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Original Article

Genetic Landscape of *DMD* Gene Mutations in Iranian Patients and the Applicability of Molecular Therapies

Ali Khanbazi[1,2#], Farzane Zare Ashrafi[1,2#], Masoumeh Akbari Kelishomi[1], Raziye Rezvani Rezvandeh[1,2], Maryam Azad[1], Fatemeh Ahangari[1], Mahsa Fadaee[1], Shima Dehdahsi[1], Mina Makvand[1], Banafsheh Salmani[1], Zohreh Vahidi[1], Mahdieh Kooshki[1], Atiyeh Ahmadpour[1], Elmira Shiuokhi[1], Armita Ghaderi[1], Mahsa Tahmasebivand[1,2], Fariba Afroozan[1], Khadijeh Noudehi[1], Fatemeh Fatehi[1], Shima Zamanian Najafabadi[1], Elahe Jafarnataj[2], Shahriar Nafissi[3], Haleh Habibi[4], Maryam Taghdiri[5], Bahram Haghi Ashtiani[6], Gholamreza Zamani[7], Farzad Fatehi[8], MohammadKazem Bakhshandeh[9], Farshid Parvini[10], Payman Jamali[11], Kimia Kahrizi[2], Ariana Kariminejad[1], Hossein Najmabadi[1,2*]

[1]Kariminejad - Najmabadi Pathology & Genetics Center, Tehran, Iran
[2]Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran
[3]Department of Neurology, Neuromuscular Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
[4]Hamedan University of Medical Science, Hamedan, Iran
[5]Shiraz Genetic Counseling Center, Welfare Office, Shiraz, Iran
[6]Department of Neurology, Firoozgar Hospital, Iran University of Medical Sciences, Tehran, Iran
[7]Tehran University of Medical Sciences, Children's Medical Center, Tehran, Iran
[8]Iranian Center of Neurological Research, Neuroscience Institute, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran
[9]Pediatric Neurology Center of Excellence, Department of Pediatric Neurology, Mofid Children Hospital
[10]Department of Biology, Faculty of Basic Sciences, Semnan University, Semnan, Iran
[11]Mashhad Medical Genetic Counseling Center, Mashhad, Iran

Abstract

Introduction: Mutations in the X-linked dystrophin gene (*DMD*) cause Duchenne (DMD) and Becker muscular dystrophies (BMD), severe and milder forms of inherited progressive muscle deterioration, for which genetic testing is essential to confirm diagnosis, genetic counseling, and eligibility for molecular therapies.

Objective: This study aimed to elucidate the pattern of *DMD* mutations in a large cohort of Iranian patients and assess the applicability of mutation-specific therapies.

Methods: We retrospectively analyzed 2162 individuals referred to the Kariminejad - Najmabadi Pathology & Genetics Center (KNPGC) for *DMD* mutation testing. The samples consisted of 1093 males for diagnostic testing, 946 females for carrier detection, and 123 fetuses for prenatal assessments. Most cases were first examined using multiplex ligation-dependent probe amplification (MLPA), with negative results further evaluated by next-generation sequencing (NGS) or Sanger sequencing.

Results: Among 1093 males tested for diagnostic purposes, 608 (55.63%) were positive for *DMD* mutations. Among these positive cases, 470 deletions (77.30%) and 81 duplications (13.32%) were identified. Point mutations were identified in 55 patients (9.05%), with nonsense and frameshift variants being the most prevalent. Mutations predominantly affected exons 44–55 (44.17%) and 3–27 (35.75%). Notably, 69.09% of point mutations were located within the proximal 40 exons of the gene. Additionally, DP427 isoforms were disrupted in all patients, while shorter isoforms (DP116, DP71) were rarely affected. Applicability of mutation-specific therapies indicated that 49.34% of patients could theoretically benefit from exon-skipping strategies, 29.60% were eligible for currently approved therapies, and 3.45% with nonsense mutations could benefit from a read-through therapy.

Conclusion: This study provides an overview of *DMD* mutations in a large cohort of patients referred for testing, highlighting mutation patterns and the potential applicability of molecular therapies.

