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Mutation spectrum of 260 dystrophinopathy patients from Turkey and important highlights for genetic counseling

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Abstract

We genetically evaluated 260 dystrophinopathy patients from Turkey. Karyotyping as an initial test in female patients, followed stepwise by multiplex ligation-dependent probe amplification and by targeted next-generation sequencing of *DMD* revealed definitive genetic diagnoses in 214 patients (82%), with gross deletions/duplications in 153 (59%), pathogenic sequence variants in 60 (23%), and X-autosome translocation in one. Seven of the gross and 27 of the sequence variants found novel. *In silico* prediction, co-segregation and transcript assays supported the pathogenic nature of the novel silent (p.Lys534=) and the splice site (c.4345–12C>G) alterations. From a total of 189 singleton cases, 154 (82%) had pathogenic alterations. From 138 of those who had maternal carrier testing, 68 out of 103 (66%) showed gross and 11 out of 35 (31%) showed small pathogenic variants. This suggests that the *de novo* occurrences in DMD appear approximately 2.1 times more frequently in meiotic unequal crossing-over than in uncorrected replication errors. Our study also disclosed three mothers as obligate gonadal mosaic carriers. Family-based investigation of dystrophinopathy patients is crucial for the ascertainment of novel or rare variants and also for counseling and follow-up care of the families.

Keywords: Dystrophinopathy; Duchenne muscular dystrophy; Becker muscular dystrophy; MLPA; NGS; cDNA.

1. Introduction

Dystrophinopathy is a group of X linked muscle diseases caused by pathogenic *DMD* variants that manifest in a clinical spectrum ranging from asymptomatic hyperCKemia and muscle cramps with myoglobinuria at the mild end to Becker muscular dystrophy (BMD, MIM 300376), *DMD*-associated dilated cardiomyopathy (XL-DCM, MIM 302045) and Duchenne muscular dystrophy (DMD, MIM 310200) at the severe end. Establishing precise identification of those pathogenic *DMD* variants not only expatiate our understanding of the molecular pathology underlying dystrophinopathies, but also helps with clarifying phenotype-genotype correlation, which is important for genetic counseling and rendering families' options for therapeutic interventions [1].

Reports of DMD incidents in male births varies from 1-in-3300, 1-in-4215, to 1-in-5618, while BMD is much less common (1-in-18,500) [2–4]. Dystrophin is present at the internal face of sarcolemma in many tissues, including skeletal, cardiac, various central nervous systems and smooth muscle cells. It forms the dystrophin-glycoprotein complex (DGC) which ties the membrane to intracellular and extracellular structures in association with a number of proteins via its domains [5]. Recently, *in vivo* assays have indicated that membrane binding domains act independently,

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suggesting a new model for dystrophin membrane binding functionality [6].

DMD is the largest gene encoding the eighth biggest transcript of the human genome following (in descending order) *TTN*, *NEB*, *RYR2*, *EPPK1*, *RYR1, ANK2*, *VPS13B*. Expression analyses revealed that *DMD* differentially spliced to 20 diverse variants, the largest accommodating 79 exons with 13,956 base pairs (bp) in nucleotide length (Dp427m) and encoding a 3685 amino acids in peptide length. Allelic heterogeneity of *DMD* gene, with the presence of gross deletions and duplications and considerable multiplicity of the sequence variants, necessitates a comprehensive testing algorithm in genetic diagnosis of dystrophinopathies. Presently, multiplex ligation-dependent probe amplification (MLPA) [7] and high density-comparative genomic hybridization array techniques [8] are commonly used methods for the recognition of gross variants. The majority of the deletions involve exons 45–50 while duplications involve exons 2–11, typically nonrecurrent with different sizes with overlapping regions or end grouping types [9–11]. Clusters of interrupted repeats located in the intronic regions are suggestive causes of these incidents. A number of investigations on breakpoint junctions support the possibility that a non-homologous end joining (NHEJ) event underlines these occurrences [12]. The imprecision of ligation of double-strand breaks in NHEJ is known to create variants which may be attributed to *de novo* instances shown in one-third of the cases [13]. Replication-based mechanisms are also considered for the patterning of complex *DMD* rearrangements, although these are seldom described [14]. Reports on pathogenic variant frequencies of DMD disclose that single or more exon deletions are identified in ~50–65%, ~65–70%, pathogenic sequence variants in ~20–35%, ~10–20% and gross duplications in ~5–10%, ~10–20% for DMD and BMD patients, respectively [15].

Nowadays advancement in molecular technologies have permitted sequencing of many patients for multifold targeted regions by next generation sequencing (NGS) in a single run, dramatically reduced labor and cost in comparison to Sanger sequencing (SS), for detecting small pathogenic variants. Notwithstanding the benefits of these technologies, they unfortunately do not capture deeper pathogenic intronic variants that may affect RNA expression, processing and splicing. Patients with unidentified causative variants in the targeted investigations and who have consistent phenotype for dystrophinopathy could be referred for conventional RNA sequencing or next generation sequencing based RNA transcript analysis (RNAseq) if a muscle biopsy is available [16]. While computational modeling provides invaluable support for the predictions of the pathogenicity of the variants, further investigations in the families previously not associated with disease are vital.

Our study involved family based investigation of DMD, BMD, manifesting females (MF) and cases with chronic elevated serum creatinine kinase (CK) levels for *DMD* variants in successive steps; gross deletion/duplication by MLPA followed by targeted sequencing of *DMD* by NGS for cases with unidentified causative variants by MLPA. Karyotyping was the antecedent test for MF group. mRNA sequencing via cDNA is performed when RNase protected muscle biopsy material was achievable.

1.1. Patients and material

1.1.1. Patients

Study cohort embraced a total of 260 patients (165 DMD, 60 BMD, 20 CKemia, 14 MF and one *DMD* related cardiomyopathy) with clinical suspicion of a dystrophinopathy determined using standard clinical diagnostic criteria. Referrals came from eight pediatric neurologists, three internal neurologists and three medical genetic clinics in Turkey to the Medical Genetics Department, Istanbul Medical Faculty, for molecular genetic testing and genetic counseling. Immunohistochemistry of the muscle biopsy for dystrophin staining was available in 82 DMD, 39 BMD, 11 MF and 6 patients with asymptomatic high CK levels. Immunohistochemistry of 5 DMD, 16 BMD, 3 MF and 3 CK elevated patients were normal for all three epitopes of dystrophin (*dys1* encompassing encoding region of exon 26–30, *dys2* encompassing encoding region of exon 77–79 and *dys3* encompassing encoding region of exon 10–12). Detailed medical family histories were recorded and pedigrees were drawn.

Peripheral blood samples of 2 ml were collected from 260 patients and 220 other family members (199 mothers, one healthy father, five healthy sisters, one chronic CK elevated brother, three healthy brothers, six healthy maternal aunts, one affected male cousin, one affected maternal uncle, one healthy maternal uncle, one healthy maternal grandmother and one healthy maternal grandfather) upon written consent, as part of regular procedures before genetic testing. DNA was extracted (MagnaPure, Roche) and stored for downstream reactions.

All female cases were karyotyped before any further tests were initiated. One male case was also karyotyped following *DMD* sequencing.

