Splicing mutation of a gene within the Duchenne muscular dystrophy family

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ABSTRACT. The aim of this study was to identify the mutation site and phenotype of the Duchenne muscular dystrophy (DMD) gene in a DMD family. The DMD gene is by far the largest known gene in humans. Up to 34% of the point mutations reported to date affect splice sites of the DMD gene. However, no hotspot mutation has been reported. Capture sequencing of second-generation exons was used to investigate the DMD gene in a proband. Sanger sequencing was performed for mutation scanning in eight family members. Scale-invariant feature transform and PolyPhen were applied to predict the functional impact of protein mutations. A hemizygous splicing mutation IVS44ds +1G-A (c.6438 +1G>A) that induces abnormal splicing variants during late transcription and produces abnormal proteins was located in intron 44. Four missense mutations (p.Arg2937Gln, p.Asp882Gly, p.Lys2366Gln, and p.Arg1745His) that are known multiple-polymorphic sites were found in the coding region of the DMD gene. A heterozygous c.6438
+1G>A mutation was detected on the X chromosome of the proband’s mother and maternal grandmother.

**Key words:** Capture sequencing of second-generation exons; Duchenne muscular dystrophy; Gene; Mutation; Pedigree study

**INTRODUCTION**

Pseudo-hypertrophic muscular dystrophy (PHMD), first described by Duchenne et al. in 1868, is an inherited and lethal X-linked recessive neuromuscular disorder. PHMD can be divided into two distinct types: Duchenne muscular dystrophy (DMD, OMIM310200), and Becker muscular dystrophy (BMD, OMIM300376). In 1953, Becker initially described BMD as a benign X-linked variant of DMD. DMD and BMD are caused by mutations of the dystrophin gene (also known as the *DMD* gene) on the X chromosome at position Xp21.2. Three different types of mutation exist: 1) a partial gene deletion (55-65%); 2) a gene duplication (5-15%); and 3) a point mutation (35%). Approximately 70% of PHMD diagnoses result from carrier mothers and 30% of these cases occur as a result of spontaneous mutation (Aartsma-Rus et al., 2006; Flanigan et al., 2009; Takeshima et al., 2010). Both DMD and BMD are characterized by progressive skeletal muscle weakness, muscular dystrophy, Gower’s sign, possible intellectual disability, or cardiomyopathy. The progressive muscle weakness of DMD usually starts by age 5. The ability to walk may be lost by age 12. Young patients with DMD typically live into their twenties; however, they typically have breathing difficulties and heart disease. BMD is less severe than DMD; with BMD, the ability to walk may be lost after age 16.

PHMD (including DMD and BMD) is an inherited and lethal X-linked recessive neuromuscular disorder. DMD affects approximately 1 in 3500 male births worldwide (Hoffman et al., 1988). Meta-analysis of international prevalence revealed that the pooled prevalence values for DMD and BMD were 4.78 (95%CI = 1.94-11.81) and 1.53 (95%CI = 0.26-8.94) per 100,000 males, respectively. The incidence of DMD ranged from 10.71 to 27.78 per 100,000 males (Mah et al., 2014). Although theoretically DMD does not occur in women, both male and female DMD patients have been reported in clinical practice; this might be caused by a secondary mutation or failure in epigenetic regulation in female carriers (Yoon et al., 2011; Brioschi et al., 2012). Therefore, this occurrence in women highlights the importance of conducting genetic testing for differential diagnoses between autosomal recessive limb-girdle muscular dystrophy and DMD. The goal of the present study was to determine the mutation site and phenotype of the *DMD* gene in a Chinese family.

**MATERIAL AND METHODS**

**Patients**

One male patient diagnosed with DMD at the First Affiliated Hospital of Fujian Medical University (also known as Fujian Provincial Hospital) and eight members of his family were included in this study (Figure 1). The study was approved by the Medical Ethics Committee of Fujian Provincial Hospital in Fuzhou, Fujian, China. Written informed consent was obtained from all participants and guardians of minors.
Clinical studies

All the family members included in the study were evaluated by cardiac ultrasound examination, X-ray investigation, computed tomography, electrocardiography, and blood, urine, and biochemical tests. Electromyography and magnetic resonance imaging of muscles were carried out on the proband.

