Title: Pathogenic Duplications in Tumor Suppressor Genes Cause Aberrant mRNA Splicing

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Background: Recent studies have shown that most intragenic gross duplications in tumor suppressor genes (TSG) occur in tandem1 . Armed with this evidence, recent publications have suggested presuming duplications occur in-tandem and create aberrant mRNA splicing when performing variant assessment2,3 . However, complexities surrounding the prediction of splice defects in tandem duplications warrant the addition of RNA analysis to DNA genetic testing. Lack of RNA evidence may lead to miss-classification in the following circumstances: duplications with mid-exonic breakpoints, cooccurrence with splicing variants and/or alternative splicing, and pseudogene gene conversions.

Previous work showed that massively parallel RNA sequencing (RNA-seq) was able to identify novel transcripts arising from tandem duplications in MSH2, a gene that is susceptible to acquiring structural variants due to its enrichment with intronic Alu elements1,4,5 . In the current work, we have expanded upon this study to investigate other duplications in additional TSG using RNA-seq.

Methods: Germline duplications were identified in patients using next generation sequencing (NGS) and multiplex ligation-dependent probe amplification (MLPA) during multi-gene panel testing (MGPT), as described previously5 . RNA-seq and RT-PCR were performed as described previously on RNA isolated from whole blood of patients identified with germline duplications4 . Patients were included in this study if they were available to submit an additional blood sample for RNA analysis. Reads supporting aberrant splicing of the involved exons were used as one line of evidence for variant classification based on ACMG/AMP guidelines6 . All study participants consented to RNA genetic testing on a research basis. This study was approved by the Western Institutional Review Board.

Results: RNA-seq provided evidence for reclassification of 20 variants in the following genes: APC (n=1), BARD1 (n=1), BRCA1 (n=4), BRIP1 (n=1), CHEK2 (n=1), DICER1 (n=1), MLH1 (n=3), MSH2 (n=1), PALB2 (n=3), PMS2 (n=2), PTEN (n=1), RAD50 (n=1). A total of 15 duplications were reclassified from VUS to clinically actionable pathogenic/likely pathogenic variants (LP), with all variants having reads supporting aberrant splicing on RNA-seq. Three variants remained VUS due to unknown structural impact of the observed abnormal transcripts, as they were in frame and predicted to escape nonsense mediated decay (NMD). One duplication in RAD50 harboring a mid-exonic breakpoint in two unrelated probands was reclassified to likely benign (LB) after RNA-seq showed no evidence of aberrant splicing. RNA-seq also provided clarification for a pseudogene gene conversion in PMS2 that appeared as a single exon copy number gain on MLPA, as there was no evidence of abnormal splicing. Further DNA analysis showed a single nucleotide gene conversion at the position analogous to the MLPA probe ligation site in PMS2CL.

Conclusion: A vast majority of gross duplications occur in-tandem and lead to aberrant mRNA splicing. RNA analysis of the resulting transcript is a further, confirmatory step in variant interpretation using ACMG/AMP guidelines6 and can provide clarification for complicated cases that would have been

misclassified without it. Individuals performing variant classifications should be cautious when interpreting evidence for duplications that have mid-exonic breakpoints, that co-occur with splicing variants and/or may be impacted by alternative splicing, and that are located in regions prone to pseudogene gene conversions.

References:

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