1.1.2. MLPA

DMD-MLPA reaction kits (SALSA P034/P035) targeting 79 exons in *DMD* (NM_004006) were used according to the manufacturer's protocol. Data analysis was performed using Coffalyser. Net software [17].

1.1.3. DMD *sequencing*

In-house designed primer pool for targeted regions of largest *DMD* gene (NM_004006), covering all the coding sequences, 20 bp up to splicing donor and splicing acceptor sites, regulatory region encompassing c.−54T>*A* [18], intronic regions flanking c.31+36947G>A in intron 1 [19], c.93+5590T>A in intron 2 [20], c.265–463A>G in intron 4 [21], c.3432+2036A>G and +2240A>G in intron 25 [22], c.6614+3310G>T in intron 45 [23], c.8217+18052A>G in intron 55 [24], c.9225–160A>G, −285A>G, −287C>A, −647A>G and −648A>G in intron 62 [19,25–27], c.9563+1215A>G, c.9564–30A>T, −427T>G in intron 65

[21,28,29] is used for sequencing on Ion Torrent PGM platform including 28.6 kb region in a total of 148 amplicons by 99.76% coverage. All small pathogenic variants (known, novel, rare), single exon deletion/duplication regions revealed by MLPA and family members for co-segregation were sequenced by SS. *In silico* analysis programs (Mutation Taster, PolyPhen and SIFT) were used for prediction of deleteriousness of unknown or rare variants [30–32]. Variant frequencies of the rare occurrences were acquired from the Exome Aggregation Consortium (ExAC) database [33]. Phenotypic impact of rare variants annotated with reference SNP numbers were searched in ClinVar [34]. Variants are classified as "pathogenic," "likely pathogenic," "variant of uncertain significance (VUS)," "likely benign" and "benign" whichever relevant [35].

1.1.4. Total RNA isolation and cDNA synthesis

40-70 mg muscle tissue is sliced in RNase free phosphate buffer on ice and homogenized (BulletBlender Homogenizer, Next Advance) via beating with 2.8 mm stainless steel beads of molecular biology grade (Next Advance) in 1 ml of TRItidy G (AppliChem) reagent for five min. and extracted with 200 μl of chloroform (Merck). Upper phase is transferred to clean micro centrifuge tube and mixed with equal volume of 70% ethanol (Merck). Liquid phase, collected in the bottom via quick centrifugation, is removed into spin cartridge with collection tube and steps of manufacturer's manual followed for washing and elution (PureLink RNA mini kit, Ambion). cDNA is synthesized from 5 μl of RNA isolate by reverse transcriptase by using hexameric primer mix according to manufacturer's protocol (Revert Aid First strand cDNA synthesis Kit, Thermo Fisher). 1 μl of cDNA is used for downstream reactions for amplification and sequencing with exonic primer pairs.

2. Results

Clinical referrals and the genetic test results of the cohort are fully listed in **Supplementary Table**

MLPA is applied to 259 subjects since one female patient (case-0007) was found to have chromosomal aberration revealed when karyotyping. Gross variants were identified in 153 patients (58.8%) in total cohort. According to clinical fractions, gross deletions and duplications were identified in 87 (52.7%) and in 19 (11.5%) of DMD group ($n=165$), in 24 (40.0%) and in five (8.3%) of BMD group ($n=60$), in 14 (70%) and in one (5.0%) of CK'emia group ($n=20$), respectively. Only three gross deletions (21.4%) and no gross duplications in MF group ($n=14$) were identified (**Supplementary Fig. 1**).

Apparently conserved Open Reading Frame (ORF) with deletions of exon 45-47 (3 DMD and 5 BMD patients) and exon 45-51 (2 DMD and 1 BMD patients) and apparently disrupting ORF with deletions of exon 48-50 (5 DMD and 2 BMD patients) and exon 48-54 (1 DMD and 1 BMD patients) were addressed in both DMD and BMD cohort.

Among gross variant group ($n=153$), 105 were multi exon deletions, 23 were single exon deletions, 18 were multi exon duplications, and seven were single exon duplications. Exon 44 and exon 2 were the most recurrent single exon deletions and duplications, respectively (**Supplementary Fig. 2A** and **2B**). Four gross deletions and a gross duplication in DMD group, a gross duplication in BMD group and a gross deletion in CK elevated group were novel (Fig. 1).

Three novel sequence alterations in DMD and a known pathogenic variant in BMD patients were disclosed during SS application, performed whenever single exon deletion and duplication are observed by MLPA in order to prevent false positive recognition (Fig. 2).

DMD sequencing on NGS platform was performed with a mean coverage depth of ~350 for all targeted regions for 102 patients that had normal results in previous tests. Among these subjects 68 patients were revealed with 70 sequence variants (case-0053 carrying two different variants, one novel missense and a known nonsense in the same allele and case-0078 compound heterozygous in homologous alleles for two rare variants, one intronic and one missense). 15 of the frame shift, seven of the nonsense, three of the splice site, one of the silent and one of the missense variants were reported for the first time in this study (Table 1).

In frame deletion insertion (c.8729_8734delAGGTCAins TGGTCG) identified in singleton DMD case-0067, strikes two adjacent codons, 2910 and 2912, leading to p.Glu2910Val (rs41305353) and p.Asn2912Asp (rs1800278), each independently reported as benign, but co-occurrence was associated with DMD (reference 31 in supplementary Table). Mother was carrier of this alteration though none of the other family members were found carriers (sister, maternal aunt, healthy maternal uncle and healthy maternal grandfather).

One six-year-old male DMD (case-0033) with normal karyotype (46,XY) and CK level of 24,099 IU/L without family history was found to be mosaic for a known nonsense variant (c.1033=/C>T; p.Gln=/345*) in exon 10. The coverage depth was 86, presenting 36% C and 64% T. SS revealed one-third smaller size for peak area ratio of the mutant to wild type allele.

Out of 14 MF only one patient (case-0007) had X/autosome translocation (46,X,t(X;9)(p.21.1;q12)) in which the break point was traversing the *DMD* locus, leading to phenotype compatible with dystrophinopathy. In this group, case-0132 with gross deletion and case-0012 with nonsense variant were available for maternal carrier testing, and both revealed *de novo* status (Table 2). In this group, case-0078 with two rare variants is presented below in detail.