Genomic DNA extraction

DNA samples from the proband (15 mL peripheral blood) and seven family members (2 mL peripheral blood) were extracted using standard techniques with an Omega EZNA® Tissue DNA Kit, (Omega Bio-Tek, Doraville, GA, USA).

The proband’s DNA sample was analyzed by Illumina sequencing (He et al., 2011; Wu et al., 2011), and bioinformatic analysis (Li et al., 2008) was carried out using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA); a 3-µg sample was used for DNA fragmentation analysis. The DNA was cut using the Covaris method and produced 250-bp DNA fragments. Next, an end repair reaction was performed and end repaired products were linked with a screening-specific adapter. The amplification reaction was performed according to the general adapter primer binding sites with further purification. Target gene enrichment was carried out using a DNA capture chip for sequencing by the Illumina 2000 sequencer (Hiseq2000, Macrogen, Seoul, Republic of Korea). Original images were analyzed using the Illumina base-calling Software 1.7 to collect 90-bp reads. Sequencing analysis was performed after deletion of poorly qualified reads and adapter sequences. The simple object access protocol (SOAP) software was used to analyze the copy number, polymorphism and insertion/deletion, and screening for genes suspected of being pathogenic. SIFT (http://sift.jcvi.org/) and PolyPhen softwares (http://genetics.bwh.harvard.edu/pph/) were used for protein function prediction. These procedures were carried out in the Shenzhen Genomics Research Institute, Shenzhen, Guangdong, China.

Figure 1. Pedigree of a family with Duchenne muscular dystrophy (DMD) genes. I = 1st generation, II = 2nd generation, III = 3rd generation, arrow = proband.
Sanger sequencing

*DMD* gene sequences were obtained from GenBank (NM_004006). The Primer Premier 5 software was used to design exons 44-45 of the *DMD* gene. Primers were synthesized by Beijing Liuhe Hua Genetic Technology Co., Ltd., Beijing, China, including F: 5’-CTAACAGAAGCTGAACAGTTTCTCAG-3’, and R: 5’-TGTTACGATGCTTCCCTCTGTCACAG-3’. Polymerase chain reaction (PCR) was carried out in a reaction volume of 25 µL containing 10X Ex Taq buffer 2.5 µL, dNTP (2.5 mM) 2 µL, forward primer (3 mM) 3 µL, reverse primer (3 mM) 3 µL, DNA template 1 µL, Ex Taq 0.2 µL, H₂O 18.3 µL for further amplification using PTC-200 PCR (BIO-RAD, Hercules, CA, USA). After a 5-min denaturation at 94°C, PCR amplification was carried out using the following cycle profile: denaturation at 94°C for 40 s; 35 cycles of annealing at 57°C for 40 s and extension at 72°C for 60 s; and extension at 72°C for 10 min to produce 359-bp PCR products. An EZNA™ Gel Extraction Kit (Omega Company) was used to purify the PCR products. Collected PCR products were directly used for a sequencing reaction according to standard protocols (BigDye Terminator v1.1 Kit, Applied Biosystems, Foster City, CA, USA). Comparison of the analyzed sequence with that of a normal sequence was performed using DNAman Version 5.2.2 software (Lynnon Biosoft, Quebec, Canada). The PCR products were then cloned into the pGEM®-T Easy (Promega, Madison, WI, USA) vector to detect heterozygous deletions or insertions.