Keywords: Duchenne muscular dystrophy, *DMD* gene mutations, Iran, MLPA, Molecular therapy

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Introduction

Duchenne muscular dystrophy (DMD) and its milder allelic form, Becker muscular dystrophy (BMD), result from mutations in the *DMD* gene (MIM:300377) and are characterized by progressive muscle weakness, with respective prevalence rates of 4.78 and 1.53 per 100,000 males.[1-3] The major factor influencing these two phenotypes is the translational reading frame rule: out-of-frame mutations that disrupt the mRNA reading frame generally result in severe DMD, whereas in-

*Corresponding Author: Hossein Najmabadi, Email: hnajm12@yahoo.com
#These authors contributed equally to this manuscript.

frame mutations that preserve the reading frame lead to the milder BMD phenotype.[4] The *DMD* gene is one of the longest genes in humans, harboring 79 exons and spanning 2.4 Mb of genomic DNA; it encodes multiple dystrophin isoforms using different promoters. The *DMD* gene produces at least seven major dystrophin isoforms through distinct promoters. Three full-length isoforms, Dp427m (muscle), Dp427c/b (cerebral), and Dp427p (Purkinje), originate from upstream promoter regions. Dp427m and Dp427c are broadly expressed in the brain, particularly in the cortex, basal ganglia, hippocampus, and amygdala, with minimal expression in the cerebellum. Four shorter isoforms, Dp260, Dp140, Dp116, and Dp71, are also generated; Dp140 and Dp71 are mainly expressed in the brain, while Dp260 and Dp116 are restricted to the retina and peripheral nerves, respectively.[5-7] The extensive size of the *DMD* gene contributes to a high mutation rate, with approximately one-third of variants representing *de-novo* mutations.[4,8] According to data from international DMD mutation registries such as the TREAT-NMD DMD Global database[4] and the UMD-DMD database,[8] large mutations (deletions/duplications involving one or more exons) represent the largest proportion of cases (75%), whereas small mutations (smaller than one exon) or, more rarely, deep intronic CNVs and small mutations account for the remaining cases. Non-contiguous mutations, involving separate deleted or duplicated exons with intact regions in between, are also occasionally observed due to complex genomic rearrangements. Generally, deletions between exons 45–55 and duplications within exons 2–20 are known mutational hotspots in the *DMD* gene.[4,8,9] Studies from Iran have also reported these exons as their mutation hotspots.[10-12] However, variations in the *DMD* mutation profile have been reported across different populations and ethnic backgrounds.[2,9,11] In recent years, several molecular strategies have been designed and examined as possible therapeutic approaches to restore dystrophin production and alleviate the DMD phenotype, including gene replacement therapy, exon skipping, stop codon readthrough, and genome editing.[5,13-15] Given the growing evidence of the applicability of molecular therapies for DMD, genetic testing for these patients is beneficial for genetic counseling and determining mutation-specific therapies for patients with specified variant types, either approved for use or in clinical trials, such as exon skipping therapies or readthrough therapy.[16] FDA-approved exon-skipping therapies for *DMD* target exon skipping-amenable mutations, variants that can be corrected by exon skipping to restore the reading frame, including Casimersen (exon 45), Eteplirsen (exon 51), and Golodirsen and Viltolarsen (both targeting exon 53). These antisense oligonucleotides function by skipping specific exons in the dystrophin gene, restoring the reading frame, and enabling the production of a shortened but partially functional dystrophin protein,[15,17] In addition, read-through therapy has emerged as a mutation-specific approach for DMD patients with nonsense mutations, allowing the ribosome to bypass premature stop codons and produce functional dystrophin.[18] The aim of this study was to contribute to expanding the current overview of the genetic landscape of *DMD* mutations in Iranian patients by using a larger sample size in a retrospective study of patients referred to the Kariminejad - Najmabadi Pathology & Genetics Center (KNPGC) over the past 15 years and investigating the potential suitability of molecular therapies when approved or novel therapeutic strategies become available in our country.

Materials and Methods

We conducted a retrospective study involving 2162 subjects who were referred by healthcare centers, various genetic laboratories, or clinicians throughout Iran to the Kariminejad - Najmabadi Pathology & Genetics Center between 2009 and 2024 (continuously) for genetic diagnosis, carrier detection, and prenatal diagnosis of *DMD*-related molecular analysis. Subjects were eligible for inclusion if they met any of the following criteria: a clinical suspicion of DMD/BMD, a family history of DMD/BMD for carrier testing, or referral for prenatal diagnosis due to a known familial *DMD* mutation.

In most cases, initial evaluations were performed using multiplex ligation-dependent probe amplification (MLPA) to screen for large deletions or duplications. Cases evaluated using multiplex PCR during earlier diagnostic workflows were not included in this study to ensure methodological consistency, given its limited ability to detect duplications and accurately characterize mutations in female carriers. Cases with negative MLPA results were subsequently analyzed to detect potential small mutations using next-generation sequencing (NGS) or Sanger sequencing. In a subset of cases, NGS or Sanger sequencing was performed as the first-line test based on a direct request from the referring physicians. The methods employed in this study followed the relevant guidelines and regulations. Furthermore, this study was conducted and reported in compliance with the Strengthening the Reporting of Genetic Association Studies (STREGA) checklist.

Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex ligation-dependent Probe Amplification was used to analyze the referred individuals and fetuses to detect large deletions and duplications, based on the manufacturer's instructions as outlined on the MRC Holland website (https://www.mrcholland.com/). The MLPA analysis utilizes SALSA MLPA probemixes P034 DMD-1 and P035 DMD-2 kits with 97 MLPA probes, including probes for all 79 exons of the *DMD* gene (NM_004006.3) and also one additional probe for exon 1 of the DP427c isoform (NM_000109.4). Each probemix contains 45 distinct probes amplifying sequences between 129 and 490 nucleotides, plus 10 control probes that generate shorter fragments (< 120 nucleotides). The final ratios were determined by comparing each sample to reference samples to compute the copy numbers. Internal validation was conducted with 16 DNA samples

from healthy individuals to ensure a standard deviation of ≤ 0.10 for all reference probes. Amplification products were detected and quantified by capillary electrophoresis using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Data analysis was performed using the Cofalyser.Net™V220513.1739 Software (MRC Holland, Amsterdam, The Netherlands).

Gene Panel Sequencing (GPS), Whole Exome Sequencing (WES) and Data Analysis

GPS or WES was performed in a number of cases with negative MLPA results or cases with direct requests from their clinicians. Genomic DNA was extracted from patient samples and subjected to GPS or WES analysis. For GPS, a custom-designed SureSelect panel covering 685 known disease-associated genes was used, while WES employed Agilent SureSelect Human All Exome Kits (V4–V7) (Agilent Technologies, Inc., Santa Clara, CA, USA) or Twist Human Core Exome (Twist Bioscience, San Francisco, CA, USA) libraries to capture exonic regions. Libraries were sequenced on Illumina platforms (HiSeq 2000/2500/4000, NovaSeq 6000) (Illumina, San Diego, CA, USA) with paired-end reads. Sequencing reads were aligned to the GRCh37/hg19 reference genome using Burroughs Wheeler Aligner (BWA),[19] and subsequent BAM processing, quality control, and coverage assessment were performed with Picard tool and the Genome Analysis ToolKit (GATK) according to the GATK best-practice recommendations.[20] Variant calling was conducted using the GATK Haplotype Caller, achieving mean coverage depths of 522 × and 126 × for GPS and WES, respectively, with ≥ 97.5% of the target regions covered at ≥ 10 ×. Variants were annotated and filtered using ANNOVAR [21] and an in-house bioinformatics pipeline, excluding common polymorphisms (≥ 1% frequency) and recurrent artifacts based on population databases (gnomAD (https://gnomad.broadinstitute.org) and Iranome (http://www.iranome.ir)) and internal datasets. Synonymous and deep intronic variants (> 10 bp from exon boundaries) were removed, and the remaining variants were prioritized according to inheritance patterns, phenotype concordance, population frequency, variant type, and *in-silico* predictions tools.

Illumina Dragen genome-wide depth-based CNV caller and AnnotSV [22] were used for variant calling and annotation of Copy number variants (CNVs) respectively. For common CNVs filtering, gnomAD, DGV (http://dgv.tcag.ca), and dbVar (https://www.ncbi.nlm.nih.gov/dbvar/) were used.

Final candidate variants were classified according to the ACMG/AMP guidelines[23] and ClinGen recommendations,[24] with validation and interpretation performed by an in-house expert committee.[25]

Sanger Sequencing

Sanger sequencing was performed on cases with negative MLPA results, as well as in families with a previously identified *DMD* mutation. Following purification using Exo SAP-IT (Affymetrix), sequencing was performed using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems Genetic Analyzer, following the manufacturer's instructions.

Data Analysis

Data filtering, descriptive statistical analysis and plotting were carried out using the RStudio software, version 2024.12.1+563 (R version 4.3.3 (202-02-29)). The effect of deletions and duplications on the *DMD* reading frame was assessed using the LOVD exonic deletions/duplications reading-frame checker.[26] Potential molecular therapies for patients with large deletions were evaluated using the DMD Open-access Variant Explorer (DOVE).[27]

Chi-square test was used to compare mutational hotspots between our cohort and international *DMD* mutation registries, including TREAT-NMD and UMD-DMD registries. Fisher's exact test was applied to compare the distribution of mutation types between our study and other cohorts. Statistical significance was set at $P < 0.05$.

Results

In total, 2162 individuals, including 1093 males and 946 females, with a median age of 21 years (IQR:7-30), were tested in KNPGC for genetic diagnosis and carrier detection, and 123 fetuses were assessed for prenatal diagnosis.

