analyzed the data using SPSS software (version 20.0).

3. Results

The genetic analysis through MLPA and NGS was employed to evaluate 305 cases of DMD/BMD. The ages of the patients ranged from three months to 38 years, with a mean of 9.14 years. The mean age of patients at the time of diagnosis was 53.89 months.

Information was collected on 284 DMD (93.1%) and 21 BMD cases (6.9%). According to laboratory data, the mean CPK level was 11,041.

An MLPA test was performed on each patient to identify the types of underlying variants. In 221 patients (72.5%), MLPA was positive, whereas in 84 cases (27.5%), MLPA did not detect any exonic deletion or duplication. MLPA analysis of 305 patients revealed 201 deletions (65.9%) and 20 duplications (6.6%). Moreover, 84 patients (38%) had deletion or duplication of a single exon, whereas 137 patients (62%) had deletion or duplication of multiple exons.

Two mutational hotspots were thought to be responsible for DMD. The results revealed that the distal hotspot (exonic region 44 to 55) was responsible for 70.9% of the dystrophin gene exonic deletion cases, while the proximal hotspot accounted for only 9.1% of the cases.

Analysis of the frequency of deletions and duplications in patients

Table 1
Number of identified variants.

Type of variant	DMD patients	%	Number of variants
Nonsense	27	46.5	18
Frameshift	18	31	17
Splice site	4	6.9	3
Missense	6	10.4	5
Synonymous	3	5.1	3
Total samples	58	100	46

with BMD and DMD revealed that exon 48 was the most frequently affected region (7.3%), occurring 35 times in total (32 deletions and three duplications). In addition, exon 49 (27 deletions and seven duplications) and exon 51 (27 deletions and four duplications) constituted the second affected region (Fig. 1).

One of the patients in this study was a 4-year-old boy with a single exon deletion mutation that was inconsistent with the patient's clinical phenotype. The patient exhibited early clinical symptoms, muscle weakness, and frequent falls at 18 months of age. In addition to proximal muscle weakness (Gower's sign), the patient had hypertrophy of the leg muscles. Myopathy appeared on the electromyogram (EMG), and biochemical analysis revealed an abnormal level of creatine phosphokinase (CPK) enzyme (5966 U/L). The patient's novel frameshift mutation (NM 004006: c.998–1088 del) was detected using NGS.

In order to follow up on the 84 patients with negative MLPA results, 58 patients underwent NGS, which revealed 25 novel variants (43.10%) and 33 known variants (56.89%). There were 27 stop codon nonsense variants (46.5%), 18 frameshift mutations (31%), six missense mutations (10.4%), four splice site mutations (6.9%), and three synonymous mutations (5.1%) (Table 1; Fig. 2).

3.1. Novel mutation

Twenty-one novel variants were discovered in total, including five nonsense variants, 15 frameshift mutations, two missense mutations (Table 2).

All variations were examined for their presence in the Leiden Open Variation Database (LOVD, http://www.dmd.nl), ClinVar, and dpSNP, Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk), and their potential pathogenicity was confirmed using the Mutation Tester. Standards established by the American College of Medical Genetics and Genomics (ACMG) were used to analyze the novel variants. The pathogenicity of novel variants was assessed using immunohistochemistry on muscle biopsies containing dystrophin protein.

3.2. Genotype-phenotype correlations

We stratified our patient population based on their clinical presentation and the Duchenne population amenable to exon skipping, paying special attention to the age of symptom onset.

