A rare missense Duchenne muscular dystrophy gene variant in a family with muscular dystrophy from Turkey

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ABSTRACT

Objectives: Duchenne and Becker muscular dystrophies (DMD/BMD) are muscle diseases that show X-linked recessive inheritance. The disease occurs depending on large mutations, deletions/duplications, small mutations, point mutations and mid-intronic mutations of the gene encoding the protein called dystrophin. Therefore, in this study, we aimed to investigate the pathogenic variants of DMD in the affected family.
Methods: A 23-year-old male who had weakness in the muscles, difficulty climbing the stairs, frequent falls at the age of seven was referred to the Medical Genetics department for an initial diagnosis of DMD/BMD. His siblings also suffered from similar symptoms. Therefore, eight individuals from the same family were included in the study. MLPA analysis was performed to evaluate deletion/duplication and variants of the DMD gene were evaluated by targeted NGS. Sophia DDM algorithms were used for the bioinformatics analysis of data, and the pathogenicity of the mutations was evaluated based on in silico prediction tools.
Results: Allelic variants were identified in 8 individuals of the family including two suspected patients and six suspected obligatory carriers. NGS analysis revealed that proband and his nephew were hemizygous for pathogenic c.10018T > C (p.Cys3340Arg, C3340R) mutation and mother, two sisters and niece were carriers.
Conclusions: C3340R mutation was first reported in a Taiwanese BMD patient among the 23 different pathologic changes. This variant identified as pathogenic because of being highly conserved cysteine substitution in the dystroglycan-binding domain of dystrophin. This study has the importance of reporting an infrequent pathogenic mutation, C3340R, in two patients and four suspected obligatory carriers of a Turkish family.
Keywords: Duchenne muscular dystrophy, next-generation sequencing, multiplex ligation-dependent probe

Dystrophinopathies refers to a group of X-linked muscle diseases caused by an absent or deficient sarcolemmal protein, dystrophin. Duchenne muscular dystrophy (DMD) gene mutations cause Dystrophinopathies. The DMD gene is one of the largest genes with 79 exons.

There are two main phenotypes. A severe form is DMD, and a mild form is Becker muscular dystrophy (BMD). It has been shown that DMD is 1:3500, whereas BMD is 1:20000 male births and one-third of the mutations occur de novo [1]. DMD is usually characterized in early childhood, whereas BMD is later

onset with the symptoms, including the skeletal muscles' proximal weakness that causes a frequent fall, difficulty climbing stairs, jumping, running, shuffling gait, and getting up from a lying or sitting position. Clinical appearance ranges mild (increase in the concentration of creatine kinase (CK) and muscle cramps) to severe (progressive muscle disease, wheelchair dependence, cardiac involvement, etc.) Typically BMD patients have milder symptoms, later-onset skeletal muscle weakness, and slower progression than DMD patients. The distinction between DMD and BMD is made depending on the age of wheelchair addiction: DMD occurs before age 13, and BMD is after age 16 [2].

The disease occurs due to defects in the DMD gene consisting of 79 exons that have been localized to the band Xp21 and encoding the dystrophin protein. The full-length dystrophin protein has four functional domains: N-terminal actin-binding domain (exons 2–8), central rod domain (exons 8-61), cysteine-rich domain (exons 63-69), and C-terminal domain (exons 70-79) [3]. Since the discovery of the gene in 1986, thousands of genetic alterations have been described worldwide [4, 5].

Deletions or duplications cause approximately two-thirds of the DMD/BMD, but point mutations, especially missense mutations, are rare. Deletions account for 60-70% of all DMD cases, whereas 80-85% of BMD cases [6] and point mutations are responsible for approximately 26% of DMD and 13% BMD cases [7]. However, Aartsma-Rus *et al*. [8] showed that DMD-related in-frame deletions/duplications were present in 7% of BMD-associated out-of-frame variants in 2% of all patients, reporting that 9% of mutations did not conform to the reading frame rule. It is also recommended that these discrepancies be confirmed at the RNA level. Depending on the variable levels of alternative insertion, one or more exons can restore or disrupt the reading frame [8].