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA analysis confirmed the diagnosis in 553 of 1051 (52.62%) male patients, while the remaining cases were negative (Figure 1). Among the positive cases, 470 (84.99%) had deletions, 81 (14.65%) had duplications, one showed a non-contiguous duplication, and one exhibited a non-contiguous deletion/duplication. Deletion of exons 45–47 represented the most frequent deletion (6.60%), followed by deletions of exons 45–50 (5.74%) and exons 45, 45–48, and 45–52 (each 4.47%) (Figure 2). Duplication of exon 2 (11.11%), exons 3–7 (6.17%), and exons 8-9 (4.94%) represented the most common duplications among the patients (Figure 3). In total, 192 distinct variations among patients, comprising 137 large deletions and 53 duplications were observed. In addition, two non-contiguous mutations were identified: a non-contiguous duplication involving exons 1–2 and 45–57, and a non-contiguous deletion/duplication affecting exon 53 and exons 48–50. Of all 137 distinct deletions, 98 (71.53%) were out-of-frame, 38 (27.74%) were in-frame, and one (0.73%) was nonsense (deletion of exon 1). Of the 53 distinct large duplications, 40 (75.47%) were frameshift and 13 (24.53%) were in-frame.

Additionally, 203 of 911 (22.28%) females tested by MLPA were identified as carriers of *DMD* gene mutations, of whom 177 (87.19%) had deletions and the remainder had duplications. Notably, 125 of all females were mothers of MLPA-positive patients, with 65 (52.00%)

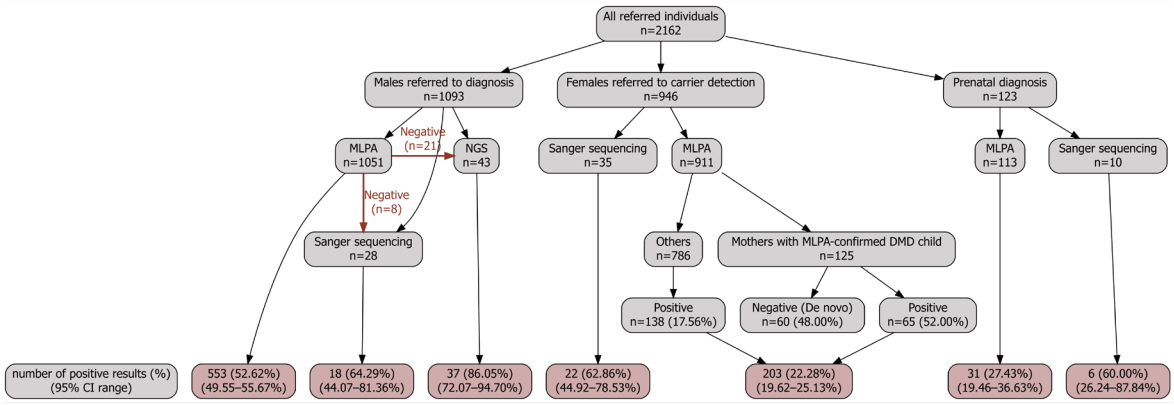

Figure 1. Flowchart of studied individuals

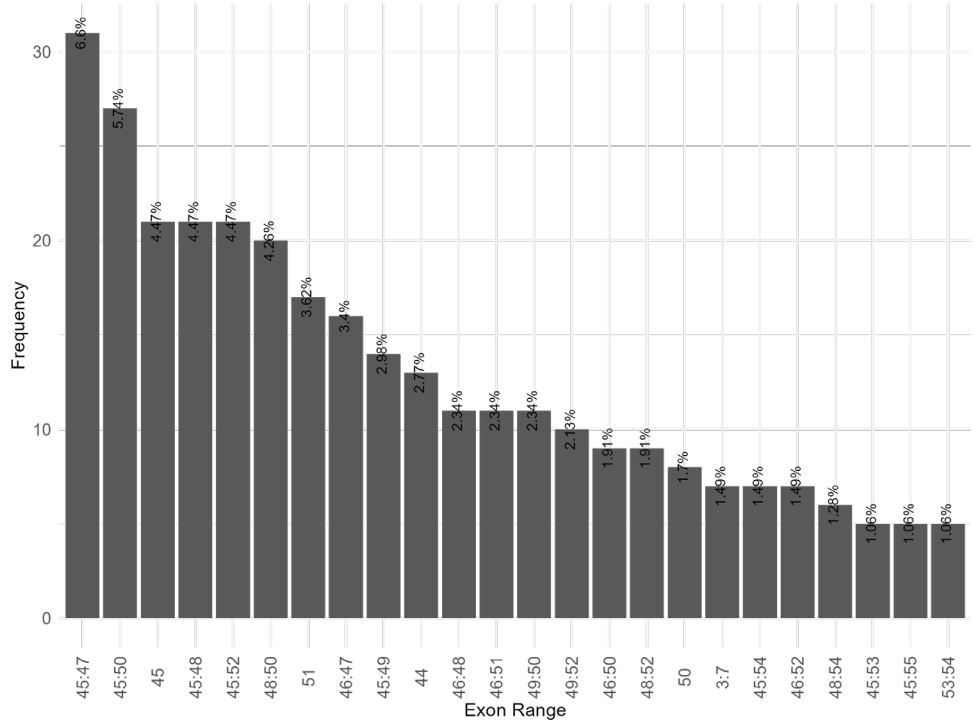

Figure 2. Frequent deletions in the *DMD* gene among patients

carrying the same mutation as their affected offspring, while the remaining mothers had no detectable mutation, indicating *de-novo* mutations in the patients (Figure 1).