Five female and 15 male cases were referred for genetic testing for dystrophinopathy due to chronically elevated CK levels (Table 3). Immunohistochemistry test results were available for six of these subjects. Two male (case-0275 with exon 3-9 deletion, and case-0021 with missense variant) and one female (case-0063 with no identified pathogenic variant) subjects were reported with normal staining. Another three patients, all-female, were reported with either of three *Dys*

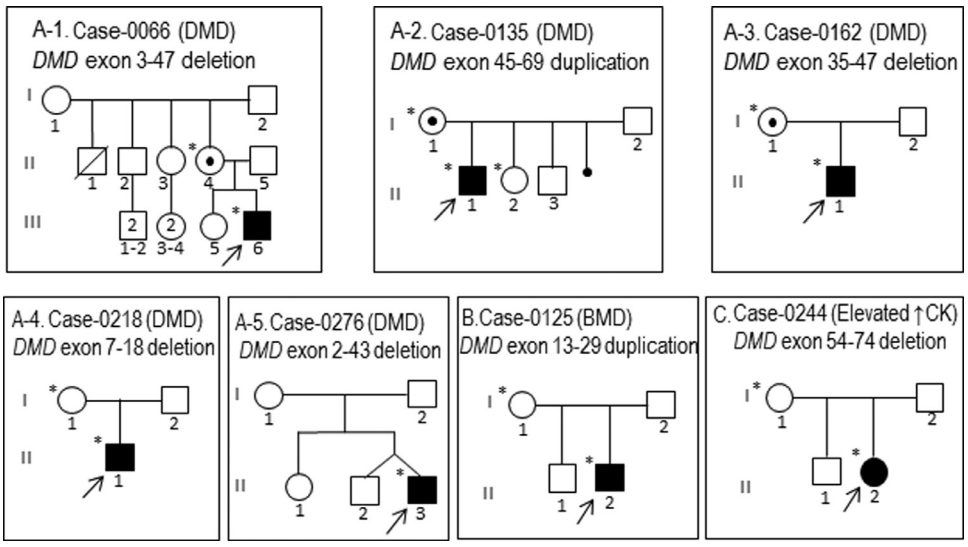

Fig. 1. Seven different novel gross deletions and duplications were identified by MLPA testing in our cohort of DMD (A), BMD (B) and CK elevated (C) cases. Affected individuals are represented by black shading and arrows point out the index cases. Tested individuals are shown with asterisk and carrier mothers are represented with a black dot in the female symbol. Plain symbols with asterisk indicate non-carrier status.

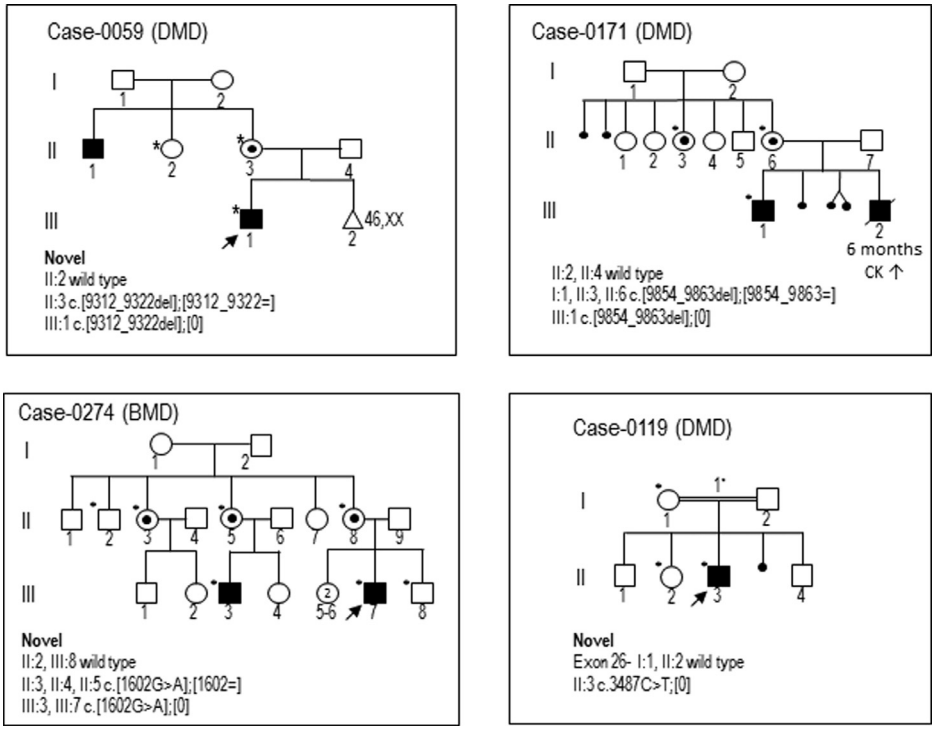

Fig. 2. Four cases with apparently single exon deletion with MLPA presented to have pathogenic sequence alterations disclosed when relevant exon is Sanger sequenced. Three of these alterations identified in Case-0059, −0119, and-0274 were novel. Carrier mothers are represented with a black dot in the female symbol. Plain symbols with asterisk indicate non-carrier status.

epitopes negative; case-0054 with exon 51-53 deletion, case-0225 with exon 1-2 deletion and case-0273 with unidentified causative variant. Two missense variants in this group (case-0021 and case-0163) were referred for incidental finding of elevated CK, besides DMD maternal cousins in latter, discussed in more detail below.

A single case referred for *DMD*-associated dilated cardiomyopathy at the age of 14 (case-0122), was found to carry novel nonsense variant (c.5632C>T; p.Gln1878*) and his mother was a carrier. He had mild muscle pain, a floppy mitral valve on echocardiogram myopathy on electromyography (EMG), and his CK level was 8811 IU/L.

Table 1
Novel alterations identified in our dystrophinopathy cohort.

Case No	Clinic	Supported w/ Dystrophin immunohisto-chemical staining of muscle biopsy	Region	Nucleotide NM_004006	Peptide NP_003997	Type	Maternal Carrier Status
0053	DMD	Yes	Exon 5	c.287G>A[(1)]	p.S96N [(1)]	Missense	+
0123	DMD	Yes	Exon 6	c.366_367insT	p.V123Cfs*15	Frame shift	+
0036	DMD	Yes	Exon 6	c.522_523delTA	p. H174Qfs*3	Frame shift	–
0051	DMD	Yes	Exon 12	c.1389G>A	p.W463*	Nonsense	+
0274	BMD	NP	Exon 13	c.1602G>A	p.K534=	Silent	+
0085	DMD	Yes	Exon 16	c.1940_1941delTT	p.F647Cfs*6	Frame shift	+
0020	DMD	Yes	Exon 20	c.2599A>T	p.K867*	Nonsense	+
0072	DMD	Yes	Exon 22	c.2887_2888delTC	p.S963Hfs*5	Frame shift	–
0077	DMD	Yes	Exon 22	c.2941G>T	p.E981*	Nonsense	+
0097	DMD	NP	Exon 22	c.2941G>T	p.E981*	Nonsense	–
0158	DMD	NP	Exon 24	c.3170_3173delTACA	p.I1057Kfs*3	Frame shift	+
0087	BMD	Yes	Exon 25	c.3370_3371delGC	p.A1124Ffs*4	Frame shift	+
0119	DMD	Yes	Exon 26	c.3487C>T	p.Q1163*	Nonsense	–
0165	DMD	Yes	Exon 34	c.4693_4694delCA	p.Q1565Vfs*10	Frame shift	+
0122	DMD-RC	NP	Exon 40	c.5632C>T	p.Q1878*	Nonsense	+
0027	DMD	NP	Exon 41	c.5893_5894delCA	p.Q1965Vfs*14	Frame shift	+
0281	DMD	NP	Exon 43	c.6256_6257insG	p.E2086Kfz*28	Frame shift	NI
0049	DMD	Yes	Intron 50	c.7309+5G>C	?	Splice-site donor	NI
0013	DMD	Yes	Exon 51	c.7467delT	p.D2490Ifs*4	Frame shift	+
0138	DMD	NP	Exon 56	c.8221_8221delC	p.L2741Sfs*23	Frame shift	+
0137	DMD	Yes	Exon 58	c.8575G>T	p.E2859*	Nonsense	NI
0014	DMD	NP	Exon 61	c.9153delC	p.L3053Ffs*36	Frame shift	+
0174	DMD	Yes	Exon 63	c.9228_9229insA	p.E3077Rfs*21	Frame shift	+
0059	DMD	NP	Exon 64	c.9312_9322delAGCTTATAGGA	p.A3105CFs*23	Frame shift	+
0093	DMD	NP	Intron 66	c.9649+5G>A	?	Splice-site donor	–
0109	DMD	NP	Intron 67	c.9808-2A>C	?	Splice-site acceptor	+
0167	DMD	NP	Exon 74	c.10478delA	p.Q3493Rfs*3	Frame shift	+

