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Becker Muscular Dystrophy Due to an Inversion of Exons 23 and 24 of the *DMD* Gene

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Abstract

The use of hybridization-based methods for DMD mutation analysis is increasingly common. We report a case of Becker Muscular Dystrophy in which discrepant results between a PCR-based (SCAIP) and a comparative genomic hybridization assay incompletely characterized the mutation (an inversion of exons 23 and 24). These results demonstrate the limits of sensitivity and specificity of both tests, and highlight the need for more detailed analysis when intronic deletions are detected by comparative genome hybridization methods.

Keywords

Becker muscular dystrophy; inversion mutation; SCAIP; MLPA; CGH

Becker muscular dystrophy (BMD) and the more severe Duchenne muscular dystrophy (DMD) are both caused by mutations in the *DMD* gene that can be detected in genomic DNA samples in 93-96% of cases ^{1,2}. Approximately 65% of *DMD* mutations are deletions of one or more exons, and another 5% are exonic duplications that require methods for detecting exon dosage. Most non-deletion/non-duplication mutations are point mutations, including nonsense mutations, subexonic frameshifting insertions and deletions, and rarely missense mutations ^{3,4}.

For many years the standard diagnostic test utilized multiplex PCR of a limited number of exons within deletion "hotspots", where 98% of deletions occur ⁵. Relatively inexpensive and reliable for detection of deletions in hemizygous males, the multiplex PCR test has been largely replaced by methods that interrogate all exons and also readily allow dosage measurements in female carriers. These include multiplex ligation-dependent probe amplification (MLPA) that detects exon copy number ⁶, and comparative genomic hybridization (CGH) arrays that may also detect copy number changes in non-exonic regions ⁷⁻⁹. Meanwhile, detection of point mutations has become feasible through the routine use of direct sequencing methods from genomic template DNA ³ or chip-based resequencing. Sequencing of cDNA derived from muscle mRNA remains useful for detecting all mutation classes and for validating the implications of certain mutations detected at the genomic level (such as putative splice site mutations). In addition, cDNA sequencing is necessary for detecting the small percentage of mutations, such as deep

intronic point mutations, that are not readily detectable with current genomic screening methods but result in inclusion of intronic sequence as pseudoexons within the mRNA transcript¹⁰.

Here we report the characterization of an unusual *DMD* mutation that led to discrepant results between PCR-based and CGH array-based diagnostic methods. Both methods were performed accurately, and their combined results gave a clue to the presence of the actual mutation, an inversion of exons 23 and 24 within the gene.

Case Report

The patient was initially found to have an incidental elevation of serum transaminases that led to a gastroenterological evaluation around age 18 months. By age 5, he was found to have an elevated serum creatine kinase (1035 iu/L by report; exact upper limit for the lab used unknown). At age 12 (his most recent examination), he has myalgias with exertion and decreased stamina compared to peers, but only minimal limb-girdle weakness (modified Medical Research Council grade 5-/5 in hip flexors and deltoids, with strength otherwise 5/5).

At age 5 he underwent a clinical diagnostic *DMD* mutation analysis that included multiplex PCR and Southern blot analysis using eight cDNA probes covering the *DMD* coding region. The clinical report noted that (1) Southern blot with probe 30-2 (covering exons 20 through 30) revealed a band shift of the HindIII fragment that normally contains exons 22-25 to a position that corresponds with a slightly lower molecular weight, and (2) exons 22 through 25 were not included in the multiplex PCR reaction.

Based upon the clinical features and this result, the patient (DR43488) was eligible for enrollment in the United Dystrophinopathy Project database (<http://dystrophy.genetics.utah.edu>). Under an Institutional Review Board approved protocol and following informed consent, his initial testing in our lab was performed under the UDP protocol using the SCAIP method, as described elsewhere³. Amplification of all exons (as the first step in SCAIP analysis³) from a genomic DNA template revealed the absence of an amplification product for exon 24 alone. As per standard laboratory protocol, amplification was repeated using alternate internal primer sets flanking exon 24 in order to exclude the possibility of polymorphisms at the primer hybridization site. No product was seen by agarose gel electrophoresis using the standard (LxRx) or three alternate exon 24 primer configurations (Figure 1A).

In order to reconfirm the results, and to ascertain the mother's carrier status, genomic DNA samples from the boy and his mother were tested at a clinical laboratory using a *DMD* Oligonucleotide Array Comparative Genomic Hybridization array⁹. This revealed a normal gene dosage signal for all exons, including exon 24 in both the mother and the patient. However, it revealed a deletion in intron 22 in a heterozygous fashion in the mother, and a hemizygous fashion in the patient. This 1.1 kb deletion included nucleotides 32,487,572 through 32,488,656 of the genomic sequence (chrX reference sequence from GRCh37 assembly); this intron 22 segment is found approximately 1.6 kb from the 3' end of exon 22, and 0.7 kb from the 5' end of exon 23.

We presumed that both results were performed in a technically correct fashion and postulated that a complex mutation comprised of an inversion of exons 23 to 24 and an intron 22 deletion could account for both results. Repeat blood samples were obtained, and another round of diagnostic testing was therefore undertaken in our laboratory. MLPA analysis¹¹ revealed the presence of both exons 23 and 24 in the patient and his mother, with normal dosage for all *DMD* exons. PCR amplification with primers 23-Rx and 24-Rx

resulted in a 1.6 kb amplification product that would be expected only in the setting of an inversion (data not shown). We then undertook long range PCR (Roche) using the manufacturer's recommended conditions with primers flanking exon 22 (22-Lx) and exon 24 (24-Rx), and successfully amplified a band of approximately 8 kb from DNA from both the boy and his mother, and a band of approximately 9 kb from a wild-type control and the boy's mother (see Figure 1B).

