Sequencing of entire 2.2 MB *DMD* gene facilitates diagnostic testing and aids selection of patients for therapeutic intervention

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INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is an X-linked inherited neuromuscular disorder caused by pathogenic variants in the *DMD* gene. The variant spectrum of *DMD* is unique including 60% intragenic deletions, 5% duplications and 35% sequence variants. The clinical presentation of DMD includes progressive weakness of the skeletal muscles that leads to loss of ambulation by age 10-12 years. Affected individuals also have significantly elevated creatine kinase (CK) levels in blood. Present mean age of diagnosis of DMD is about 5 years; however, it can be diagnosed as early as immediately after birth through newborn screening (NBS) by measuring serum CK levels followed by confirmatory molecular testing. Molecular diagnosis for Duchenne and Becker muscular dystrophies (DMD/BMD) involves a two-tiered approach for detection of deletions/duplications using MLPA or array CGH, followed by sequencing of coding and flanking intronic regions to detect sequence variants, which is time-consuming and expensive.

MATERIALS AND METHODS

DMD NGS assay was designed to cover all *DMD* variant types including deletions, duplications, and single-nucleotide variants including deep intronic variants in the single assay. A custom Agilent Sureselect *DMD* targeted sequence capture was used to enrich the entire 2.2 Mb *DMD* gene, which includes coverage for all 79 exons, introns, and promoter regions, followed by NGS on the Illumina NovaSeq 6000 with 100-base pair paired-end reads. Alignment to the human reference genome (hg19) was performed and annotated variants are identified in the targeted region. Deep intronic baits are very useful to achieve a uniform coverage across the entire *DMD* gene, which ultimately helps in the detection of deletion–duplication breakpoints more precisely and also permits detection of deep intronic pathogenic variants.

Sequence variants were assessed by our proprietary analysis and interpretation pipeline, Ordered Data Interpretation Network (ODIN). Variants were called at a minimum coverage of 8× and an alternate allele frequency of 20% or higher. Variant interpretations and classifications were performed using the American College of Medical Genetics (ACMG) standards and guidelines for the interpretation of sequence variants. More than 100 known intronic variants in the *DMD* gene were curated from the Human Gene Mutation Database (HGMD) and 30 intronic variants were tagged as pathogenic or likely pathogenic in ODIN after further curation using ACMG guidelines. Deletions and duplications (CNV) in the *DMD* gene were assessed using the NxClinical 5.1 software (BioDiscovery). Male and female reference sets were created from healthy controls using NGS data with the help of BAM Multiscale Reference (MSR) Builder module.

RESULTS

We have developed a comprehensive next-generation sequencing (NGS)-based single-step assay to sequence the entire 2.2 Mb of the *DMD* gene to detect all copy number and sequence variants in both index males and heterozygote females. A total of 772 individuals were tested using different sample types including whole blood, saliva, and dried blood spots. Intragenic deletions and duplications (single-exon or multi-exon) were detected in 60% and 14% of the index cases, respectively. Definitive molecular diagnosis was established in 86% of the index cases, while rest of the sample were reflexed for further expanded molecular testing.