The results indicated a correlation between variant type and onset age. The mean age at disease onset was 1.33 years in the "exon 52 amenable skipping" subgroup, which had a more severe phenotype, compared to 5.5 years in the "deletions amenable to skipping exon 44"

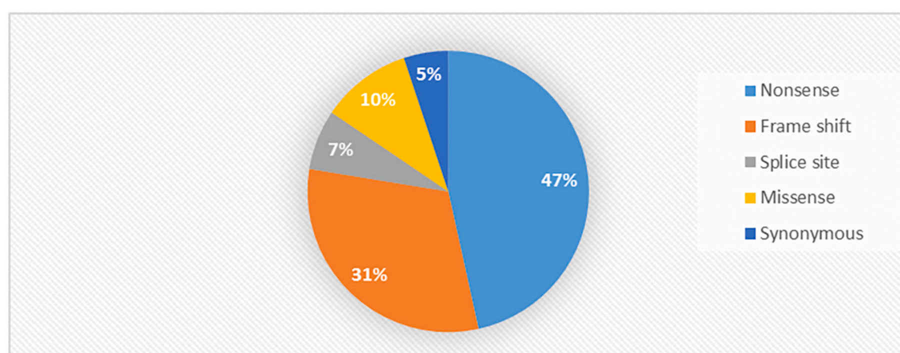

Fig. 2. Distribution of identified point variants by NGS.

Table 2
Novel variants.

	Type of variant	Location	Nucleotide change	Protein effect	ACMG classification
1	Nonsense	Exon25	NM_004006.3:c.3414G > A	Trp1138Ter	Pathogenic
2	Nonsense	Exon 12	NM_004006.3:c.1480A > T	Lys494Ter	Pathogenic
3	Nonsense	Exon 8	NM_004006.3:c.826C > T	Gln276Ter	Pathogenic
4	Nonsense	Exon19	NM_004006.3:c.2323A > T	Arg775Ter	Pathogenic
5	Nonsense	Exon 21	NM_004006.3:c.2698A > T	Lys900Ter	Pathogenic
6	Frameshift	Exon 10	NM_004006.3:c.998_1088del	s333fs ter	Pathogenic
7	Frameshift	Exon 73	NM_004006.3:c.10363_10364insA	Leu3455HisfsTer3	Pathogenic
8	Frameshift	Exon 37	NM_004006.3:c.5269delA	Ile1757SerfsTer24	Pathogenic
9	Frameshift	Exon 30	NM_004006.3:c.4169_4176dup	Leu1393ThrfsTer28	Pathogenic
10	Frameshift	Exon 31	NM_004006.3:c.4317_4318dup	Val1440GlufsTer18	Pathogenic
11	Frameshift	Exon 39	NM_004006.3:c.5544_5548del	Ile1849ThrfsTer9	Pathogenic
12	Frameshift	Exon 69	NM_004006.3:c.10027dup	Ser3343PhefsTer9)	Pathogenic
13	Frameshift	Exon 6	NM_004006.3:c.464delA	Asn155MetfsTer2	Pathogenic
14	Frameshift	Exon 31	NM_004006.3:c.4317_4318dup	Val1440GlufsTer18	Pathogenic
15	Frameshift	Exon 73	NM_004006.3:c.10363_10364insA	Leu3455HisfsTer3	Pathogenic
16	Frameshift	Exon 37	NM_004006.3:c.5269delA	.Ile1757SerfsTer24	Pathogenic
17	Frameshift	Exon 67	NM_004006.3:c.9672_9679delTTCAACAG	Ser3224ArgfsTer4	Pathogenic
18	Frameshift	Exon 30	NM_004006.3:c.4169_4176dup	Leu1393ThrfsTer28	Pathogenic
19	Frameshift	Exon 74	NM_004006.3:c.10447dup	Ser3483PhefsTer8	Pathogenic
20	Missense	Exon 26	NM_004006.3:c.3598A > G	Met1200Val	Likely Benign
21	Missense	Exon 58	NM_004006.3:c.8548G > T	Ala2850Ser	Likely Pathogenic