Next-generation Sequencing (NGS)

Forty-three males were analyzed using the NGS method (Figure 1). The small mutations identified were as follows: 24 pathogenic (55.81%), 13 likely pathogenic (30.23%), 2 variants of uncertain significance (VUS) (4.65%). Additionally, four CNVs, including two likely pathogenic and two VUS variants, were detected. Since NGS CNV analysis is considered a screening tool, and only one of these CNV samples was available for further validation using MLPA, the three CNVs that were not validated were excluded from the final mutation count. Among all pathogenic and likely pathogenic mutations detected, 19 (51.35%), 14 (37.84%), 3 (8.11%), and 1 (2.70%) were nonsense, frameshift, splicing, and missense mutations, respectively.

Sanger Sequencing

Among 63 individuals analyzed by Sanger sequencing, 28 (44.44%) were males, of whom 18 (64.29%) revealed pathogenic and likely pathogenic variants, one revealed a VUS, and in the remaining cases, no variants were detected (Figure 1). The most common mutation types among pathogenic and likely pathogenic variants were nonsense (nine, 50.00%), frameshift (six, 33.33%), and splicing variants (two, 11.11%). Additionally, in one case, a small deletion was detected at the intron 7 and exon 8 junction. Among 35 females referred for carrier detection, 22 (62.86%) were carriers of pathogenic and likely pathogenic variants, one had a VUS variant, and the remaining were found to have no mutation.

Genetic landscape of DMD Mutations in Patients

Taken together, among the 608 identified mutations, 470

large deletions (77.30%) were the most prevalent, followed by 81 large duplications (13.32%), 28 nonsense mutations (4.61%), and 20 frameshift mutations (3.29%) (Figure 4). Mutations were primarily concentrated within exons 44-55 (44.17%) and exons 3–27 (35.75%). Large deletions were mainly distributed across exons 44–55 (52.98%) and exons 3–27 (29.73%), while large duplications occurred predominantly within exons 3-27 (58.59%), particularly across exons 2-11 (31.46%). Additionally, 69.09% of point mutations were identified within the first 40 exons of the *DMD* gene (Figure 5).

Dystrophin Isoform Defects

Assessment of the distribution of dystrophin isoform defects based on *DMD* mutation locations revealed that DP427 isoforms were affected in all patients. DP260 and