[(1)] "This variant is identified in the same allele with known nonsense mutation in *cis* configuration; c.[287G>A;4723A>T], p.[Ser96Asn;Lys1575*], his mother was carrier of both; + shows carrier mothers; - shows non-carrier mothers; NI: not investigated; NP: not performed; DMD-RC: *DMD*-related cardiomyopathy.

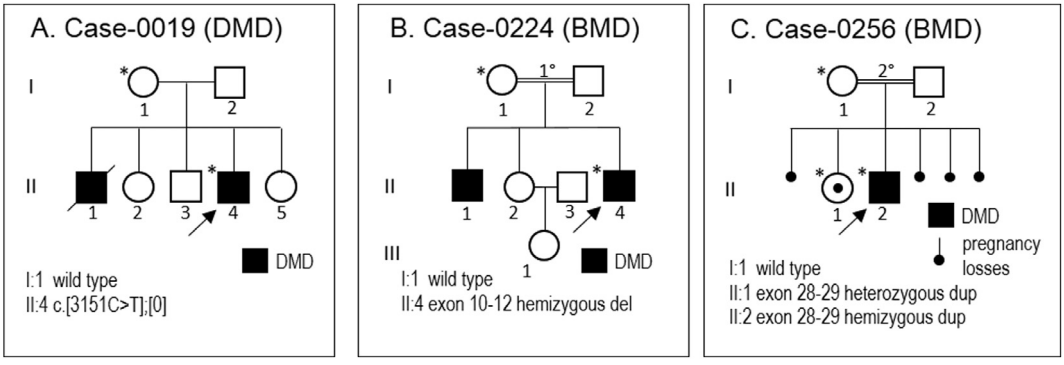

Fig. 3. Families with maternal gonadal mosaicism. Case-0019 with DMD and cases-0224 and −0256 with BMD.

His three brothers, not tested genetically, were diagnosed with cardiac insufficiency and one reported to be operated for heart transplantation.

A pedigree analysis of one DMD (case-0019) and one BMD (case-0224) subjects, with non-carrier mothers but clinically similarly affected brothers, were suggestive of gonadal mosaic mothers, while pathogenic variant carrier status of a sister indisputably supported the gonadal mosaic mother in singleton BMD case-0256 (Fig. 3).

13 cases from sequencing group; five with missense (cases −0021, −0047, −0053, −0116, and −0163), one with co-occurrence of missense and intronic (case-0078), five with intronic (cases-0028, −0049, −0058, −0080 and −0093), and two with silent alterations (cases −0064 and −0274) were initially classified as VUS; those needed family based investigation and transcript analysis when relevant for further delineation of the disease causing status as described below

Table 2a
Manifesting female (MF) patients referred for molecular genetic diagnosis.

Case No	Age at referral (Year)	Consanguinity	Karyotype	CK level at last measurement U/L (Age as year)	Immunohistochem. staining for Dys I, II and III (Age as year)
0006	10	1°	46,XX	555 (10)	Normal (8)
0007	$8^{4/12}$	1°	46,X,t(X;9) (p.21.1;q12)	14 690 (7)	Compatible with dystrophinopathy carrier status (7)
0012	$12^{9/12}$	–	46,XX	10 540 (12)	Compatible with dystrophinopathy carrier status (12)
0045	$12^{9/12}$	1°	46,XX	3611 (10)	Dys I, III normal, II weak (12)
0068	$14^{10/12}$	1,5°	46,XX	2829 (14)	Dys I, III normal, II weak (10)
0078	$7^{4/12}$	1,5°	46,XX	9165 (5)	Normal (7)
0090	$1^{9/12}$	–	46,XX	964 (8/12)	Dys I, III normal, II weak (8/12)
0091	40	–	46, XX	1000-2000 (40)	NI
0094	$7^{8/12}$	–	46,XX,9qh+	8564 (7)	Compatible with dystrophinopathy carrier status (7)
0111	46	–	46,XX	1740 (42)	NI
0132	18		46,XX	7967	Compatible with dystrophinopathy carrier status (6)
0251	3	? (*)	46,XX	4000 (3)	Dys I, II and III weak (3)
0262	49	–	46,XX	845 (47)	NI
0269	27	–	46,XX	1375 (25)	Normal

(*) foster child; NI: not investigated.

2.1. Missense VUS group

Case-0021 was a 10-year-old boy with elevated CK level (2000 IU/L-6000 IU/L) (Table 3). This subject's muscle biopsy presented with myopathy findings, lipid accumulation and immunohistochemistry staining of dystrophin revealed normal signal for three of the *dys* epitopes at the age of eight. The boy's medical history reported hydrocephaly, EMG showed myogenic involvement. c.1988C>T (p.Ala663Val) identified in exon 16, addressed as rs41309715. The mother of the subject was a carrier; the healthy maternal uncle was not a carrier (Fig. 4**A**). ClinVar reported this as VUS (SCV000335267.2). Three *in silico* analysis programs (MutationTaster, PolyPhen and SIFT) predicted benign and ExAC data reported three alleles out of 87,430 with no homozygous and hemizygous notification. Achievable segregation analysis neither ruled out nor sufficiently confirmed its association with phenotype and sustained as VUS

Case-0047 with c.1513G>C (p.Val505Leu, rs140340626) was an 18-year-old intellectually disabled male who was the offspring of a second-degree cousin marriage. Beginning at age four, the subject frequently fell and eventually, at age ten became wheelchair bound. A muscle biopsy performed at age four presented chronic myopathy with normal merosin staining. His echocardiogram was normal and EMG presented myogenic involvement. Medical history revealed a similarly affected six-year-old brother who was not available for genetic testing. Identified alteration gave different predictions; disease-causing (Mutation Taster) and benign (PolyPhen and SIFT). ClinVar listed this variant as benign (SCV000112359.8) and likely benign (SCV000235827.13, SCV000560863.2 and SCV736222.2). ExAC reports 74 heterozygous females and 25 hemizygous males out of 84,296 alleles. Under these conditions disease-causing status of c.1513G>C was not convincing and classified under "likely benign" status.

Case-0053 carried novel missense alteration (c.287G>A; p.Ser96Asp) in *cis* configuration with nonsense (c.4723A>T; p.Lys1575*) allele. Although *in silico* analysis predicted disease causing by Mutation Taster and tolerant by PolyPhen and SIFT, independent effect of this missense occurrence could not be assessed. His mother was found to carry both of these alterations.