DMD/BMD is inherited in an X-linked recessive manner. The diagnosis of DMD/BMD is based on the characteristic clinical findings and by identifying a pathogenic variant. Still, it may be necessary to use many different genetic analysis methods due to various pathogenic variants. Approximately two-thirds of the muscular dystrophies are caused by deletions or duplication in the DMD gene. The remaining variants are insertions or deletions, splice site changes, and single nucleotide variants. Among these changes, pathogenic missense variants are infrequent.

Here we report a family with a rare pathogenic missense variant, c.10018T > C (p.Cys3340Arg) in exon 69 of the DMD gene with the clinical appearances of affected males the carrier females.

METHODS

Eight individuals from the same family were included in the study. A 23-year-old male proband was referred to the Medical Genetics department for an initial diagnosis of DMD/BMD. He was born after an uneventful pregnancy at 40 weeks of gestation by standard vaginal delivery. Her parents were non-consanguineous but originated from the same small village. The neuromotor development of the patient was normal until the age of 7 years. The patient then started to suffer from weakness in the muscles, difficulty climbing the stairs, frequent falls, and difficulty getting up from a lying or sitting position. Electromyography (EMG) examination at eight years old revealed myopathic findings in both extremities. The echocardiography of the patient was normal at the age of 9 years. Respiratory function tests showed severe restrictive pulmonary function at the age of 19. The patient was tested for genetic analysis for DMD/BMD (22 exons by multiplex PCR were performed) at the age of 10 in an external center and was found to be normal. In the family history; it was learned that his older brother, the son of his older sister and the son of the other older sister who suffered from similar symptoms, died at 14 years old, 22 years old, and 21 years old (the last one died while our genetic analysis was performing) respectively. We have been informed that nobody has similar family symptoms, especially in his grandmother's family. The Ethics Committee approved all protocols used in this study of Pamukkale University Hospital with the number E-60116787-020-9709. The procedures performed in this study involving human participants were applied according to the ethics committee's standards.

MLPA Analysis
The multiplex ligation-dependent probe amplification (MLPA) was performed using the MLPA DMD kit (Salsa MLPA kit DMD/Becker, MRC Netherlands)

following the manufacturer's protocol. The SALSA MLPA Probemix P034-B2 DMD-1 contains 49 MLPA probes, while the SALSA MLPA Probemix P035-B1 DMD-2 contains 48 MLPA probes.

Targeted Next-Generation Sequencing (NGS)

Eight individuals from the family (I:2 grandmother, II:1 mother, III:3 older sister, III:4 older sister, III:5 older sister, III:6 affected male PROBAND, IV:5 affected male nephew, IV:7 niece) were included to NGS study. DNA library was prepared using the DMD master kit (Multiplicom). First, PCR was performed using a 20ng genomic template to amplify all coding regions of the DMD gene and control amplicons in four separate plexes with multiplex PCR amplification reactions containing 118 amplicons per sample. Second, PCR has performed to label the amplicons with molecular descriptors. Each pooled amplicon was purified using Agencourt AMPure XP beads. The library was prepared from each amplicon (280-400 bp) using equimolar concentration. Fluorometric quantitations of DNA samples and purified libraries were determined using Qubit dsDNA Kit (ThermoFisher MA). Sequencing was performed on the Miseq instrument by using Illumina MiSeq Nano Kit v2.

Statistical Analysis

Sophia DDM® (v4.3.1) platform was used for the bioinformatics analysis of data, enabling SNV and Indels (Sophia Genetics SA, Switzerland). Generated FastQ files were then aligned against the reference sequence specified in a manifest file and trimmed to remove primer sequences. Besides, SOPHIA DDM® (v4.3.1) holds a CNV module allowing the CNV analysis for amplicon-based NGS. The pathogenicity of the mutations was evaluated based on in silico prediction tools (SIFT, MutationTaster, Polyphen2) the inheritance (OMIM), database entries (ClinVar, HGMD), and ACMG recommendations.

RESULTS

For the initial diagnosis of DMD/BMD, we first performed deletion/duplication analysis (79 exons) for the DMD gene by MLPA technology, and we did not detect deletion or duplication. The patient was subjected to laboratory and radiological testing. The serological analysis showed creatine kinase (CK) level to be elevated to 933 U/L and lactate dehydrogenase to 252 µg/dl. Polyphasic motor unit action potential (MUAPs) were detected in the left tibialis anterior, left vastus lateralis, right gastrocnemius muscles in the needle electromyography examination.

