The identification of a novel splicing mutation in the DMD gene of a Chinese family

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Abstract

The Duchenne Muscular Dystrophy (DMD) gene variants are associated with the disease phenotypes. The pathogenic mutation, c.2293-1G>C, was detected in DMD gene in the proband and the fetus, which has not been reported in the literature.The minigene expression in vitro confirmed that c.2293-1G>C is responsible of aberrant splicing.

1. Introduction

Duchenne Muscular Dystrophy (DMD) is a X-linked recessive lethal disorder found in approximately 1/3,500 live male births. The symptoms or signs of the disease is characterized by early-onset, rapidly progressive muscle degeneration, muscle weakness, and being wheel-chair-dependent before age 13 and even dead of cardiopulmonary failure at age 20 (Birnkrant et al., 2018).

The incidence of DMD is correlated with genetic variants of the DMD gene, which is one of the biggest genes (OMIM: 310200) on chromosome Xp.21.2 (Muntoni F, Torelli, &Ferlini, 2003). The DMD gene spans over 2.22Mb, more than 99% of which is intronic sequence (Keegan, 2020). The coding sequence of its largest isoform with totally 11,058 bases across 79 exons harbors a 14kbp transcript encoding a 427 KD protein product (Keegan, 2020) . The protein is a major component of the dystrophin-glycoprotein complex that maintains the structural integrity of the different muscle tissue (such as skeletal, cardiac and smooth muscles) by linking the muscle contractile cytoskeleton with the extracellular matrix (Chevron, Girard, Claustres, &Demaille, 1994). Mutations in the *DMD* gene usually results in disrupting the reading frame, responsible of generating truncated/dysfunctional protein (Monaco, Bertelson, Liechti-Gallati, Moser, &Kunkel, 1998). Previous reports have summarized the DMD gene variation spectrum, containing deletion, duplication, small rearrangement, and point mutation. Large deletion in the DMD gene has been observed in more than 70% of diagnosed patients, while large duplication was seen in more than 10% (Ankala, et al., 2012). Current evidence demonstrated that the deletion spots are usually identified at exons 45–52 and 8–13 but the gene deletion pattern differs between cases (Baudat, et al., 2010). In our study, we found a novel splicing mutation in the proband, which is not seen in Clinvar or Human Genome database and undescribed in current clinical reports. The novel splicing mutation found in the proband was not only detected in the patient's parents but also confirmed in a prenatal diagnosis at the second pregnancy of the patient's mother; Further, the effect of the splicing mutation on damaging biofunction of the coded protein was investigated to confirm its putative role. The findings could be new evidence for diagnosing prenatal case and for preventing the birth defect incidence since there is not yet an effective cure of DMD to date (Hirst, McCullagh, &Davies, 2005).

2. Materials and Methods

2.1 Study patient and family

The patient is a five-year-old boy diagnosed with DMD, who is as wheelchair user suffering from slow walking, difficult squatting, and muscle weakness of both lower limbs. In the study, his mother had a second pregnancy, and the prenatal examination showed that the value of NT (nuchal translucency) of the fetus was normal and the results of non-invasive prenatal test was low risk.

2.2 DNA extraction

Five ml of whole blood sample was individually taken from the proband and his parents, and 10 ml of the amniotic fluid was collected from the pregnant mother in an amniocentesis test under ultrasound. Genomic DNA was extracted from obtained blood samples or amniotic fluid using a DNA extraction kit (TianGen, Beijing, China) and stored at -20 for analysis later.

2.3 Mutation screening

The genomic DNA extracts of the proband and parents were used for screening the DMD gene by the WES analysis. The detected variants were further evaluated by the Polyphen and SIFT software to determine pathogenicity.