Case-0078 was referred due to muscle weakness at eight years of age (Table 2). Her muscle biopsy revealed normal staining. She was found to be compound heterozygous, for a rare missense change (c.8762A>G, p.His2921Arg, rs1800279) in exon 59, inherited from her healthy homozygous mother and a deep intronic point alteration (c.8217+18016T>A, rs948507469) in intron 55, inherited from her healthy hemizygous father. Her healthy sister was similarly compound heterozygous. Segregation assay in this family excluded the disease association status of these two variants.

Case-0116 with missense alteration, c.5438A>G (p.Asp1813Ser, rs767254304) was found to inherit this rare alteration from subject's healthy maternal grandfather, which supported variants' benign nature.

Case-0163 was referred due to elevated CK (4267 IU/L) at the age of one-year and four-months, revealed with c.9938G>A in exon 68, resulting missense change (p.Cys3313Tyr, rs886043597). The child's mother was found to be a carrier. ClinVar reported two individuals; one with unknown (SCV000340931.2) and one with DMD (SCV000625998.1) clinic. Pedigree data for the family presented two maternal uncles: a 20-year-old who is wheelchair bound was found carrier of this alteration, and an older one who was also wheelchair bound, died due to respiratory failure at the age of nine (Fig. 4B). A healthy older brother, with normal CK level, and healthy sister were not carriers of this variant. Co-segregation assay in this family supported the pathogenic nature of p.Cys3313Tyr. Three different missense alterations at the same amino

Table 2b
Manifesting female (MF) patients referred for molecular genetic diagnosis.

Case No	Remonstrance	ECO (Age as year)	EMG (Age as year)	MLPA	DMD seq.	Maternal Carrier status
0006	Muscle weakness since 6 years of age	Normal (10)	Myogenic involvement (10)	–	–	NA
0007	Muscle weakness since 2 years of age	Normal (8)	Myogenic involvement (8)	–	–	NA
0012	Shoulder pains and muscle cramps since 11 years of age	Normal (12)	Myogenic involvement (12)	–	c.1261C>*T* (p.Q421*)	–
0045	Pain and weakness in legs since 10 years of age	NI	Normal	–	–	NA
0068	Frequent falling and difficulty climbing stairs (1)	Normal (12)	NI	–	–	NA
0078	Muscle weakness since $2^{6/12}$	Normal (7)	NI	–	c.[8217+18016T>*A*]; [8762A>*G*]	c.[8762A>*G*]; [8762A>*G*]
0090	Hypotonia	NI	NI	–	–	–
0091	Fatigability since younger age	NI	NI	–	–	NA
0094	Fatigability since 2 years of age	NI	NI	Ex. 44 del.	NA	NI
0111	Back and shoulder pains, difficulty climbing stairs since younger age	Normal (46)	Myogenic involvement (42)	–	–	NA
0132	Walking difficulty started at the age of 8 and bound to wheel chair since age of 16	NI	NI	Ex. 3-43 del.	–	–
0251	Muscle weakness started at age 3	Normal (3)	NI	Ex. 8-44 del.	NI	NA
0262	Muscle cramps and pain	Mild left ventricular cardiomyopathy (48)	Normal (48)	–	–	NA
0269	Muscle cramps and pain	Normal (27)	Normal (27)	–	–	NA

(1) Three years young sister similarly affected; ECO: echocardiogram; EMG: electromyography; NI: not investigated; –: normal result; NA: not applicable.

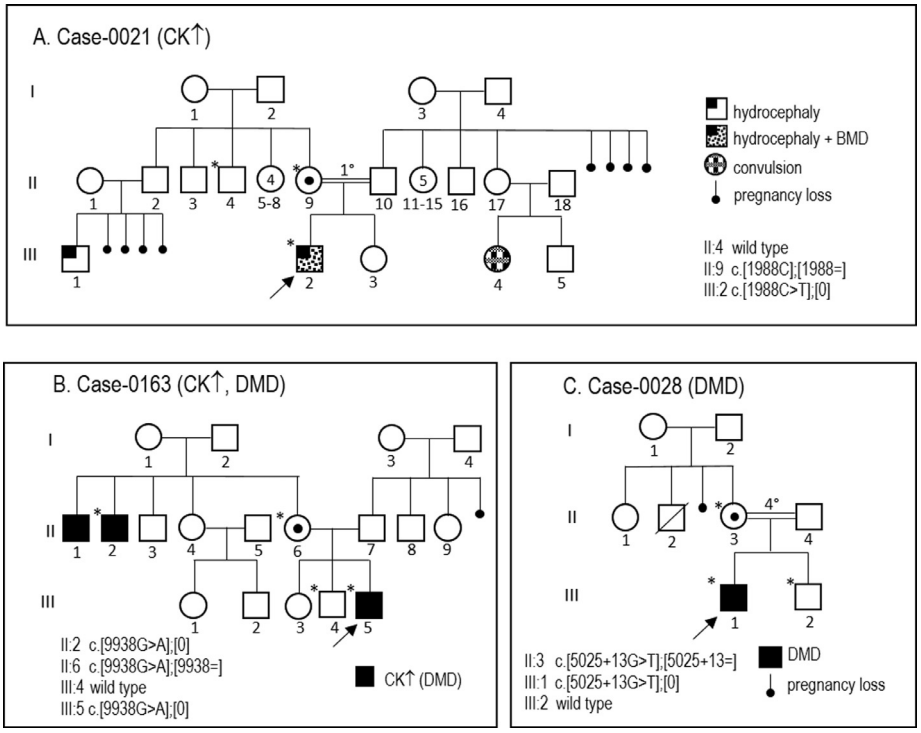

Fig. 4. Pedigrees of families with rare known alterations previously not associated with dystrophinopathy. Segregation analysis of missense variant in case-0021 (A), in Case-0163 (B), and intronic variant in Case-0028 (C).

Table 3
Patients referred for chronic CK elevation (DMD?/BMD?).

Case	Age as year	Gender / Karyotype	Symptoms	EMG	ECO	Maternal Carrier	MLPA	Identified variant
0021	$9^{3/12}$	M	Epilepsy [1]	Diffuse myopathy	NI	+	–	p.A663V (rs41309715)
0050	$^{4/12}$	M	Asymptomatic	NI	NI	+	Ex. 46-50 del	NA
0054	6	F/46,XX	Asymptomatic [2]	NI	Normal	+ [3]	Ex. 51-53 del	NA
0063	36	F/46,XX	Asymptomatic [1]	Normal	Normal		–	–
0139	$^{8/12}$	M	Asymptomatic	NI	normal		Ex 48-50 del	NA
0163	$1^{4/12}$	M	Asymptomatic	NI	NI	NI [4]	–	p.C3313Y (rs886043597)
0178	$3^{8/12}$	M	Asymptomatic	NI	NI	+	Ex. 17-44 del	NA
0183	$^{7/12}$	M	Asymptomatic	NI	NI	–	Ex. 45-55 del	NA
0186	$2^{2/4}$	M	Asymptomatic	NI	NI	–	Ex. 31-43 del	NA
0201	$2^{5/12}$	M	Asymptomatic	NI	normal	–	Ex. 45-54 del	NA
0207	1	M	Asymptomatic	normal	NI	–	Ex. 48-50 del	NA
0223	5/12	M	Asymptomatic [5]	NI	NI	–	Ex. 46-51 del	NA
0225	$8^{6/12}$	F/46,XX	Asymptomatic	Normal	Normal	–	Ex. 1-2 del	NA
0275	19	M	Asymptomatic [1]	Normal	normal	+	Ex. 3-9 del	NA
0278	4	M	Asymptomatic	Diffused myopathy	NI	–	Ex. 3-9 dup	NA
0231	$4^{4/12}$	M	Calf pseudohypertrophy	NI	normal	+ [6]	Ex. 45-49 del	NA
0240	$8^{6/12}$	M	IUGR [7]	NI	NI	–	Ex. 48-51 del	NA
0244	$2^{2/12}$	F/46,XX	Spina bifida	normal	NI	–	Ex. 54-74 del	NA
0267	$7^{2/12}$	M	Asymptomatic	Mild myopathy	NI	–	–	–
0273	$6^{7/12}$	F/46,XX	Asymptomatic [8]	NI	NI	NA	–	–