Genetic data for the family are shown in Fig. 1. Allelic variants were identified by NGS analysis in 8 individuals of the family (demonstrated in the pedigree) who were suspected patients (n = 2) and suspected obligatory carriers (n = 6). The rare pathogenic mutation we detected, c.10018 T > C (p. Cys3340Arg; C3340R), was found in 6 out of 8 individuals (hemizygous in males (III:6, IV: 5) and heterozygous in fe-
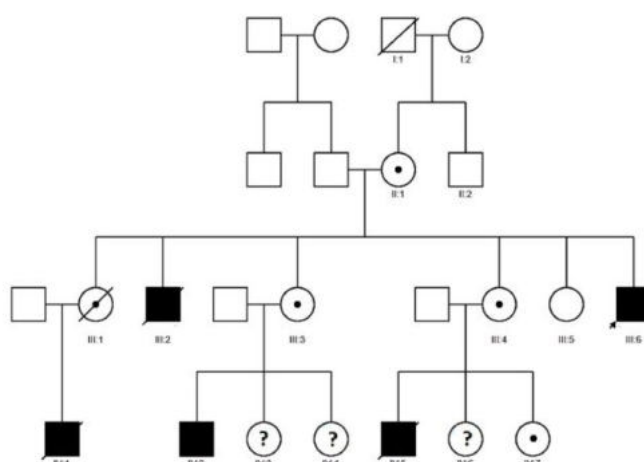
Fig. 1. Pedigree of the family with muscular distrophy: Proband and his nephew (III:6, IV:5) were hemizygous and mother, two sisters and his niece (II:1, III:3, III:4, IV: 7) were heterozygous for C3340R mutation. Eight individuals from the family (I:2 grandmother, II:1 mother, III:3 older sister, III:4 older sister, III:5 older sister, III:6 affected male PROBAND, IV:5 affected male nephew, IV:7 niece) were included to NGS study.
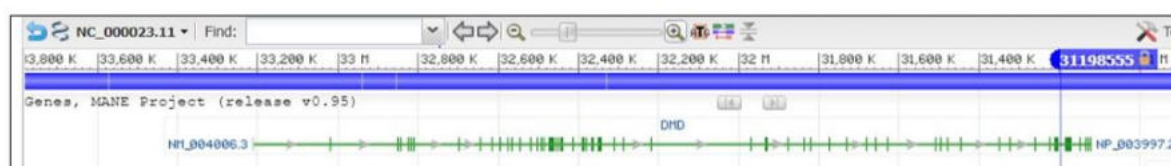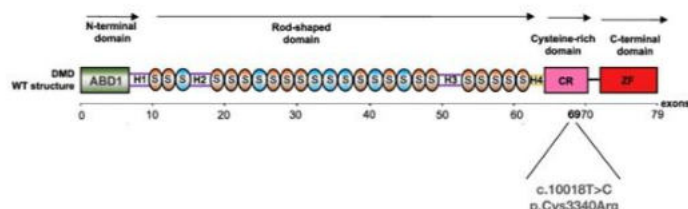
Fig. 2. Overview of the functional domains the Dystrophin protein (A) and genomic and location (B) of c.10018T > C mutation.

males (II: 1, III: 3, III:4, IV: 7) except in grandmother and elder sister. The coding sequence and genomic location of c.10018 T > C mutation is represented in Fig. 2.

DISCUSSION

Muscular dystrophy is a collection of more than 30 genetic diseases causing progressive weakness and degeneration in the skeletal muscles. DMD and BMD are X-linked recessive inherited muscular dystrophies. DMD/BMD symptoms are similar, such as the proximal muscles' weakness that cause a waddling gait, frequent falls, running, jumping, difficulty climbing stairs, and getting up from a lying or sitting position. Affected men use the Gower maneuver to rise from the prone position, using their arms to support the weak pelvic girdle muscles. Calf muscles are hypertrophic and intact during palpation, and calf pain is sometimes observed. There are myopathic changes in EMG examination and increased serum CK levels. Respiratory complications and progressive cardiomyopathy are the most common causes of death. However, BMD is distinguished from DMD by the dependence on the wheelchair after 16 years of age (before age 13 in DMD), the late onset of symptoms, and milder course.