2.4 Nested polymerase chain reaction (PCR) of pcMINI-gene and pcMINI-C gene

The nested PCR was initially conducted using two sets of primers (4705-DMD-F/7551-DMD-R and 5063-DMD-F/7188-DMD-R listed in Table 1) with the genomic DNA used as template. A second round of nested PCR was followed to obtain the amplicons of DMD gene. Subsequently, the PCR with the primer set of pcMINI-DMD-KpnI-F and pcMINI-DMD-EcoRI-R was conducted to generate wild-type (wt) amplicon (840bp) using the PCR product from the second round of nested PCR as template. By the same protocol, the mutant fragment 1 was amplified with the primer set of pcMINI-DMD-KpnI-F and DMD-MUT-R, whilst the mutant fragment 2 was amplified with the primer set of DMD-MUT-F and pcMINI-DMD-EcoRI-R. The amplicons of mutant fragment 1 and mutant fragment 2 were mixed with ratio of 1:1. The primer set of pcMINI-DMD-KpnI-F and pcMINI-DMD-EcoRI-R were used for amplifying pcMINI mutant type (mut) fragment (840bp). The sizes of pcMINI-C (wt/mut) amplicons are the same. The primer sequences are listed in Table 1.

TABLE 1 The primers used in qPCR

Name	Primer sequence(5'- -3')
4705-DMD-F	CTTTTTCTCTGCAACTATTTCTGAT
5063-DMD-F	CAATCACAGTTAACACTGGTCACA
7188-DMD-R	TGACATGCACAACAAAAACATT
7551-DMD-R	CAGTTTCTAGGGGGAACTTACA
DMD-MUT-F	TGCTCTCATGCTGCACGCCATAGAGCGAGAA
DMD-MUT-R	TTCTCGCTCTATGGCGTGCAGCATGAGAGCA
pcMINI-DMD-KpnI-F	GGTAGGTACCGTTATGTGACACTTTATCTT
pcMINI-DMD-EcoRI-R	TGCAGAATTCATGACGGTAAAACATTCCAT
pcMINI-C-DMD-KpnI-F	GGTAGGTACCAAATGAAGTCTTAAAAAATA
pcMINI-C-DMD-BamHI-R	TAGTGGATCCCATTCACCATCTGTTCCACC

2.5 Plasmid construction and transfection

The PCR amplicons were ligated into eukaryote expression vector pcMINI using restriction enzymes Kpn I and EcoR I to construct the recombined pcMINI-DMD-wt/mut plasmids, which were transformed into *E. coli*competent cell DH5α individually. The plasmid containing *E. coli*colonies were screened and the presence of inserted DMD gene fragments were determined by colony PCR using primer sets of pcMINI-DMD-KpnI-F and pcMINI-DMD-BamHI-R. The positive colonies were used for extracting plasmid by Rapid Mini Plasmid Kit (Simgen, Hangzhou, China) and plasmid was subsequently sequenced to confirm the inserts. The

confirmed plasmids were followingly introduced into human embryonic kidney 293T cells and MCF-7 cells. The 293T cell line was cultured in DMEM medium containing 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin at 37°C in the **5** % CO_2 atmosphere. The MCF-7 cell line was cultured in MEM medium containing 10% FBS, 0.01mg/mL insulin, and 1% penicillin/streptomycin at 37°C in the **5** % CO_2atmosphere. Liposomal Transfection Reagent (Yeasen, Shanghai, China) was used to transfect cell lines with pcMINI-DMD -wt/mut or pcMINI-C- DMD -wt/mut. After 6 hours of transfection, the transfect cells were resuspended in fresh media and cultured for 48 hours. Total RNA was extracted from the enriched transfect cell lines and used for analysis later.

2.6 minigene transcription analysis

Extract total RNA from cell samples according to the instructions of the kit. After concentration determination, cDNA synthesis was performed with the same amount of RNA. Reverse Transcription (RT-PCR) was carried out with primers **(** the forward primer: CTAGAGAACCCACTGCTTAC; the reverse primer TAGAAGGCACAGTCGAGG; The primers were the same in the pcMINI- DMD -wt/mut or pcMINI-C-DMD -wt/mut**).** Total RNA was reverse-transcribed using the PrimeScript RT reagent Kit (Yeasen company, Shanghai, China) to generate the minigene constructs for analysis. The size of gene transcription band was detected by agarose gel and sequenced.