[1] Immunohistochemical detection of the gastrocnemius muscle is normal for *Dys1*, *Dys2* and *Dys3* epitopes.
[2] Immunohistochemical detection of the gastrocnemius muscle is weak for *Dys1*, *Dys2* and *Dys3* epitopes.
[3] Fatigability in the mother age of 34.
[4] 20 years old uncle with DMD in the family with wheelchair.
[5] Immunohistochemical detection of the gastrocnemius muscle is normal for *Dys1* and *Dys2*, weak for *Dys3*at the age of $8^{6/12}$.
[6] Brother at the age of $1^{10/12}$ years with same mutation.
[7] IUGR: intrauterine growth retardation, normal karyotype (46,XY).
[8] Immunohistochemical analysis of the gastrocnemius muscle is normal for *Dys1* and *Dys3*, weak for *Dys2* at the age of 2 years.F: female; M: male; NI: not investigated; NA not applicable; EMG: electromyography; ECO: echocardiogram.

acid residue (p.Cys3313Arg, p.Cys3313Gly, p.Cys3313Phe) were previously associated with DMD [26,36,37]. These data including the information already existed in ClinVar, placed this variant into "pathogenic" status according to ACMG/AMP 2015 Guideline [35].

2.2. Intronic VUS group

Case-0028 was a 14-year-old DMD boy, bound to wheelchair with normal intellectual capabilities. He had been suffering from significant muscle wasting since age seven. ECO showed mild cardiomyopathy. Muscle biopsy at the age of eight revealed absent dystrophin. c.5025+13G>T (rs779484327) identified in intron 36 (Fig. 4C) predicted benign and ExAC reported a total of eight haplotypes -three in males and five in females- out of 86,779 alleles. His mother was a carrier of the variant while the boy's healthy brother was not a carrier. Segregation analysis for this variant neither ruled out nor sufficiently confirmed its disease association, and classified under VUS.

Case-0049 was a singleton DMD case carrying c.7309+5G>C predicted to disrupt splicing donor site of intron 50, interpreted as VUS One earlier study presented a case carrying c.7309+5G>A in Chinese DMD cohort [38] In a study with Cavalier King Charles Spaniel dogs with dystrophin-deficient muscular dystrophy, c.7309+5G>T alteration was reported to cause skipping of exon 50 [39].

Case-0058 was 48-year-old isolated BMD patient identified to carry rare intronic variant, c.11046+11T>C (rs754992621), predicted disease causing. One hemizygous allele reported in ExAC population out of 83,462 alleles. ClinVar reported this variant as likely benign (SCV000516044.4). Subject's muscle biopsy reported myopathy with normal dystrophin staining. He had gastrocnemius pseudohypertrophy and Gowers sign with elevated CK (5000 IU/L-6000 IU/L). Since the age of 21, the subject was reported to be having difficulty in walking and climbing stairs. None of his family members were available for segregation analysis. This variant is interpreted as "likely benign".

Case-0080 was an eight-year-old Azerbaijani DMD patient who suffered from frequent falls, difficulty climbing stairs since the age of four. Immunohistochemistry of muscle biopsy showed absent dystrophin for three epitopes rendering few revertant fibers. c.4345-12C>G identified in intron 31 annotated rs398123951 with no frequency data and classified under VUS in ClinVar. Mutation Taster analysis predicted splicing error. The boy's mother was not a carrier for the change. Transcript analysis of cDNA sequencing of the reverse transcribed total RNA from muscle biopsy (left