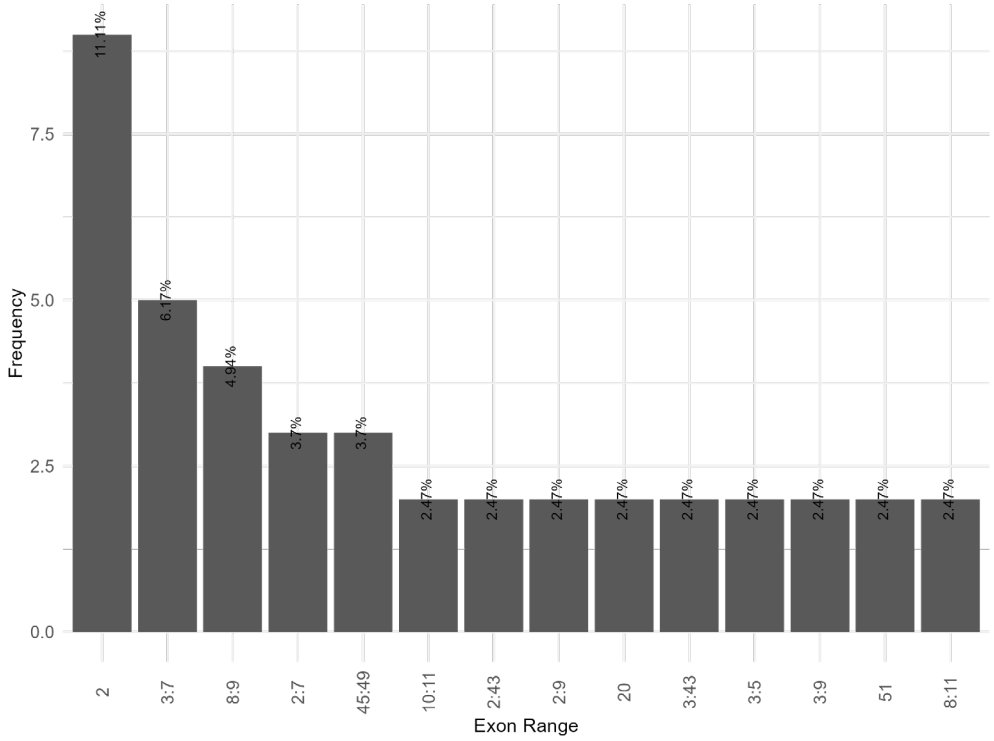

Figure 3. Frequent duplications in the *DMD* gene among patients

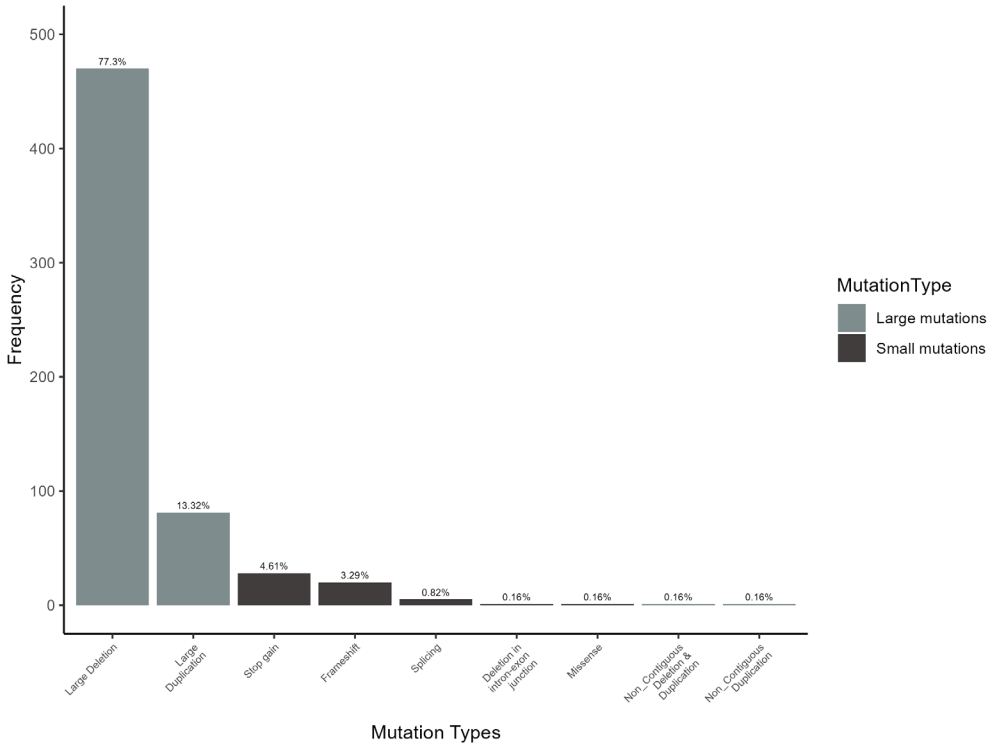

Figure 4. Mutation types among the patients

DP140 were also frequently disrupted, with deletions observed in 76.15% and 65.79% of cases, respectively. In contrast, mutations affecting DP116 and DP71 were rare, detected in only 6.09% and 3.29% of patients, respectively (Figure 6).

Eligible Molecular Treatments

Checking for potential molecular treatments indicated that 300 patients (49.34%) could theoretically benefit from exon-skipping therapies, of whom 180 (29.60%) were eligible for currently approved treatments. Furthermore, 21 patients with nonsense mutations (3.45%) could potentially benefit from read-through therapy. Table 1 presents all the possible and eligible cases for the treatment.

Prenatal Diagnosis (PND)

Among the 123 prenatal samples analyzed, MLPA was performed on 113 fetuses, identifying 31 affected (27.43%), while the remaining 10 fetuses were analyzed by Sanger sequencing, which revealed six affected (60.00%) (Figure 1).

Discussion

Based on the genetic analysis of 2162 cases from a prominent referral center (KNPGC), this study provides a more comprehensive characterization of *DMD* mutation profiles, the affected dystrophin isoforms, and their eligibility for molecular therapies with the largest sample size reported to date in Iran. Evaluation of 1093 male patients in this study yielded a diagnostic rate of (55.63%). Previous studies have indicated that large deletions are