3. Results

3.1 DNA sequencing

A heterozygous DMD splicing mutation, c.2293-1G>C on intron 18, was detected from the proband and his mother but not from the father. It is recognized as a new mutation with WES and Sanger sequencing analysis, being not found in Human Gene Mutation Database (HGMD) (Fig.1).

3.2 Identification of gene inserts in recombinant vectors by colony PCR

The positive colonies were selected and analyzed by colony PCR to determine if the gene inserts of pcMINI-DMD -wt/mut (Fig.2A) or pcMINI-C- DMD -wt/mut were present within the recombinant vertors, respectively (Fig.2B). The gene inserts were confirmed by plasmis sequencing and results were showed in Fig.3A and Fig.4A.

3.3 DMD mRNA expression in the transfected cell lines

The minigene splicing assay was to determine whether this genetic variant could result in aberrant splicing during gene expression. 293T and MCF-7 cells were transfected and a total of 8 samples were collected after 48h of transfection.

3.3.1 pcMINI-DMD -wt/muttranscriptional analysis results

A schematic diagram of the pcMINI-DMD -wt/mut construct is shown in Figure 3A. RT-PCR obtained the expressed gene inserts from the pcMINI-DMD-wt/mut vector in 293T and MCF-7 cells. The different splicing products for wild-type (band a: wt lane, 477 bps) and variant type (band b: mut lane, 470 bps) are obtained on electrophoresis gel with close bp size (Figure 3B). Sequencing results indicated that the minigene of wild-type (from pcMINI-DMD-wt) formed mRNA containing complete exon 19 (Figure 3C, D). For the expressed minigene from pcMINI-DMD-mut, the intron c.2293-1G>C caused aberrant splicing, leading to the absence of 7 bp on the left side of exon 19 (Figure 3C, D).

3.3.2pcMINI- C- DMD -wt/mut transcriptional analysis results

We further determined that mutation c.2293-1gG> C could affect the normal splicing on mRNA of DMD gene and the deletion of 7bp on the left side of Exon19 was seen in the pcMINI-C- DMD -wt/mut vectors (Figure 4A, B, C, D).

Through *in-vitro* Mini-gene splicing assay, we confirmed that the biofunction of the novel splicing mutation (c.2293-1gG> C). This gene variant is responsible of causing a frame shift of the transcripts, which could

lead to the generation of an abnormal dystrophin protein.

4. Discussion

Duchenne muscular dystrophy is a rare inherited disease due to lack of dystrophin, which is caused by the mutation of *DMD* gene. To date, there are 4690 gene mutation sites in *DMD* gene recruited in The Human Gene Mutation Database (HGMD) (Stenson, et al., 2020). HGMD (*http://www.hgmd.org)* have been collating all known gene lesions associated with DMD that have been published in the peer-reviewed literature. Mutation catalogues in HGMD are divided into 11 categories by mutation type, including gross deletions/insertions/duplications mutation (> 20 bp) (account for 52.8%), frameshift mutation (17.1%), nonsense mutation (15.6%), Canonical-splice mutation (6.8%), missense mutation (4%), splice mutation (1.7%), noncoding mutation (1.2%), inframe mutation (0.7%), synonymous mutation (0.1%), initiation mutation (0.02%), and regulatory mutation (0.02%). The intron variant of DMD gene is rarely studied since it is assumed to be less lethal than exonic variants. This study reported a proband with clinically diagnosed DMD that carried a novel splicing intronic variant in the DMD gene. The *in vitro*Mini-gene splicing assay demonstrated this new variant caused mRNA damage during gene expression, which resulting in pathological symptoms. This novel finding could be added to the DMD mutational repertoire.

