Molecular characterization and reclassification of a 1.18Mbp *DMD* duplication following positive carrier screening for Duchenne/Becker Muscular Dystrophy

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Abstract

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Abstract

A 2-month-old male harboring a duplication of *DMD* exons 1-7 classified as pathogenic by an outside institution presented with mildly elevated CK levels; molecular breakpoint analysis by our laboratory reclassified the duplication as likely benign. To date, proband continues to develop normally with decreased CK levels, further supporting our reclassification.

Key clinical message

Molecular characterization of *DMD* duplications at the breakpoint level is necessary to accurately interpret their effects on dystrophin activity and their roles in Duchenne and Becker muscular dystrophies.

Keywords

Duchenne muscular dystrophy, DMD, duplication, dystrophinopathy

Introduction

Duchenne [DMD, MIM: 310200] and Becker [BMD, MIM: 300376] muscular dystrophies are X-linked disorders which result in progressive proximal muscle weakness and degeneration, in conjunction with characteristic elevation of creatine phosphokinase (CK) in blood. DMD is rapidly progressive and typically presents in early childhood with motor delays and gait instability, while BMD is characterized by a later-onset phenotype of skeletal muscle weakness [1]. Both DMD and BMD are caused by mutations in *DMD* , a ~2.2Mbp sized gene containing 79 exons encoding the dystrophin protein. Dystrophin maintains the structural integrity of striated muscle cells through the formation of a dystrophin-glycoprotein complex, linking the cytoskeleton to the extracellular matrix and thus providing tensile strength to muscle fibers [2].

Given its size (roughly 0.1% of the human genome), a high degree of *DMD* allelic heterogeneity exists for DMD and BMD. Deletions are the most common pathogenic variants in *DMD* (~64% of cases), followed by nucleotide substitutions (22%), duplications (12%), and others (inversions, insertions, etc., ~2%) [3]. These alterations disrupt dystrophin's reading frame in diverse ways, which can lead to mutated transcripts susceptible to nonsense mediated decay, truncated unstable proteins products targeted for degradation, or reduced activity protein variants [4].

DMD duplications are up to five times under-represented compared to deletions in public databases [3]. *DMD* duplications have historically posed a technical detection challenge as exonic duplications could not be captured by PCR-based techniques, fell outside interrogated probes by multiplex ligation-dependent probe amplification (MLPA), or were under the limit of detection of chromosome microarray (CMA). Such technical difficulties are coupled with the fact that duplications can adopt different structural configurations in the genome. These include duplications in tandem direct or inverted orientations, or insertions into an entirely different chromosome, which can significantly alter the functional impact of duplications. These limitations can challenge the clinical assessment of newly identified duplications in young asymptomatic individuals based solely on genomic data; for these individuals, further molecular and protein truncation characterizations need to be performed in order to provide an accurate diagnosis.

In this report, we illustrate the molecular characterization of a 1.18Mbp *DMD* duplication spanning exons 1-7 in a 2-month-old male with mildly elevated CK levels. The duplication was found to expand up to 698Kb beyond *DMD* 's promoter regions and had a tandem configuration in direct orientation, which led to our reclassification of the variant as likely benign. Follow-up studies at 13 months of age indicated decreasing CK levels and normal development in this individual, further supporting the likely benign classification of the duplication. We hope our study prompts further characterization of *DMD* duplications not only in young asymptomatic individuals but also in symptomatic patients, to further elucidate the impact of genomic duplications on *DMD* transcription and dystrophin structure and improve our technical and interpretative capabilities for DMD and BMD diagnosis.

Results

Case presentation

A 2-month-old Caucasian male (proband, IV-3 in Figure 1) was referred for genetic testing due to a duplication of *DMD* exons 1-7 identified in his mother (III-6 in Figure 1) during prenatal carrier screening by an outside laboratory; the duplication was classified as pathogenic by that laboratory. Proband was born at 39 weeks gestation by cesarean section; upon birth he presented with jaundice, mild erythema toxicum neonatorum, and neonatal hypoglycemia that resolved with feeding. His CK levels at two months of age were 324 U/L. His mother is a 33-year-old P1G1 Caucasian female with a history of infertility. She presented

with gestational hypertension without evidence of preeclampsia during pregnancy and had normal electro-cardiogram results after prenatal screening follow-up for potential cardiovascular disorders conferred by the detected *DMD* duplication. There is no family history of musculoskeletal concerns or cardiomyopathy for either of proband's parents.