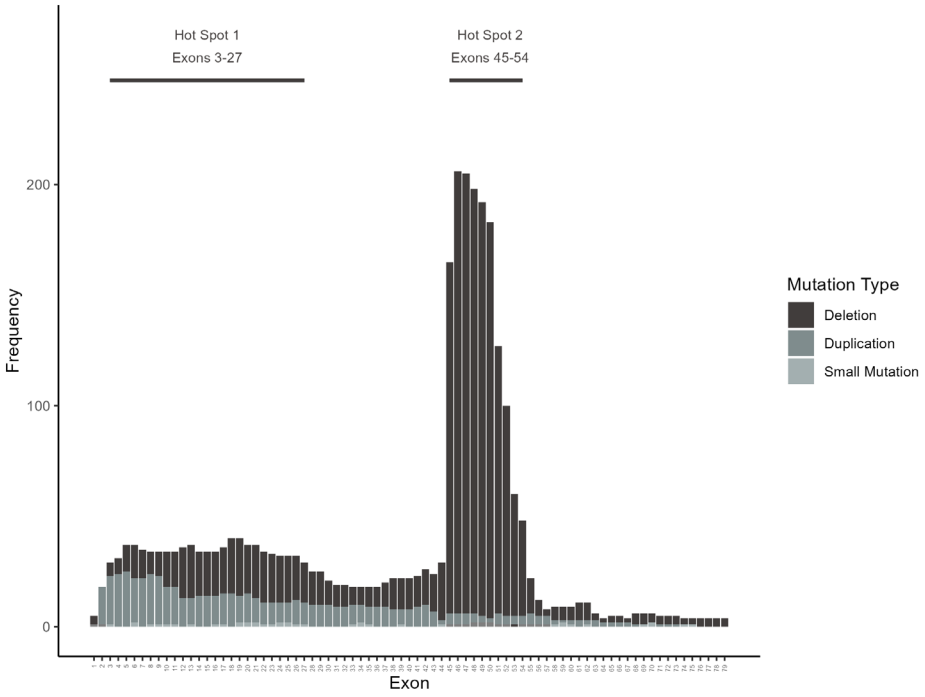

Figure 5. Plot of all engaged exons in mutations

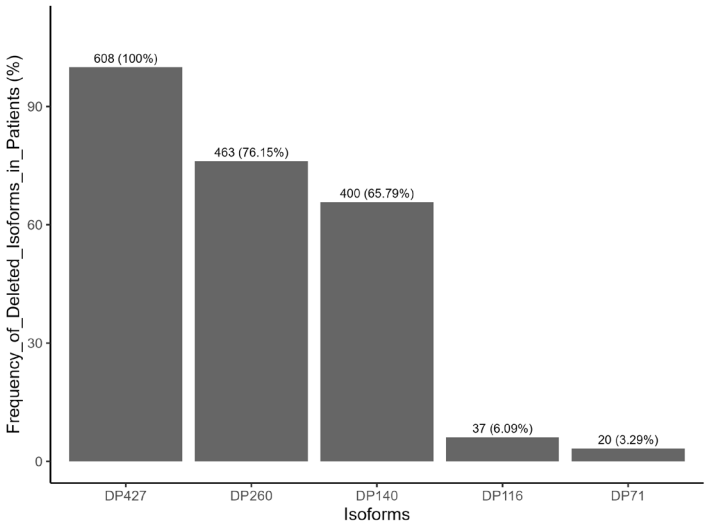

Figure 6. Frequency of deleted dystrophin isoforms in patients

most frequently observed within two major regions of the *DMD* gene: exons 2–20 and 45–55, corresponding to the proximal and distal hotspots, respectively. In our analysis, the distal hotspot aligned closely with these earlier reports (exons 44–55), whereas the proximal region showed a wider distribution (exons 3–27), even relative to earlier studies from Iran.[10,11] Table 2 presents the comparison between our study and the international *DMD* mutation registries (TREAT-NMD and UMD-DMD) for the proximal (exons 2–20) and distal (exons 45–55) hotspots. Large deletions in our cohort were significantly higher in both hotspots than in TREAT-NMD and closely matched UMD-DMD. Large duplications showed a significantly higher concentration in the proximal hotspot, while the distal hotspot frequency was consistent with TREAT-NMD. Although some Iranian studies reported *DMD* mutational hotspots, most lacked exon-level quantitative data, preventing direct statistical comparisons; thus, our hotspot analysis focused on international datasets. Within the mutation spectrum, large deletions were the most prevalent variant in our cohort, consistent with global trends, yet they occurred at a significantly higher frequency than in TREAT-NMD and UMD-DMD, closely resembling frequencies reported in other Iranian cohorts. Large duplications were the second most frequent mutation type, in line with previous studies; notably, their frequency in our cohort was higher than in other Iranian studies and comparable to larger global datasets. Small mutations in our cohort showed a distribution similar to Iranian studies but differed significantly from TREAT-NMD and UMD-DMD. Although the rank order of mutation types was consistent across studies, the overall proportions differed significantly (Supplementary Table 1). Differences in sample size may have contributed to these variations.[4,8,10,11]

Our findings regarding the identification of mutated isoforms in patients were consistent with those of previous reports,[28] indicating that mutations predominantly affect full-length isoforms, such as DP427, whereas deletions in shorter isoforms, such as DP116 and DP71, are relatively rare.