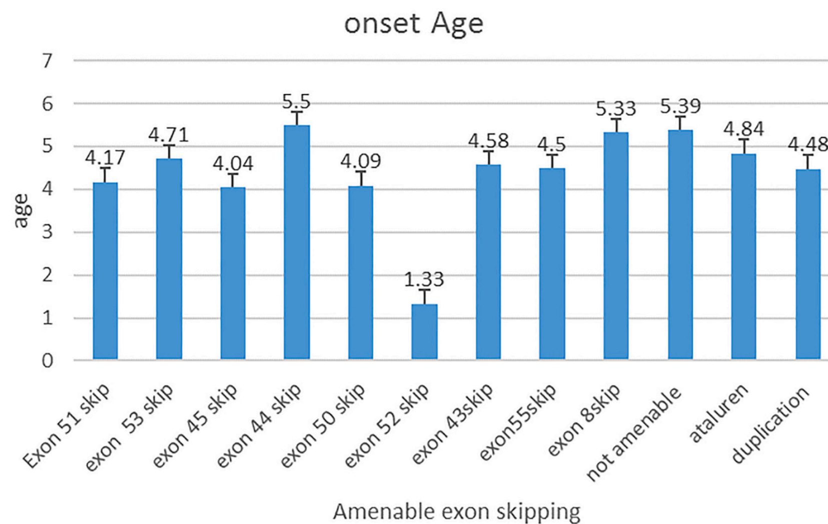

Fig. 3. Genotype-phenotype correlation. Chart showing the relationship between age of onset disease and type of variant.

subgroup, which had a less severe phenotype (Fig. 3).

4. Discussion

In total, 221 deletions and duplications were discovered after analyzing 305 DMD/BMD patients. The most exciting finding of the current study is that MLPA analysis detected deletion/duplication in 72.5% of children with DMD/BMD phenotype.

The deletion detection rates reported in other studies using MLPA and multiplex PCR varied widely across diverse populations ranging from 31% to 94% [15].

North American studies report deletion detection rates ranging from 55 to 70%, whereas detection rates in European populations range from 39 to 45% in Germany, Hungary, Former Czechoslovakia, and Spain. Patients with DMD/BMD in the Philippines have had the lowest deletion rate (approximately 33%). Deletion rates among DMD/BMD patients in Asia are 45–59% for the whole continent, 40–43% for China, 52–59% for Japan, 55% for Turkey, 86% for Thailand, 63% for Arab countries, and 71.8% for India and South Korea [2,16,17]. A multinational study of Singaporean, Vietnamese, and Japanese populations revealed lower rates of deletion detection, amounting to 40%, 32%, and 51%, respectively [18].

Other factors, such as bias concerning sample size, inclusion/exclusion criteria, numbers/locations of the analyzed exons, and race, may be responsible for these differences in deletion detection rates between ethnic groups. It is evident that the inclusion of patients with other clinically similar muscle diseases and the limited number of exons analyzed may have contributed to the lower frequency of dystrophin gene deletions detected. In the current study, all dystrophin exons (1–79 exons) were analyzed, and patients with similar clinical symptoms as determined by muscle biopsy and dystrophin immunohistochemistry were excluded. Such strict inclusion criteria allowed for the detection of deletion/duplication in 72.5% of the subjects studied, which is in line with the higher deletion detection rates found in India and South Korea [19,20]. Upon further investigation of patients with single exon deletions, the percentage of patients with exon deletion will also change in this study. We had 84 patients (38%) with single exon deletion/duplication, and the sequencing of three of them revealed a frameshift mutation. This finding suggests that a subset of patients with single-exon deletions may actually carry a point mutation. Importantly, three patients with a single exon variant of the dystrophin gene, detected via MLPA, underwent whole-exome sequencing (WES) for verification, and a novel frameshift mutation of DMD (NM-004006: c.998–1088 del) was identified in a four-year-old boy. This variant is associated with the early onset of symptoms at the age of four. Additionally, this finding suggests that additional research is required to confirm the high detection rate.