The first report of a missense mutation associated with DMD was published by Prior *et al*. in 1993 [9]. In the UMD-DMD France database, which provides up-to-date information about the DMD gene's mutations, 2898 variants have been reported [10-12]. A comprehensive study involving 1497 patients with dystrophinopathy in Japan revealed the spectrum of mutations identified in the DMD gene. According to this spectrum, exon deletions (61%), duplications 13%), and nonsense (13%) mutations are seen with the highest frequency [13]. However, in some studies, the rate of large deletions was reported to be less (nearly 43%) than generally reported. However, a higher rate for missense mutations was observed (1.4%) due to the diversity in the patients' selection [6]. In a study conducted on a Turkish family of DMD/BMD without 56 detected DMD deletions, Eraslan *et al*. [14] scanned the eighteen deletion-prone exons of the dystrophin gene using modified non-isotopic multiplex single-strand conformation analysis (SSCA). They reported that they identified five unique mutations, 2 of which were responsible for the onset of disease phenotype in DMD [14]. Flanigan *et al*. [6] reported that Point mutations account for 46% of all mutations in their study. However, contrary to previous studies, they could not observe point mutation hotspots due to the nearly evenly distribution of point mutations across the exons [6].

In our family, we found a rare missense mutation, c.10018T > C(p.Cys3340Arg) in exon 69, which is predicted as pathogenic according to in-silico databases (SIFT, PolyPhen and Mutation Taster) and the UMD-DMD France database (record ID: 1607). This mutation has not been reported previously in Turkish population and this substitution affects the highly conserved and substantial cysteine-rich domain for the functions of dystrophin isoforms. In addition, this mutation was first reported in a Taiwanese BMD patient

by Hwa *et al*. among the 23 various pathologic changes including 7 substitutions of which C3340R is the only missense mutation, 6 small deletions and 2 small insertions. This variant has been reported to be pathogenic in that dystrophin has a highly conserved cysteine substitution in the second half of the dystroglycan binding domain [15]. For this mutation, another nucleotide changes in 3340. amino acid position of the dystrophin protein (p.Cys3340Tyr and p.Cys3340Trp) was also reported as pathogenic [16, 17]. Lenk *et al*. [17] reported a G10227A transition, which resulted in a highly conserved cysteine substitution at position 3340 in a child with DMD. They concluded that the patient's mental retardation and absence of b-wave in his electroretinogram could indicate central nervous functions of dystrophin isoforms also depend on cysteine presence 3340 [17]. Second report was from India revealing that C3340R, F590R, C3207R and C3319R have a higher potential to cause DMD than other mutations [18]. Mutations with cysteine residues mutated to some other amino acids such as C3207R, C3313F, C3319R, and C3340R significantly altered the secondary structure of the dystrophin. Another interesting fact is that any mutation containing a basic amino acid instead of a relatively hydrophobic one (like F590R, C3207R, C3319R, and C3340R) has a higher potential cause DMD than other mutations.

The diagnosis of DMD/BMD depends on clinical features and genetic analysis. For genetic diagnosis, a two-step procedure as MLPA to detect deletions and duplications and sequencing of the coding region and splice sites to detect point mutations were usually performed [19]. MLPA analysis alone in DMD/BMD yields approximately 71% [20]. However, the sensitivity of MLPA in combination with sequencing is about 97.3% [19]. These rates are variable among studies. Ballo *et al*. [21] confirmed the genetic diagnosis of DMD in 42% of the affected males (by detection of deletions in the dystrophin gene). In another study from Iran, the genetic diagnosis rate was 81% in DMD [22]. MLPA analyses of our patient were normal. We performed sequencing of the DMD gene by NGS technology, depending on his clinical findings and family history. As a result, we did not detect C3340R mutation in grandmother even though there are female carriers in our family, consistent with the X-linked recessive inheritance of DMD. These situa-

