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Genomic breakpoint analysis facilitates identification of complex rearrangements and re-classification of non-tandem duplications in the *DMD* gene: Implications for prenatal testing and new therapeutics.

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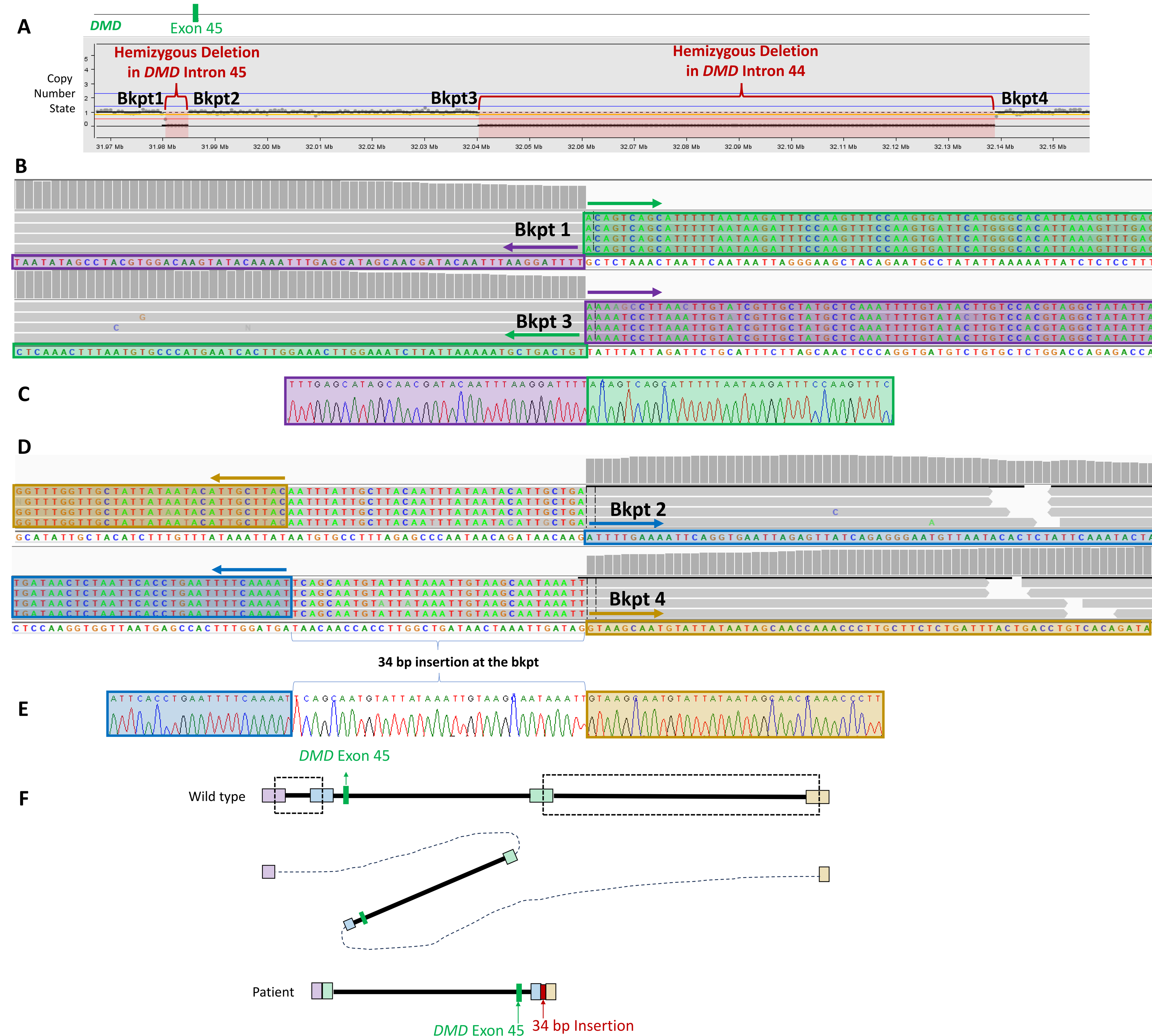
1 Introduction

Duchenne (DMD) and Becker Muscular Dystrophy (BMD) are X-linked recessive disorders with a frequency of 1 in 5000 male infants caused by pathogenic variants in the dystrophin gene. Copy number variants (CNVs) such as intragenic deletions (65%) and duplications (10%) are the most frequent type of variants observed in Duchenne (DMD) and Becker Muscular Dystrophy (BMD). The remaining variants include nonsense, indel variants, and complex rearrangements. Most duplications located within the *DMD* gene are in tandem resulting in disruption of the dystrophin gene, hence these duplications are typically classified as pathogenic. However, in rare cases the duplicated region of the *DMD* gene is inserted elsewhere in the genome, rendering such duplications non-pathogenic. In addition, intronic CNVs in the *DMD* gene are typically not disease-causing. The identification of the location of duplications and the significance of intronic CNVs is not routinely explored due to the technical limitations of conventional molecular techniques such as MLPA and microarray. We used breakpoint junction sequence analysis from next-generation sequencing data (NGS) to identify complex rearrangements in the *DMD* gene which enabled re-classification of non-tandem duplications which are generally interpreted as pathogenic using traditional testing methods like MLPA and microarray due to a lack of precise breakpoints.