RESULTS

Demographic and clinical studies

A 12-year-old male presented progressive bilateral lower extremity weakness and soreness evolving over 2 years. He could walk, but his ability to squat, stand up, and climb stairs was limited. Physical examination revealed intellectual impairment, mild dystrophy of the bilateral proximal lower extremities, and enlargement of the calf muscles. In the lower extremities, the patient’s strength was 3+/5 proximally and 5-/5 distally. Abnormal laboratory findings included creatine kinase (11,930 IU/L), CK-MB isoenzyme (320 IU/L), aspartate aminotransferase (253 IU/L), alanine aminotransferase (299 IU/L), lactate dehydrogenase (827 IU/L), serum uric acid (622 µM), serum creatine (13 µM), troponin I (0.28 µg/L) and erythrocyte sedimentation rate (2.0 mm/h). Electrocardiography indicated sinus tachycardia with a heart rate of 140 bpm and abnormal Q waves (I, aVL, V3-V6). The electromyography results showed myopathic changes. The pulmonary computed tomography and cardiac sonography examinations were negative. Previous multiplex ligation-dependent probe amplification reported absence of *DMD* DNA copy number variations. In the muscular magnetic resonance imaging, several muscles including obturatorius, sartorius, gluteus maximus, rectus femoris, semimembranosus, semitendinosus, lateral, adductor, biceps femoris, and thigh muscles showed different degrees of atrophy, and the gracilis muscle had mild hypertrophy. The presence of subcutaneous fat increased significantly with fascia connective tissue hyperplasia and hypertrophy.

Sequence capture

Figure 2 shows the results of sequence capture of the *DMD* exon for the proband. The coverage ratio for exon 79 was over 99%. The mean and median depth had similar values demonstrating random sequencing.
Splicing mutation of a DMD family gene

Figure 2. Exon sequencing capture array of the Duchenne muscular dystrophy (DMD) gene. Mean depth (X): average depths at each base in the exon; median depth (X): the depth separating the higher half of all depths; coverage (%): the percentage of bases in all targeted regions.

Mutation analysis

In individual III 1 (proband), DMD exon sequencing analysis revealed no large deletions or duplications of the DMD gene. A hemizygous splicing mutation IVS44ds +1G-A (c.6438 +1G>A), located in intron 44, created abnormal splicing variants during late transcription, and these produced abnormal proteins. This might represent a potentially pathogenic mutation because it rarely occurs in a normal population (Figure 3). Four missense mutations (p.Arg2937Gln, p.Asp882Gly, p.Lys2366Gln, and p.Arg1745His) that are known multiple-polymorphic sites were found in the coding region of the DMD gene.

Figure 3. Sequencing of the region between exon 44 and intron 44 splicing site of the Duchenne muscular dystrophy (DMD) gene in the DMD family. A. A hemizygous splicing mutation IVS44ds +1G-A (c.6438 +1G>A, X chromosome) of the proband’s DMD gene. B. A heterozygous c.6438 +1G>A was carried on the DMD gene of the proband’s mother. C. Normal sequencing of the proband’s father and brother.
Sanger sequencing in the eight family members reported that the hemizygous mutation IVS44ds +1G-A (c.6438 +1G>A) was found on the X chromosome in individual III 1. A heterozygous c.6438 +1G>A mutation was carried on the X chromosome of individuals I 2 and II 2 but not on that of individuals I 1, II 1, 3, 4, and III 2 (Table 1). These finding raised the possibility of a splicing mutation, IVS44ds +1G-A (c.6438 +1G>A), in the region between the exon 44 and intron 44 splicing site of the DMD gene as causative mutations in the DMD family.

**DISCUSSION**

The DMD gene was initially located on the short arm of the X chromosome (Xp21) by Lindenbaum et al. in 1979. In 1983, Kingston et al. found that the BMD gene had the chromosomal locus Xp21. Brown et al. (1985) revealed that linkage analysis of the DNA polymorphisms in relation to the two disorders showed similar genetic distances. Koenig et al. (1987) used molecular cloning of full-length complementary DNA (cDNA) to confirm that DMD and BMD had different mutations in the DMD gene. The DMD gene is by far the largest known gene in humans, spanning 2400 kb in 79 exons and 78 introns. This gene comprises almost 0.1% of the human genome or about 1.5% of the entire X chromosome. cDNA sequences that code for the protein dystrophin account for just 1% of the genome. Dystrophinopathy is characterized by a spectrum of muscle diseases caused by DMD mutation-induced protein dystrophin deficiency. Four clinical types of dystrophinopathy exist: DMD, BMD (including asymptomatic high creatine kinase hyperlipidemia, muscle cramps and myalgia, quadriceps myopathy), X-linked dilated cardiomyopathy, and asymptomatic or symptomatic carriers.