According to the findings of this study, 70.9% of dystrophin exon deletion/duplication clusters were detected in the distal hotspot, while only 9.1% were detected in the proximal hotspot. The deletion hotspot regions of our cases were primarily concentrated in the central region, with a distribution rate of approximately 70.9%. This finding is consistent with the findings of other studies, which reported 70–80% of detected deletions in the distal hotspot between exons 44 and 55 [21]. With respective deletion rates of 6.8%, 5.7%, and 5.7%, exons 48, 49, and 51 were the most frequently affected regions. With a frequency of >16%, however, exon 50 was the most frequently deleted exon in a study of the Indian population. Compared to Indian studies, the frequency of exon 44 deletion was surprisingly higher in the Turkish population [22].

This discrepancy may be linked with the hypothesis that different ethnic groups have distinct variations in a particular intronic region of the dystrophin gene. Based on this hypothesis, the diverse intronic sequence makes this region more susceptible to breakpoint deletions [2].

Due to insufficient information, we only examined 172 of the 305 patients regarding the mothers' carrier status. There were 32% (55/172) de novo mutations, while 45 mothers were not analyzed. Therefore, genetic counseling for DMD requires an efficient and rapid diagnostic method and a systematic pedigree analysis.

Point variants were determined for the MLPA-negative individuals who underwent NGS. The NGS identified frameshift, missense, and splicing variants in 18 (31%), six (10.3%), and four (6.9%) patients, respectively; nonsense was the most prevalent mutation observed in 27 (46.5%) patients. The rate of nonsense variants was higher than in other studies [23]. Five nonsense variants were novel and unreported in previous studies. The variants were predicted to result in a premature stop codon in the protein's coding sequence and a nonfunctional dystrophin protein, which leads to DMD.

Frameshift was the second most prevalent type of point variant. It was identified in 18 patients (31%), nearly at the same prevalence rate reported in China (28.29%) and Eastern Europe (29%), and at a higher prevalence rate than in the Turkish target population (6%) [24–26]. Micro deletion and insertion altered the open reading frame and caused early termination of the amino acid coding, which can lead to the production of truncated dystrophin protein, typically associated with the DMD phenotype. Fourteen of these variants were novel and located in

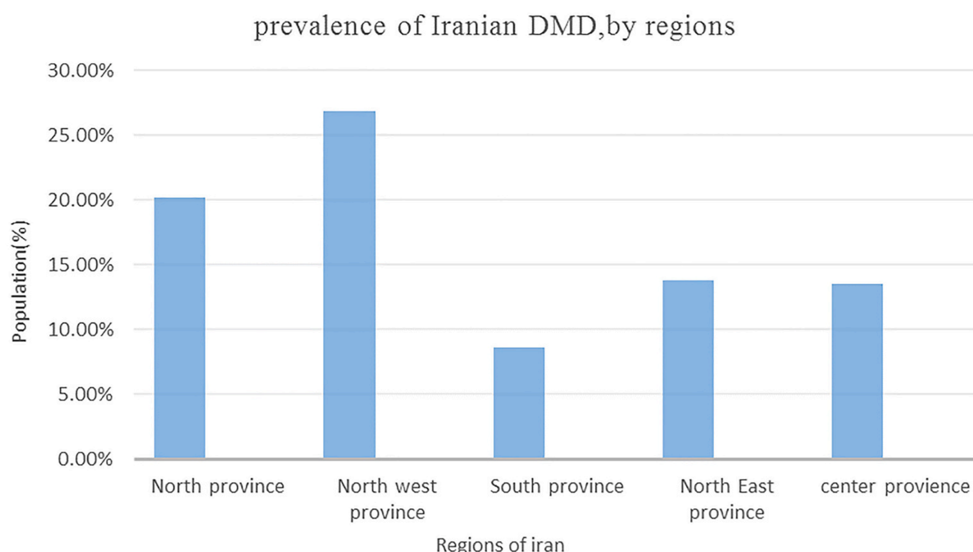

Fig. 4. In this study, DMD patients from all Iranian provinces were registered. The prevalence rate was highest in the northwestern provinces.

different exons. Studies conducted in France, Japan, and Iran indicate a low frequency of missense mutations [23,27,28]. However, six patients (10.34%) were identified as having this variant. Two missense variants were reported as benign or likely benign in this study.