Currently, there is no effective treatment for DMD (Li, et al., 2012; Ebrahimzadeh-Vesal, Teymoori, Azimi-Nezhad, &Hosseini, 2018). It is important to develop accurate molecular diagnosis for this fatal disease as preventing birth defect is the main approach to manage DMD. Therefore, the investigation of gene variant in DMD gene is necessary for providing the basic knowledge to design and invent the mutation-specific gene therapy (Takeshima, et al., 2010). For this disease, the genetic diagnosis usually starts with the proband. In our study, the proband is a five-year-old boy who has been diagnosed DMD, being characterized by slow walking, difficult squatting, weakness of both lower limbs and dependent on wheelchair. A novel heterozygous DMD splicing mutation, c.2293-1G>C on intron 18, was detected in the proband and his mother but not the father by WES and Sanger sequencing. The splicing mutation was predicted to be deleterious by multiple computational methods. Complying with the guideline of American College of Medical Genetics and Genomics (ACMG) (Richards, et al., 2015) and ClinGen Sequence Variant Interpretation (SVI) (Ahmad N Abou Tayoun, et al.,2018; Biesecker, &Harrison, 2018; Ghosh, Harrison, Rehm, Plon, &Biesecker,2018), it is suggested that the c.2293-1G > C mutation of gene DMD is a pathogenic mutation site and associated with X-linked recessive disease Duchenne muscular dystrophy (DMD).

Further, our study successfully constructed pcMINI- DMD- wt/mut and pcMINI-C-DMD-wt/mut vectors, and then transfected 293T and MCF-7 cells. In vitro experiment of mini-gene splicing assay verified that the mutation c.2293-1G > C on intron 18 alters the normal splicing of DMD mRNA. The variant in both pc-MINI-DMD and pc-MINI-C-DMD vectors resulted in the deletion of 7bp on the left side of Exon19, with a codon frameshift resulting in an abnormal dystrophin protein as Fig 3 and 4. Because of the deletion of 7bp on the left side of Exon19, the variation of cDNA is c.2292_ 2299del and the variation of protein is p. ala765ser FS * 26. We concluded it would produce a truncated body protein with a size of 789aa, or may take the NMD pathway (nonsense mediated mRNA degradation) on account of the deletion of 7bp on the left side of Exon19 by the software (*http://www.mutationtaster.org/*). Hence, we deduced that the novel splice site mutation on intron 18 was a pathogenic variant and influenced the normal splicing of mRNA leading to alternative on the DMD translation products**.**

Another valuable data included in the study is that we collected the amniotic fluid (10ml) of the fetus at the second pregnancy of the studies family. The splicing mutation c.2293-1G>C was reproduced in the prenatal investigation and the sex of this fetus was male as well, indicating that the new genetic variant could be closely linked with the incidence of DMD. Besides of knowing DMD as a severe X-linked recessive muscle-wasting disease, DMD is also characterized by pseudohypertrophy in the calf muscle and the rapidly progressive degeneration and necrosis of the proximal muscles (Birnkrant et al., 2018). DMD patients are commonly first diagnosed before age of 5 and many died of respiratory or cardiac failure around age of 20. Consistently, the fetus in our study was also diagnosed with this new DMD gene variant. After genetic counseling and an accurate prenatal diagnosis, this couple decided to terminate pregnancy at 18 weeks of

gestation.

In conclusion, we found a novel DMD splicing mutation c.2293-1G>C on intron 18 and confirmed that the mutation was pathogenic in gene expression level. Our finding enriches the DMD mutation spectrum. and provide scientific tool to achieve an effective prenatal diagnosis for the studied family.

Compliance with Ethical Standards

Ethical approval

The study was approved by the Research Ethics Committee of Tongji Hospital affiliated to Tongji Medical College of Huazhong University of Science and Technology. Informed consents were obtained from all the participants.

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Conflict of interest

The authors declare that they have no conflict of interest.

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