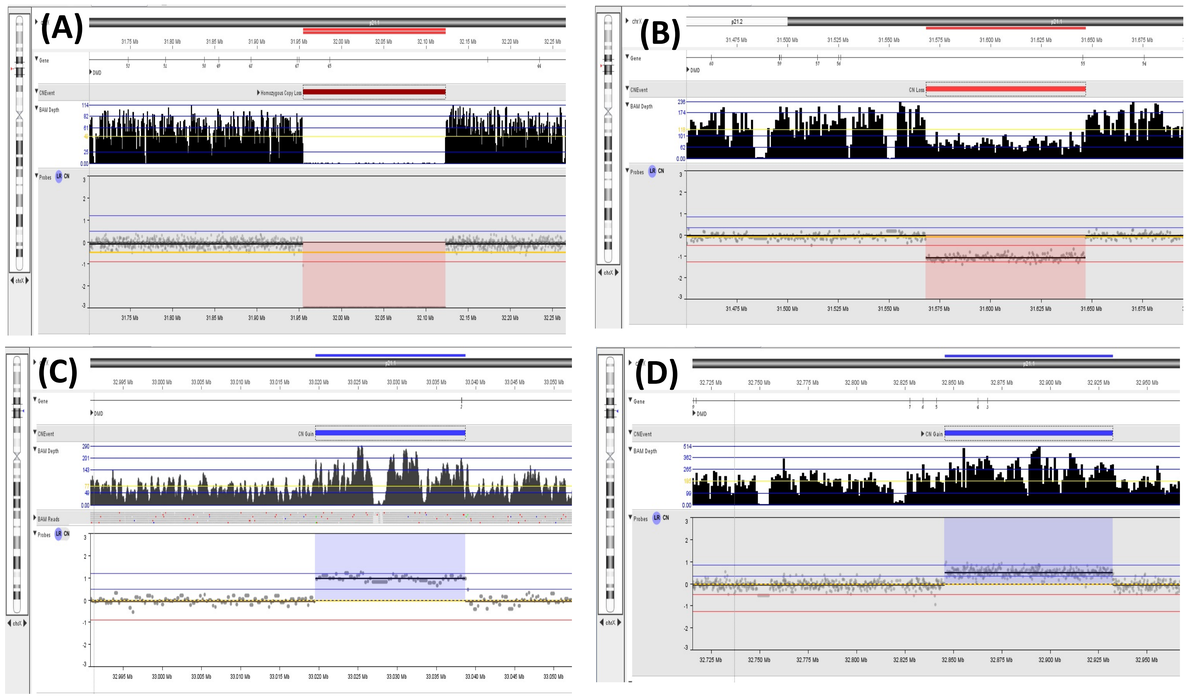

Figure 1. (A) Male DMD patient with single exon 45 deletion. (B) Female carrier with single exon 55 deletion. (C) Male DMD patient with single exon 2 duplication. D) Female DMD carrier with exon 3-4 duplication

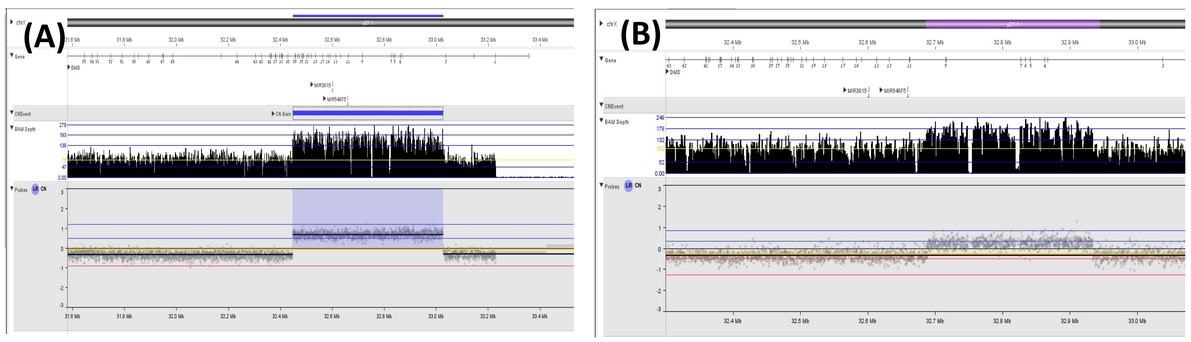

Figure 2. (A) Male DMD patient with exon 3-29 duplication (B) Female DMD carrier with exon 3-9 duplication

CONCLUSION

- NGS-based assay detects CNVs and single-nucleotide variants in one assay and eliminates the need for reflex testing. Ultimately, it saves cost and time, leading to an early definitive diagnosis.
- Sequencing of the entire 2.2 Mb genomic sequence of the *DMD* gene facilitates a uniform coverage across the gene, which ultimately helps detection of CNVs with accurate breakpoints. Molecular diagnosis with near-precise identification of breakpoints ultimately helps in enrollment DMD patients in the new therapeutic intervention.
- The availability of the adjacent intronic sequence facilitates identification of binding sites for interventions such as CRISPR-Cas9 and possibility of any off -target hits. We have developed a library of adjacent sites sequences which are suitable for such binding.
- Innovative variant specific personalized therapies are emerging in recent years for DMD including read-through, exon skipping, vector mediated gene therapy and genome editing. Definitive molecular diagnosis is required for appropriate clinical care and enrollment to clinical trials and personalized treatments. Genome editing is an important gene therapy approach to correct a genetic defect on a permanent genomic basis. DMD is an ideal target for genome editing, because of diverse variant spectrum, X-linked inheritance and the preserved function of internally truncated BMD-proteins.