2 Methods

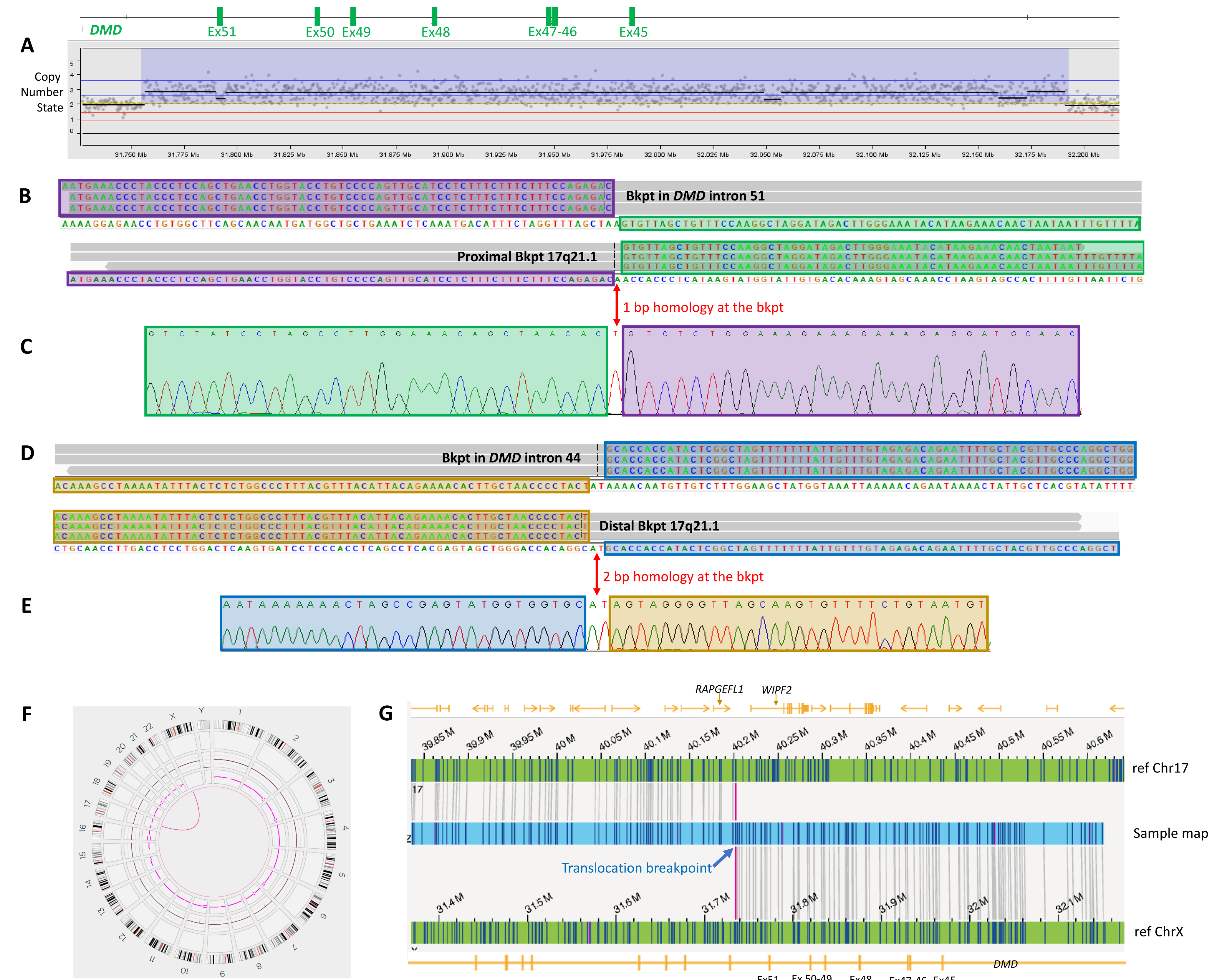
The Agilent Sureselect *DMD* targeted sequence capture method was used to enrich the entire *DMD* gene, which includes all exons and introns from the genomic DNA. DNA was analyzed by next-generation sequencing (NGS) on the Illumina MiSeq or NovaSeq™ 6000 with 2x150 paired-end reads. CNVs within the *DMD* gene were assessed using BioNano's NxClinical v6.1 software. Split reads were visualized in Integrative Genomics Viewer v2.8.0.7. Sanger sequencing was performed to confirm the breakpoint junctions wherever possible using case-specific primers across the breakpoints. Optical genome mapping was performed by loading labeled ultra-high molecular weight gDNA on Bionano's Saphyr chip for linearization and visualization on the Bionano Saphyr instrument.