Nearly half of the patients (49.34%) in our cohort carried mutations amenable to exon-skipping therapy, indicating significant potential for this approach in the Iranian DMD population. About one-third of them (29.60%) were eligible for currently approved exon-skipping drugs. Additionally, 3.45% of patients had nonsense mutations that could benefit from read-through therapy. It should be noted that although therapy availability is restricted in countries with limited healthcare resources, such as Iran, and therapy eligibility percentages must be considered alongside factors such as regulatory approvals, costs, and accessibility, limited access to genetic testing further complicates timely diagnosis and treatment of this condition. Therefore, mutational screening and understanding genetic variability in DMD/BMD are the primary keys to precision treatments and better outcomes.

Conclusion

In conclusion, this study provides a detailed characterization of *DMD* mutations in Iranian patients, based on a large cohort, which can improve genetic counseling and help identify patients eligible for mutation-specific therapies.

Acknowledgments

The authors sincerely thank all the family members for their participation in this study, as well as physicians, healthcare centers,

Table 1. Eligible and approved treatment for our cohort patients.

Treatment			Number of Cases Eligible for the Treatment	Percentage (Among all 608 Patients)
Exon skipping therapy	Approved treatments	Exon 45 skipping	55	9.05%
		Exon 51 skipping	72	11.84%
		Exon 53 skipping	41	6.74%
		Exon 45 & 51 Skipping	9	1.48%
		Exon 51 & 53 Skipping	3	0.49%
		Sum	180	29.61%
	Possible treatments (not approved)	Exon skipping in exons except for 45/51/53	120	19.74%
	Sum		300	49.34%
Readthrough therapy			21	3.45%
Sum			321	52.80%

Table 2. Comparison of mutation hotspots of our cohort with TREAT-NMD and UMD-DMD databases

Hotspots	Population	Proximal-hotspot-large-deletion	*P*-Value	Proximal-hotspot-large-duplication	*P*-Value	Distal-hotspot-large-deletion	*P*-Value	Distal-hotspot-large-duplication	*P*-Value
Proximal (2–20) Distal (45–55)	Our study	83 (17.7%)	—	58 (71.6%)	—	352 (74.9%)	—	15 (18.5%)	—
	TREAT-NMD	673 (13.8%)	0.024	394 (50.3%)	<0.005	3218 (65.8%)	<0.005	120 (15.3%)	0.55
	UMD-DMD	213 (15.2%)	0.227	77 (35.8%)	<0.005	1053 (75%)	1	NA	-

and genetic laboratories throughout Iran who referred families to our laboratory.

Authors' Contribution

Conceptualization: Hossein Najmabadi
Data Curation: Ali Khanbazi, Farzane Zare Ashrafi, Raziye Rezvani Rezvandeh, Masoumeh Akbari Kelishomi
Formal Analysis: Ali Khanbazi, Masoumeh Akbari Kelishomi, Maryam Azad, Fatemeh Ahangari, Mahsa Fadaee, Shima Dehdahsi, Mina Makvand, Banafsheh Salmani, Zohreh Vahidi, Mahdieh Kooshki, Atiyeh Ahmadpour, Elmira Shiuokhi, Armita Ghaderi, Mahsa Tahmasebivand, Elahe Jafarnataj
Funding Acquisition: Hossein Najmabadi
Investigation: Farzane Zare Ashrafi, Ali Khanbazi, Raziye Rezvani Rezvandeh, Masoumeh Akbari Kelishomi
Methodology: Hossein Najmabadi, Maryam Azad, Fatemeh Ahangari, Mina Makvand
Project Administration: Hossein Najmabadi
Resources: Hossein Najmabadi, Ariana Kariminejad, Kimia Kahrizi, Fariba Afroozan, Khadijeh Noudehi, Fatemeh Fatehi, Shima Zamanian Najafabadi, Shahriar Nafissi, Haleh Habibi, Maryam Taghdiri, Bahram Haghi Ashtiani, Gholamreza Zamani, Farzad Fatehi, Mohammadkazem Bakhshandeh, Farshid Parvini, Payman Jamali
Software: Ali Khanbazi
Supervision: Hossein Najmabadi
Validation: Hossein Najmabadi
Visualization: Ali Khanbazi
Writing - Original Draft: Farzane Zare Ashrafi, Ali Khanbazi
Writing - Review & Editing: Hossein Najmabadi, Ariana Kariminejad, Kimia Kahrizi, Farzane Zare Ashrafi, Ali Khanbazi

Competing Interests

The authors declare no conflict of interest.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available in supplementary data.

Ethical Approval

This study was approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran (IR.USWR.REC.1404.081). Written informed consent was obtained from each patient or their guardian. The study was conducted in accordance with the declaration of Helsinki.

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Supplementary Files

Supplementary file 1 contains Table S1.

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