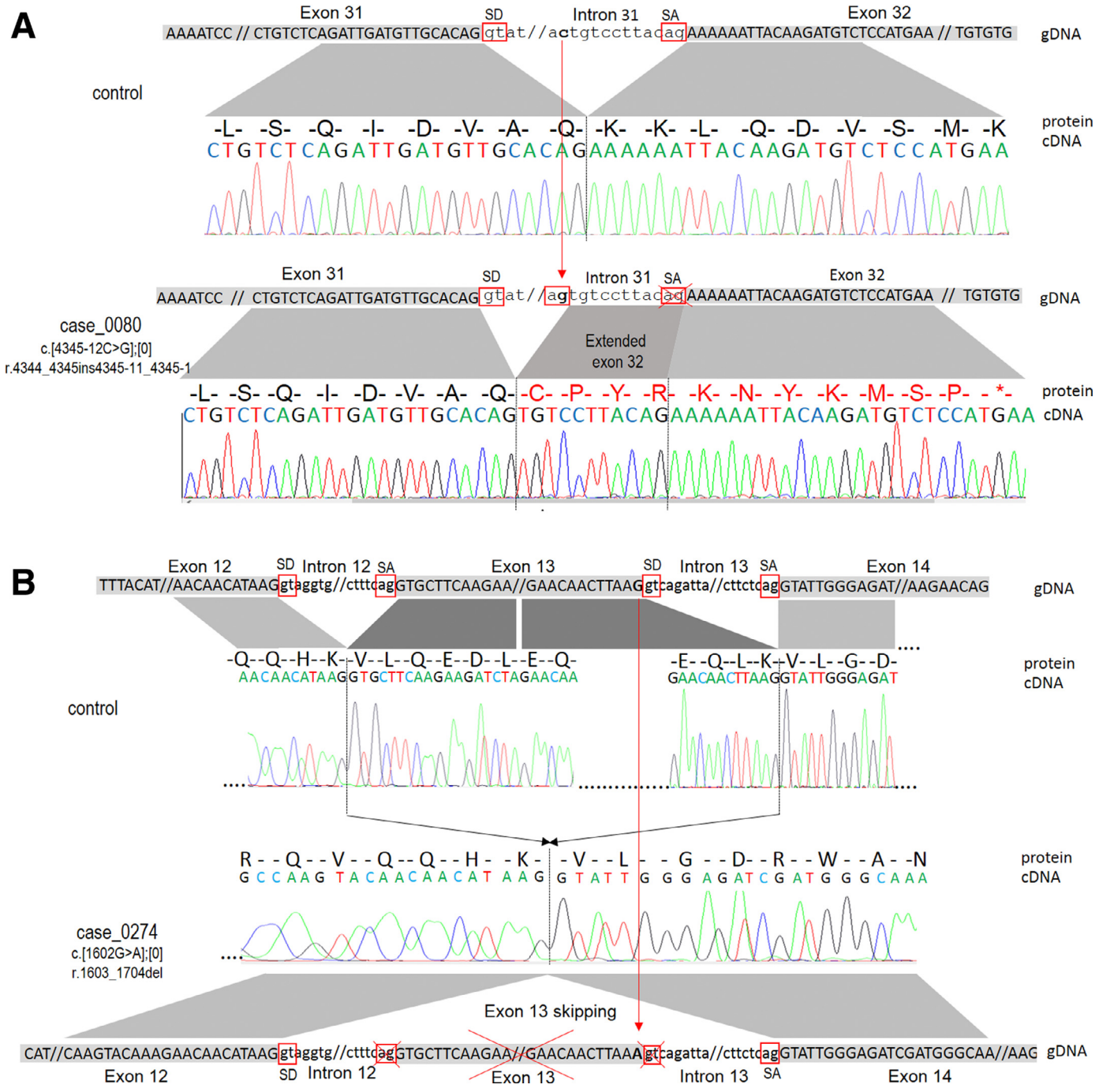

Fig. 5. cDNA sequences reverse transcribed from total RNA of muscle biopsy of control (in the upper diagrams of A and B), case-0080 (lower diagram of A) and case-0274 (lower diagram of B). Sequences of exons are shown with uppercase and introns with lowercase letters, presented discontinuously with coding amino acids with intermittent lines. Retention of 11 nucleotides from intron 31 to exon 32 for case-0080 and loss of exon 13 in case-0274 are observed in cDNA sequences. Altered nucleotides are shown by red vertical arrows for each case. Splicing donor (SD) and splicing acceptor (SA) sites are shown in red boxes. Disruption of SA of inton 31 in case-0080 and disruption of canonical SD of intron 12 and SA of intron 13 in case-0274 are crossed. Pathogenic variants are stated under each case for DNA and RNA level.

deltoid) showed that c.4345-12C>G change created a stronger splice acceptor (SA) site in intron 31 and revealed the insertion of 11 bp from intron into exon 32 leading to a change of lysine to cysteine at codon 1449 and termination at 12th codon (p.Lys1449Cysfs*12) (Fig. 5**A**).

Case-0093 was a singleton DMD case carrying novel *de novo* c.9649+5G>A, predicted to alter splicing donor in intron 66. He had two healthy sisters, who did not consent genetic testing for searching possible gonadal mosiacism in them. Previously same nucleotide change of G to T was

associated with DMD [40]. This variant placed under "likely pathogenic" status.

2.3. Silent VUS group

Case-0064 was a 27-year-old isolated BMD male, living wheelchair bound since the age of 14. His echocardiogram at the age of 24 was reported normal. c.6132T>C alteration in exon 43, annotated as rs761338933 (p.Ser2044=), predicted disease-causing (Mutation Taster). The ExAC database reports in heterozygous form in two females and hemizygous form in three males. In ClinVar data, GeneDx reported this change as likely benign. Unfortunately, no other family members were available for segregation test. Pathogenic nature of this variant could not be supported and classified under "likely benign".

A novel silent variant (c.1602G>A; p.Lys534=) identified in a 7-year-old BMD male (case-0274) predicted to disrupt splicing donor site of intron 13. Subject's healthy brother was not carrier, but affected maternal cousin was carrier. Co-segregation with phenotype supported disease causing status. Furthermore, cDNA sequencing of the reverse transcribed total RNA from muscle biopsy (gastrocnemius) revealed primarily the disruption of SA site of intron 13 but also apparently the canonical splice donor (SD) site of intron 12 leading to the skipping of exon 13 (Fig. 5**B**). Exclusion of exon 13 does not disrupt the translational open reading frame, leading to the loss of 44 amino acids, coherent with the BMD clinic.

3. Discussion

In this study, we present the molecular genetic algorithm test results of 260 dystrophinopathy cases with the finding of novel gross and small variants from Turkey. Initially 211 cases revealed with definitive pathogenic variants and subsequent transcript assays of silent (c.1206G>A, p.Lys534=) and splice site (c.4345-12C>G) alteration and co-segregation of missense (c.9938G>A, p.Cys3313Tyr) variant, lead to 214 cases (82.3%) with variants supporting their clinical phenotype.

In DMD cohort, gross deletion of 52.7% (87/165), duplication of 11.5% (19/165) and pathogenic sequence alterations of 29.1% (48/165) revealed a definitive molecular result in 93.3% (154/165).

In BMD cohort, gross deletions of 40.0% (24/60), gross duplications of 8.3% (5/60) and sequence variants of 15.0% (9/60) revealed a definitive molecular result in 63.3% (38/60). Deletion and duplication rates in our BMD cohort were found to be lower than other populations, as a possible result of a number of patients being directed for the differential diagnosis of BMD.

Attainment of two suggestive and one definitive gonadal mosaic mothers in our cohort, presented that one out of 10 gross duplications (10.0%), one out of 12 small pathogenic variants (8.4%), and one out of 60 gross deletions (1.7%), lead to a recurrence rate of 3.6% in our families. This percentage was lower than the rate identified in a study of 318 DMD/BMD cases with *de novo* pathogenic variants in unhaplotyped families which was calculated to be 4.3% [41]. Expectation of gonadal mosaicism for the parents of *de novo* variant carriers is an important issue of genetic counseling, since as high as 50% risk for similarly affected child must be kept in mind when a second child from uncarrier mother is born to a family.

Muscle biopsy test results were available in 138 out of a total of 260 cases. 111 of these cases were presented with weak or negative immunohistochemistry staining, supporting the ascribed phenotype. Within this pool of 111 individuals, one with chromosomal aberration, 50 (X-autosome translocation cases included) with gross and 44 with small pathogenic variants lead to a total of 95 cases with associated genetic results, raising the overall genetic diagnostic rate to 85.6% for this sub group. In this pool, two variants in two cases (DMD cases-0028 and −0049) reside under VUS due to limitation in family segregation and no transcriptional evaluation. 14 further cases; four of DMD (cases −0037, −0038, −0098 and −0151), six of BMD (cases −0034, 0064, −0071, −0259, −0260 and −0263), three of MF (cases −0045, −0068 and −0090) and one of CK elevated group (case-0273) remained genetically undiagnosed. Additionally, pathogenic variants were identified in five normal staining subjects (in DMD cohort cases −0117 and −0246, in BMD cohort cases −0125 and −0280, in CK elevated cohort case-0275) of 27 cases. 114 out of 122 cases without muscle biopsy data were attained with clinically compatible variants (93.4%). Noticeably, genetic diagnostic rate for singleton patients without muscle biopsy was 92% (80/87). This underscored how even with no any family history, clinical examination provided the uppermost ground for reaching the highest genetic diagnosis. DMD patients with no pathogenic variants with normal *Dys* staining (Case-0070 and −0116 males at 3-years and 5-years respectively) may be considered as good candidates for Western blot analysis to observe if dystrophin protein is intact in size.