Fig. 1 Breakpoint analysis of case 1 harboring an inversion with flanking deletions.



A NxClinical screenshot showing whole DMD gene NGS results. The magnified region of interest shows hemizygous deletions within DMD intron 45 and DMD intron 44. B IGV viewer of short-read whole DMD gene NGS sequencing showing mismatched bases from split reads (soft-clipped reads) after breakpoints 1 and 3. The transparent colors indicate matching reverse complement sequences. C Mapping of Bkpts 1 and 3 by Sanger sequencing. D IGV viewer of short-read whole DMD gene NGS sequencing showing soft-clipped reads after breakpoints 2 and 4. The transparent colors indicate matching reverse complement sequences. A 34 bp insertion is present at the breakpoint. BLAT shows this insertion mapping at 100% identity to chromosomes 14 and 4. E Mapping of Bkpts 2 and 4 by Sanger sequencing. F Schematic of wild type and patient with deletion-flanked inversion of exon 45. (Bkpt=Breakpoint)

Fig. 2 Breakpoint analysis of case 2 harboring a DMD duplication inserted into the 17q21.1 region



A NxClinical screenshot showing whole DMD gene NGS results. The magnified region of interest shows a duplication of DMD exons 45-51. B IGV viewer of short-read whole DMD gene NGS sequencing showing soft-clipped reads after breakpoints in DMD intron 51 and the proximal breakpoint in 17q21.1. The transparent colors indicate matching sequences. C Mapping of bkpts in DMD intron 51 and the proximal 17q21.1 breakpoint by Sanger sequencing. Only the reverse direction was obtained. There is 1 bp homology at the breakpoint. D IGV viewer of short-read whole DMD gene NGS sequencing showing soft-clipped reads after breakpoints in DMD intron 44 and the distal breakpoint in 17q21.1. The transparent colors indicate matching sequences. E Mapping of bkpts in DMD intron 44 and the distal 17q21.1 breakpoint by Sanger sequencing. Only the reverse direction was obtained. There is 1 bp homology at the breakpoint. F Optical genome mapping genome-wide circos plot showing chromosomes 17 and X connected by a pink line indicating inter-translocation. G Optical genome map showing the translocation breakpoint in one direction (other direction not shown). The left part of the sample map maps to chromosome 17 and the right part to chromosome X.

3 Results

We present two cases with a complex rearrangement in the *DMD* gene. Case 1 harbors an inversion of exon 45 and case 2 carries a duplication of exons 45-51 inserted elsewhere in the genome. In case 1, an adult male with clinical symptoms of DMD since childhood was tested by comprehensive NGS analysis. Initial analysis showed two non-contiguous deletions in intronic regions, a deletion entirely within intron 44 and a deletion entirely within intron 45. Further breakpoint analysis revealed that the ~56 kb region between the two intronic deletions encompassing exon 45 was inverted, which resulted in the clinical phenotype. Case 2 was a 2-year-old female whose mother was determined to carry a duplication of *DMD* exons 45-51 by carrier screening at an outside laboratory. Duplication of *DMD* exons 45-51 has been detected in male patients with dystrophinopathy. Comprehensive NGS analysis of the daughter identified a duplication of *DMD* exons 45-51. However, breakpoint analysis and Sanger sequencing confirmed the duplicated region of *DMD* is inserted into the 17q21.1 region, rendering the classification of this duplication as non-pathogenic for DMD.

4 Conclusion

In conclusion, breakpoint junction analysis of genomic data facilitated the identification of a pathogenic inversion in a male with clinical symptoms of DMD and lack of dystrophin protein by muscle biopsy and the identification of an extragenic *DMD* duplication in a family that was previously classified as pathogenic in carrier testing.

The two cases presented here enabled precise diagnosis and classification of the variants and appropriate genetic counseling. Our results emphasize the importance of utilizing breakpoint analysis of NGS data or long-read sequencing to assess the pathogenicity of CNVs detected in the *DMD* gene, particularly in duplications identified by carrier screening or prenatal diagnosis when there is no family history of dystrophinopathy. The precise molecular diagnosis of DMD is essential in clinical evaluation, variant classification, and therapeutics.

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