In addition, four splicing variants (6.89%) were identified at a highly conserved splicing site (1–2 bp from the exon-intron boundary). This mutation has also been reported in Spain (20%), China (14.63%), and Eastern Europe (4%) [24,25,29]. Four out of all variants were found in the proximal and distal regions of the DMD gene [30], which complies with the findings of other studies.

In this study, patients were categorized by region, with the results revealing that the majority of patients came from North West provinces (East Azerbaijan, West Azerbaijan, Zanjan, and Ardabil) (26.9%), while a smal proportion of patients came from southern provinces (Sistan and Baluchistan, Khuzestan, Bushehr, Hormozgan, and Fars) (8.6%). In addition, data for 17% (52/305) of the patients was unavailable (Fig. 4).

4.1. *Novel therapeutic*

Exon skipping is a potential therapeutic approach to modifying and restoring dystrophin production. In previous research, the population variants of DMD have been categorized into several groups based on their amenability to therapeutic exon skipping, which has served as the foundation for the majority of treatments to date [31].

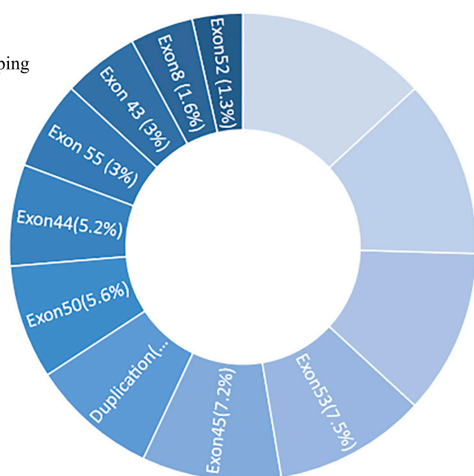

Fig. 5. Classification patients with exon deletions amenable to exon-skipping therapy. The chart was designed on study of Annemeike Aaertsma [31].

A sizable subset of patients may be candidates for novel variant-based therapies if they fall into a category with variants amenable to top exon-skipping strategies. Indeed, 13%, 8.1%, and 7.7% of patients have been assumed to be candidates for skipping single exons 51, 53, and 45, respectively [31]. However, in this study, 10.5% of patients were eligible for exon 51 skipping, 7.5% for exon 53 skipping, and 7.2% for exon 45 skipping (Fig. 5). Furthermore, 27 patients with nonsense variants that result in stop codons may benefit from nonsense suppression therapy with compounds such as ataluren that enable read-through of untimely stop codons [32].

5. Conclusions

Due to the high frequency of single-exon deletion variants in Iran [23], normal MLPA results are insufficient, as patients with DMD may have other variants. Therefore, it is vital for future research to utilize NGS for the molecular diagnosis of DMD patients. The pattern of the point variant in Iranian DMD/BMD patients with negative MLPA and dystrophin gene sequencing requires additional experimental research. These studies bolster genetic counseling, prenatal diagnosis, and the clinical management of genetic disorders. Additionally, genetic testing provides crucial information regarding the suitability of emerging therapies entering clinical practice.

Authors' contributions

KB organized this study, performed data analysis, and reviewed clinical and laboratory data; SB performed data analysis. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The ethics committee of Tehran University of Medical Sciences

approved this research (IR.TUMS.REC.1394.2076). All procedures were carried out in compliance with the applicable rules and guidelines. Moreover, written informed consent was obtained from all participants and their immediate family members for participation.

CRediT authorship contribution statement

MohammadKazem Bakhshandeh: Conceptualization, Methodology, Software, Writing – original draft, Supervision. **Samira Behroozi:** Investigation, Writing – original draft.

Declaration of Competing Interest

There are no conflicts of interest.

Acknowledgments

Not applicable.

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