A clinically mild form of dystrophinopathy is associated with the maintenance of the open reading frame (ORF), allowing the expression of semi-functional dystrophin with preserved N- and C-terminal protein-binding domains [42]. An exception to this rule is reported for alternative spliced transcripts [9]. Multiple factors have been suggested to determine the splicing pathways including the conserved *cis* regulators, polypyrimidine regions, and branch sequences, splicing kinetics, epigenetic features and structure of pre-mRNA [43]. mRNA sequencing experiments of various *DMD* alterations suggested that 3′ splicing site strength and the density of regulatory elements are determining factors of the splicing pathways in variants affecting splicing signals [44].

Number of DMD and BMD phenotypes revealed similar exon deletions/duplications in our cohort. The compliance with reading frame rule was 80.5% for deletion (70 cases of DMD with deletions complied with predicted ORF-lost versus total of 87 DMD cases with deletions) and 89.5% for

duplication (17 cases of DMD with duplications complied with predicted ORF-lost versus total of 19 DMD cases with duplication) in DMD cases, while 66.7% for deletion (16 cases of BMD with deletions complied with predicted ORF-conserved versus total of 24 BMD cases with deletions) and 60.0% for duplication (3 cases of BMD with duplications complied with predicted ORF-conserved versus total of 5 BMD cases with duplications) in BMD cases. When available, sequencing of the flanking regions expected to reveal if the break points lead to conserved or to disrupt ORF. Association of severe phenotype with apparently in-frame deletions may be due to the disrupted region important for the hinge functionality of the protein and mild phenotype with apparently out of frame deletions may be the result of correction of the frame via novel intronic splice sites.

In our cohort of 60 cases with definitive small pathogenic variants (23.1%, 60/260), exhibited 32 nonsense (12.3%), 17 frame shift (6.5%), five intronic (1.9%), four missense (1.5%), one silent (0.3%) and one delin (0.3%) changes.

In the vast majority of the subjects, all of the identified small disease causing variants were scattered throughout *DMD*, however, one novel nonsense variant (c.2941G>T; p.Glu981*) was identified in two (cases −0077 and −0097), three nonsense variants (c.3151G>T; p.Arg1051*, c.4996C>T; p.Arg1666*, c.9568C>T; p.Arg3190*) in three (cases −0019, −0052,and −0089), two (cases −0088 and −0142) and two patients (cases −0102 and −0172), respectively, implied weak aggregation of small pathogenic variants in exons 22, 23, 35 and 66.

Only one out of 20 cases referred with elevated CK level (721 IU/L) was familial (case-0275) who was referred for genetic testing at the age of 13 for pain in his legs. His 19-years old brother also had elevated CK (1600-2000 IU/L) and had a molecular genetic report showing exon 3-8 deletion by multiplex PCR testing from external laboratory. His elevated CK level was first recognized incidentally at the age of eight during preoperational investigation for middle ear infection and had been referred to neurology clinics. Immunohistochemistry against *Dys* epitopes and dystrophin-associated protein antibodies (anti-35DAG, −43DAG and −50DAG) revealed normal results. His EMG and echocardiogram at that age were reported as normal. Neurological examination revealed mild gastrocnemius, pseudohypertrophy and hyporeflexia. Both brothers had normal intellectual development. These two brothers, their mother and maternal grandmother were all found to be carriers of exon 3-9 deletion by MLPA. Similar deletion was reported previously in one Afghani boy who had isolated global developmental delay/intellectual disability with prominent language delay and no motor manifestations at the age of eight [45]. Similar deletion was further described in two BMD males [46] and recently in heterozygous form in a seven month old baby girl with drug-resistant epileptic encephalopathy who died at the age of 14 months. The deletion was reported to be inherited from her healthy asymptomatic father carrying moderately elevated CK (1016 IU/L) and normal echocardiogram [47]. Exon 3-9 deletion was considered predictive for BMD clinic according to presumptive protected reading in-frame rule.

Diagnostic utility of MLPA followed with targeted NGS for *DMD* in an algorithmic approach and in hand technique for SS, provides high detection rates for nearly the full spectrum of *DMD* variants. A fractional amount, estimated at 2% and comprising complex rearrangements or damaging deep pathogenic intronic variants, remain undetected [37]. Furthermore number of silent and intronic alterations deeper than ± 4 bp may necessitate additional clarification especially when segregation assays could not be performed or unconvincing. Three variants in this study (c.5025+13G>T, c.7309+5G>C, and c.6132T>C leading to silent change) and *Dys* negative or weak patients without any identified pathogenic variants would have benefit from RNA sequencing if muscle tissues were available. A solution to this could be to establish a parallel work for RNA isolations or tissue storage whenever diagnostic biopsy is planned. cDNA sequencing-based analysis of mRNA, extracted from muscle biopsy, may help to disclose coding region variants and more importantly splicing aberrations in transcripts that are caused by intronic variants, as long as mRNA do not go to decay. The intronic regions of *DMD* are very long and NGS with full coverage of an entire gene may be very expensive and laborious. Nevertheless, advancement of sequencing machineries and software technologies may overcome the limitation of read capacities of the repeat regions in near future and not before long will enable diagnostic genetic laboratories to sequence complete genomic *DMD* to disclose all sets of variants including break points of gross deletions, and deep intronic variants leading to splicing error, besides disclosing the positional location of duplicated sequences [48]. The challenge will be the need for considerable data helping to filter out the harmful variants from vast amounts of benign deep intronic variants. Therefore, reporting of rare and novel innocuous variants is very helpful collection of information.

Our study showed that *de novo* occurrence was higher for gross deletion/duplications than small alterations. This can be explained by a higher rate of new crossing over errors than replication errors in human genome. 35 out of 103 singleton patients with gross variants were born to carrier mothers (34.0%) (**Supplementary Fig. 3A**) while 25 out of 36 singleton patients with carrying small variants were born to carrier mothers (69.4%) (**Supplementary Fig. 3B**). There was approximately a 2.1 fold increased risk for being a carrier mother if affected offspring presented small variants. We think that meiotic unequal crossing over may contribute approximately a 2.1 time greater factor than uncorrected replication errors in the formation of *de novo* variants in *DMD*. In combined variant group, our study showed that 56.8% of the mothers were carriers whilst 43.2% were non-carriers in singleton cases (**Supplementary Fig. 3C**).

One another important point is that Limb-girdle muscular dystrophy (LGMD), a highly heterogenous muscular dystrophy group, bear clinical resemblances to DMD but inherited with autosomal recessive and autosomal dominant manner with at least 25 associated genes and occur

in both sexes [15]. Therefore, for cases with normal immunohistochemistry, when no gross or small pathogenic variant is identified in *DMD,* should be considered for LGMD under differential diagnosis.

Family-based investigation of dystrophinopathy patients is not only important for the ascertainment of novel or rare variants, but also provides valuable information and insights for carrier detection, especially mothers of singletone patients with small pathogenic variants, prenatal diagnosis, appropriate genetic counseling and management; possibly including the utilization of causative variant-specific therapeutic drugs which may be available in broader spectrum in the future. Carrier detection especially in asymptomatic males is a very valuable finding to ease the challenges in genetic counseling on young CK elevated patients for the management of the predictive issues.

Table 2a, 2b

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Supplementary materials

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