A search of the human genome database (HGMD Professional 2014.4) found that at least 2982 mutations occur in the DMD gene resulting in a DMD genotype that may include 589 missense or nonsense mutations, 257 splicing mutations, 1 regulatory mutation, 347 small deletions, 122 small insertions, 34 small insertions and deletions, 1054 gross deletions, 493 gross insertions, or 85 complex rearrangements. No mutation hotspot has been reported. Despite the challenges caused by the length of the DMD gene and its various mutations, few novel methods exist that would benefit the detection of mutations in the DMD gene, e.g., multiplex ligation-dependent probe amplification (Li et al., 2013). Li et al. (2011) studied 720 DMD cases in China and reported a detection rate of 64.9% (54.3% for gene deletions, 10.6% for gene duplications). The deletion mutation rate in exon 45-54 was 71.9% (281/391) in patients with gene deletions. Gene duplications in exon 1-40 occurred in 82.9% (63/76) of all gene duplications. In all gene deletion and duplication cases, the rate of DMD and intermediate muscular dystrophy was 90.6% (423/467), and that of BMD was 9.4% (44/467).
These results indicate that DMD is mainly caused by dystrophin gene deletion mutations. Mutations were not uniformly distributed in mutation hotspots, as would be expected. Second-generation exons capture sequencing demonstrates a high detection rate of DMD mutations.

Recent studies have shown that no correlation exists between deletion and/or duplication size of the DMD gene and clinical phenotypes and disease severity (Malhotra et al., 1988; Li et al., 2013). Microdeletions in exon 44 led to typical DMD, while gross deletions (approximately 50%) resulted in BMD (Love et al., 1991). Deletions in the central portion and distal rod-like region caused muscle cramps and myalgia, and some other mutations caused asymptomatic high creatine kinase hyperlipidemia (Melis et al., 1998). This was explained by the reading frame hypothesis (Monaco et al., 1988), which states that mutations that disrupt the reading frame (out-of-frame/frame-shift) produce shorter abnormal proteins that lack the main function region (a cysteine-rich C-terminal region), and usually lead to dystrophin deficiency, eventually causing DMD. In BMD patients, however, mutations maintained the reading frame (in-frame mutations), and generally resulted in abnormal but partly functional dystrophin. The reading frame rule explained more than 90% of DMD and BMD cases (Zhou et al., 2006).

Splicing mutations are common and account for 15% of mutations in humans (Cooper and Mattox, 1997). Up to 34% of the point mutations reported to date affect splicing sites of the DMD gene (Tuffery-Giraud et al., 1999). To the best of our knowledge, the rare splicing sites that we found in this study were similar to those found in a pedigree research study by Flanigan et al. (2009). The splicing mutation might change an RNA precursor splicing mechanism leading to abnormal splicing variants during late transcription and abnormal proteins. Recently, it has been demonstrated that mutations of pseudoexons within introns may cause activation of pseudoexons or cryptic exons (Béroud et al., 2004). A single-site mutation may activate potential splicing sites that transform introns into exons, usually leading to extra insertions into mature mRNA forming a termination codon. Translation termination occurs early when reading reaches a termination codon and eventually leads to DMD.

**CONCLUSION**

In summary, our findings suggest that the splicing mutation IVS44ds +1G-A (c.6438 +1G>A) in the DMD gene might be a potentially pathogenic mutation in the DMD family. To the best of our knowledge, these rare splice sites were found for the second time in the present study.

**Conflicts of interest**

The authors declare no conflict of interest.

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