Genetic testing

Diagnostic MLPA testing was performed on proband, targeting all*DMD* coding exons as well as its 5'UTR, 3'UTR, and DP427c regions. We identified a duplication spanning from the 5'UTR to exon 7 (g.(?_-33229574)_(32827735_32717219)) (hg19). Subsequent CMA analysis of proband revealed the duplication to be 1.18Mbp in size, overlapping the 5'UTR and exons 1-7 of *DMD* (NM_004006) (arr[hg19] Xp21.1(32,741,375-33,926,846)x3 mat) (Supplemental Figure 1). To further characterize the structural configuration of the duplication, PCR primers were designed bordering the identified duplication breakpoints, such that it could distinguish between direct tandem orientation, inverted tandem orientation, and insertion elsewhere in the genome (Supplemental Figure 2 and Supplemental Data). A long-range PCR protocol using these primers yielded a product ~1.8Kb in size (Supplemental Figure 3). Sanger sequencing of the amplified PCR product showed the duplication breakpoints to fall at Xp21.1:32,741,022 and Xp21.1:33,928,069 (hg19, Supplemental Figure 4 and Figure 2). Duplication breakpoint analysis revealed the presence of short palindromic sequences at the junction as well as microhomologies in neighboring regions (Supplemental Data).

Discussion

We molecularly characterized a novel contiguous 1.18Mbp duplication in*DMD* including its 5'UTR up to exon 7 in a two-month-old male with mildly elevated CK levels (324 U/L). The duplication was maternally inherited and initially discovered during prenatal carrier screening; the prenatal analysis reported this rear-rangement to be a pathogenic exon 1-7 duplication, with the child being at a 50% risk of developing DMD. Follow-up diagnostic analyses of proband in our laboratory included MLPA, CMA, and Sanger sequencing; we showed this duplication to be in direct tandem orientation within Xp21.1, extending further away from exon 1 and including *DMD*'s 5'UTR as well as the DP427c alternative promoter/exon1 (Figure 2).

Sanger sequencing showed the duplication to encompass Xp21.1:32,741,022-33,928,069. There are ~570Kb of intervening sequence separating *DMD*'s DP427c isoform start (neuronal and retinal isoform)[5] and the duplication end, and ~698Kb separating the duplication end from Dp427m, which is the main isoform produced in muscle and is involved in DMD and BMD (Figure 2)[5]. While we cannot rule out splicing defects in the normal *DMD* transcript caused by the presence of the duplicated*DMD* exons 1-7, the intervening ~570-698Kb of sequence between the duplication and the two main DMD promoters led us to hypothesize this duplication to be likely benign, as no promoter or regulatory regions were separated from the main gene body, and any potentially truncated *DMD* product would likely undergo nonsense mediated decay (NMD). Our hypothesis was further supported by the observation that, at thirteen months of age, new measurements of CK levels in proband showed a reduction to 211 U/L (original measure was 324 U/L). In addition, proband remains asymptomatic and shows normal development without any noticeable neuromuscular issues or motor delays. Splicing analysis remains to be performed in future experiments, as we are currently unable to complete protein truncation testing (PTT) in proband due to the requirement of a muscle biopsy which is not recommended in an asymptomatic young child [6]. Family studies are being pursued, including testing of males II-8, II-9, III-9, III-11, and IV-4 (Figure 1), to further clarify the significance of the duplication.

At the sequence level, the duplication described herein overlaps the duplication hotspot previously described for *DMD*, encompassing exons 2-20 [4]. The majority of DMD duplications have different sizes and are non-recurrent events. These findings support non-homologous end-joining as a possible mechanism of generation. Junction analysis of the 1.18Mbp duplication in proband revealed the presence of microhomology, which also suggests a possible origin through microhomology-mediated mechanisms such as fork stalling and template switching (FoSTeS)[7] and microhomology-mediated break-induced replication (MMBIR)[8]. Microhomology tracts have been previously reported in the analysis of complex *DMD* rearrangements [9], suggesting that several mechanisms can participate in DMD and BMD pathogenesis.

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We surmise the exon 1-7 duplication was given a pathogenic classification by the prenatal screening laboratory based on a previous study that had reported an exon 1-7 duplication in an individual with the Duchenne phenotype [10]. Moreover, the mild CK elevation in proband could have prompted such overdiagnosis. Currently most laboratories use a Gaussian distribution of 0-200 IU/L as the normal CK range in white individuals, with affected males having ranges >504 IU/L; however, it has been shown that CK levels in healthy populations can be skewed toward higher values, which can lead to overdiagnosis of mild abnormal values [11]. Said issue poses a dilemma to the correct diagnosis of asymptomatic infants with variants of uncertain significance (VUS) in *DMD* and mild CK elevations. In such instances the comprehensive characterization of the identified VUSs, particularly duplications, is paramount to the correct prediction and diagnosis of dystrophinopathies.