tions, like ours, were being studied for several years. Haldane was the first to suggest a mutation-selection equilibrium in X-linked recessive disorders. In DMD, affected males can not inherit their defective allele to the next generation depending on the reduced life expectancy. In every generation, one-third of mutated alleles are expected to be 'lost' from the population leading to a rapid decrease in the disease incidence. Therefore, the conclusion of Haldane [23] was if there is an equivalent rate of de novo mutations, then the selection of mutated alleles from the population can be compensated. After that, Haldane [23] enlarged on a formula for mutation-selection balance, and according to this genetic model, three ways for the inheritance of DMD mutations were accepted: (1) Mothers are female carriers because their mother is already a carrier; (2) A de novo mutation has occurred in meiosis either in the grand-parental generation (in spermatogenesis of the grandfather or oogenesis of the grandmother; or the mother (de novo mutation in oogenesis); and (3) Mitotic de novo mutations because of germline mosaics can occur in the spermatogenesis of the grandfather, in the oogenesis of the grandmother or the oogenesis of the mother [19].

Grimm *et al*. [24]reported that most deletions arise in oogenesis, while most point mutations are welded from spermatogenesis. In our family, c.10018 T > C mutation was not detected in grandmother (I: 2), and grandfather (I: 1), who died at 87 years old, did not have any muscle problem. So it was intensely estimated that he was not a DMD/BMD patient, and mutation was firstly detected in proband's mother (II: 1) due to the second or third way of the inheritance of DMD mutation.

In family studies for DMD/BMD, rates of inherited variants or de novo variants can differ due to criteria such as variant types, patient selection, or patient number. In Consistence with Haldanes' theoretical model, Ma *et al*. [11] reported a genetic analysis on mothers of 442 patients revealing that 297 (67.2%) of them owned the same mutations as their children, while 145 (32.8%) of them did not. In a study in 106 Taiwanese families with DMD/BMD in 19 mother–patient pairs, 21.1% (4/19) of cases were found to be de novo [15]. Yang *et al*. [20] also reported that DMD gene defects were inherited in 51.72% of their mothers' patients. According to Prior and Bridgeman [25], deletions in the gene's proximal part are more likely

to turn into a familial hereditary mutation. In contrast, distal deletions are more frequently seen sporadically.

Our analysis also revealed 4 missense mutations besides c.10018T > C(p.Cys3340Arg) in our family including c.2645 A > G (p.Asp882Gly), c.5234G > A (p.Arg1745His), c.8810G > A (p.Arg2937Gln) and c.7096A > C (p.Lys2366Gln). These variants are predicted to be benign according to in-silico algorithms and online databases and reported as polymorphisms in the literature [26]. But C3340R mutation is predicted as pathogenic mutation depending on the scores of SIFT, POLYPHEN2 and Mutation Taster. SIFT and POLYPHEN2 reported this variant as "affect protein function" and "probably damaging" whereas MutationTaster as "disease causing". Despite the fact that all males in our family had DMD phenotype of whom their mothers' were carriers, this variant can be considered as "pathogenic". Detection of large deletions and large duplications was performed using MLPA or multiplex PCR. Because most deletion/duplication variants cause DMD, copy number variation analysis such as MLPA is recommended as the first step. In addition, Sanger or NGS was used to identify point mutations and small insertions or duplications [27, 28]. Studies in the literature have reported that it can identify approximately 92% of dystrophin mutations through NGS [29, 30]. However, by combining MLPA and DNA sequencing analysis, the genetic diagnosis rate in DMD/BMD patients can be increased. It can be possible to detect rare variants as in our family.

CONCLUSION

As a conclusion; This study has the importance of reporting an infrequent pathogenic mutation, c.10018T > C (C3340R), for the first time in two patients and four suspected obligatory carriers in a Turkish family. The identification of new mutations could be increasing awareness among parents and physicians with early identification of DMD cases and genetic counseling.

Authors' Contribution

Study Conception: OT, BA, ÇE; Study Design: OT, BA, NK, HA; Supervision: OT, ÇE, AD, HA; Funding: OT, AD, HA; Materials: BA, ÇE; Data Collection and/or Processing: OT, NK, ÇE, AD; Statistical Analysis and/or Data Interpretation: OT, BA, NK, HA; Literature Review: OT, BA; Manuscript Preparation: OT, BA and Critical Review: OT, BA.

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