Sequencing of this ~ 8 kb PCR product from the patient revealed an inversion of exon 23 through exon 24 associated with the intron 22 deletion breakpoint (Figure 1C and D). The 5' inversion breakpoint is at the site of the 1.16 kb deletion first detected by CGH array within intron 22. The 3' inversion breakpoint is located within intron 24 between the splice donor site of exon 24 and the location of the internal PCR primer site (24-Ri). The sequence generated from the 24-Li primer aligns with intron 23, exon 24 and intron 24 (196 nt. to nucleotide 32,482,469) before aligning with intron 22 at the 5' junction of the deletion (nucleotide 32,488,694). The sequence generated from the 24-Rx primer begins in intron 24, aligning through to nucleotide 32,482,468 and then continues aligning in intron 22 beginning at nucleotide 32,487,537. These alignments are consistent with a complex deletion/inversion: intron 22 deletion (g.32487538_32488693del, 1.16 kb) plus an exon 23 to 24 inversion (g.32482469_32487537inv, 5.07 kb).

Discussion

These results show limitations inherent in CGH, MLPA, and SCAIP, methods which are in widespread clinical use. The parsimonious explanation for the discrepant results from CGH and SCAIP testing was an inversion, and an amplification using predicted primer pairs resulted in the confirmation of that explanation and characterization of the breakpoints. The reported absence of exon 24 by SCAIP is explainable by the location of the 3' break point between exon 24 and flanking PCR primer site (24-Ri).

The 5' deletion/inversion junction occurs in unique sequence, while the 3' inversion junction occurs within a 235 nt. DNA transposon-like medium reiterated frequency repeat element (MER20) located in intron 24. The nucleotides encompassing the 1.16 kb deletion are derived from intron 22, with no additional insertion of nucleotides at either the 5' or 3' inversion junction. These breakpoint sequence features suggest that the mechanism underlying this rearrangement was nonhomologous end joining (NHEJ) rather than nonallelic homologous recombination (NAHR). *DMD* deletions account for ~65% of mutations, and studies involving sequencing of dozens of deletion breakpoints have found that NHEJ appears to be the major mechanism, often with the breakpoint junction exhibiting a few nucleotides of homology between 5' and 3' flanking sequences or the addition of a few extraneous bases^{12,13}. A sequenced nonrecurrent deletion breakpoint involving the 17p11.2 Smith-Magenis syndrome region involved a MER5B element, and it was suggested that double-strand break substrates within low density MER elements may not be able to be repaired by homologous recombination in a timely manner, thus leading to error-prone NHEJ¹⁴. The *DMD* exon 23-24 inversion/deletion observed here may be another example of a complex rearrangement involving the NHEJ pathway.

This case – only the fifth intragenic *DMD* inversion in the literature¹⁵⁻¹⁸ – provides a cautionary note to the interpretation of any individual gene dosage test. Both MLPA and CGH revealed normal exon dosage. In our standard diagnostic algorithm, the absence of a detectable mutation by gene dosage and SCAIP methods leads us to recommend muscle biopsy to determine mRNA structure. In this case, a biopsy had not been performed as part of the diagnostic workup; furthermore, even after recontacting the patient and his family, we were unable to obtain tissue for mRNA or protein analysis, as they declined any tissue

sampling whatsoever. This refusal included skin biopsy, from which fibroblast cultures could have been established for MyoD-forced myogenesis and subsequent DMD expression; this option is less invasive than muscle biopsy yet still allows reliable characterization of *DMD* mRNA structure. Nevertheless, we would predict that the mRNA contains an in-frame deletion of exons 23 and 24, which would be consistent with the patient's relatively mild clinical course. Such a mild course suggests that these exons encode protein regions that of limited significance, and that exon skipping of small mutations in this region may prove beneficial. As in all of the previously reported cases, the inversion mutation was flanked by a deletion, in this case the smallest (1.16 kb) yet reported; this pattern suggests that the deletion event is intrinsic to the formation of the inversion. We emphasize that the report of a novel intronic deletion of any size on a diagnostic CGH test should lead to more detailed analysis by additional genomic or mRNA studies. Neuromuscular clinicians should have a high index of suspicion for the presence of an inversion mutation when interpreting CGH results.

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Figure 1.

PCR and sequencing confirmation of the *DMD* deletion/inversion mutation. (A) PCR amplicons from SCAIP deletion analysis of exons 23, 24 and 25 in the proband (DR43488) and a normal control. (B) PCR amplicons using primers 22-Lx and exon 24-Rx for a normal control, the mother of DR43488, and patient DR43488. (C) Locations of PCR/sequencing primers in a 9 kb interval of the *DMD* gene (coordinates chrX:32,491,000-32,482,000), including exons 22, 23 and 24. Diagrammatic alignment of sequence reads generated from primers 24-Li and 24-Rx on normal versus inverted exon 23 to exon 24 segments. The breakpoint sequence from the 22-Lx::24-Rx amplicon from patient DR43488 defines the 5' junction (sequence from 24-Li primer) and 3' junction (sequence from 24-Rx primer).