Conclusion

Altogether, we have illustrated the importance of molecular characterization of *DMD* duplications in the clinical diagnosis of asymptomatic and young individuals. While duplications may escape finer characterizations due to current technical limitations, establishing their correct sizes and orientation is paramount to delivering an accurate clinical interpretation. As next generation sequencing becomes more widely available in DMD clinical testing, further molecular characterizations of duplication junctions in *DMD* can help elucidate and predict at-risk recombination sites, and better inform our diagnostic capabilities.

Materials and Methods

Clinical *DMD* MLPA analysis was performed on proband to confirm the presence of the duplication, assess its size, and confirm a DMD diagnosis. MLPA experiments included probes for each of the 79 *DMD* coding exons, 3'UTR, 5'UTR, one probe for the alternative promoter/exon 1 DP427c, as well as control probes (MRC Holland). Capillary electrophoresis of PCR amplified ligated probe pairs was performed on ABI 3730xl (Applied Biosystems), and data analyzed with GeneMarker Software (SoftGenetics LLC, State College, PA, USA). CMA analysis was performed on proband using CytoScan HD Suite (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. CMA minimal and maximal probe positions for the duplication were used to design sequencing primers using Primer3Plus. Forward primer sequence: ctgtgttttgggccatttct and reverse primer sequence: tgggtttagccctaggacac produced a ~1.8Kbp product visualized in 2% agarose gel in a UV light box. The 1.8Kbp product was cleaned up using Exo-SAP-IT PCR Product Cleanup Reagent (ThermoFisher Scientific, Waltham, MA, USA) and Sanger sequenced on the ABI 3730xl (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Chromatogram files were analyzed in FinchTV (Geospiza, Inc.) and FASTA files exported and mapped to hg19 using BLAT in the UCSC genome browser [12].

Author Contributions

CFF and JKGA provided clinical information; AM and CZM performed additional experiments for the molecular characterization of the DMD duplication; CZM, JEB, and LH wrote the manuscript; CZM, JEB, CFF, AM, JKGA, and LH read and approved the manuscript.

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Conflict of Interest

The authors declare that they have no competing interests.

Ethical Approval

Participants were consented accordingly for release of data included in this study.

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Figure legends

Figure 1. Pedigree showing the maternal segregation of a 1.18Mbp duplication at Xp21.1 overlapping DMD. Proband is indicated with a black arrow (IV-2). There is no reported history of cardiomyopathy or dystrophinopathy in maternal and paternal families.

Figure 2. Schematic representation of *DMD* and the duplicated segment encompassing exons 1-7, 5'UTR, Dp427m, and Dp427c promoters. Notice the large intervening sequence separating the isoform promoters from the exon 7 in the duplication (dotted rectangle).
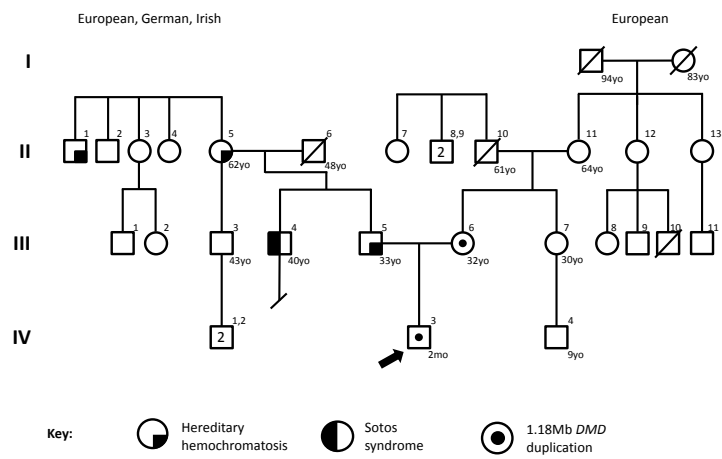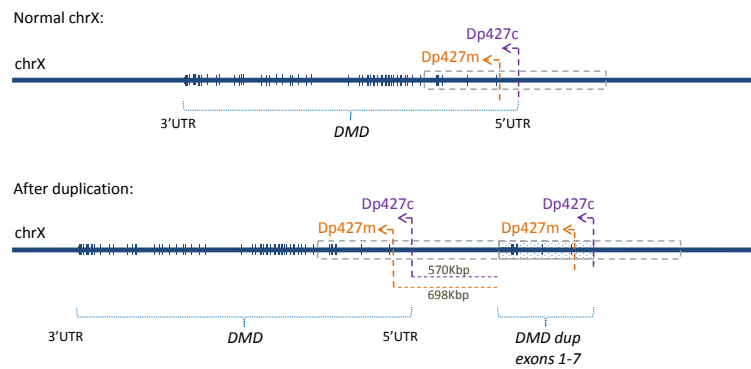
Figure 1

Figure 2