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Alternative Splicing Analysis Identifies Mutation Hotspots In Hereditary Breast and Ovarian Cancer Genes

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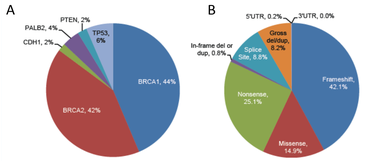
BACKGROUND

Genetic testing for hereditary breast and ovarian cancer (HBOC) is becoming increasingly widespread in the era of precision medicine. Variants of unknown significance (VUS) in the HBOC susceptibility genes *BRCA1* and *BRCA2* pose a quandary to medical providers and patients because these genes are clinically actionable[1]. A large percentage of VUS in *BRCA1/2* are predicted to affect splicing[2]. Previous efforts have focused on interrogating splicing VUS using low-throughput and/or imprecise techniques[3]. Therefore, we developed and validated a method for using RT-PCR NGS to accurately and efficiently characterize germline splicing defects.

SPLICING VARIANT DATA



Sequence data retrospectively analyzed from a cohort of 23,000 individuals tested at a clinical diagnostics laboratory for a HBOC DNA Multi-Gene Panel Test (MGPT). (A) In this cohort, 44% and 42% of pathogenic mutations were detected in *BRCA1* in *BRCA2*, respectively. (B) Among the variants that were classified as pathogenic, 8.8% have known effects on splicing.

ASSAY VALIDATION

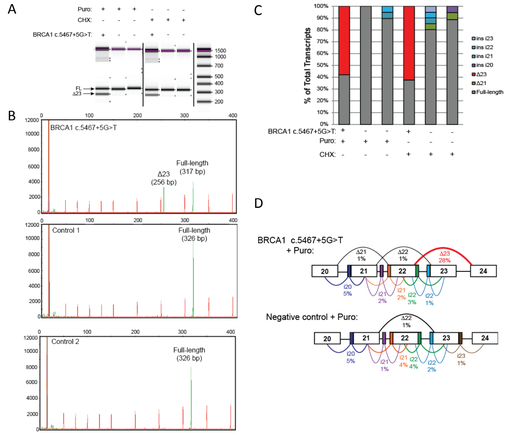


Figure 1. Splicing alterations caused by the variant BRCA1 c.5467+5G>T. LCLs with variant or controls were treated with puromycin (puro) or cycloheximide (CHX). RNA was extracted and RT-PCR performed and analyzed on TapeStation (A) or capillary electrophoresis (B). (C) Sanger sequencing of subcloned transcripts of LCLs treated with indicated NMD inhibitor. Median transcript levels over 3 replicates are shown. Transcripts must be detected in ≥2 replicates to be graphed. (D) CloneSeq results from LCLs treated with puromycin on the indicated variant. Median levels of each transcript are displayed. Elevated levels of the Δ23 transcript were seen with all 4 methods. All experiments were performed in triplicate and were highly reproducible.

RECLASSIFICATION TO VLB USING CLONESEQ

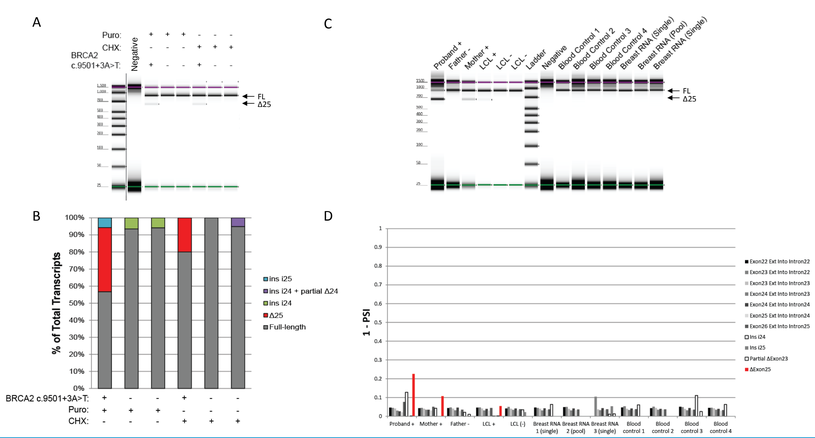
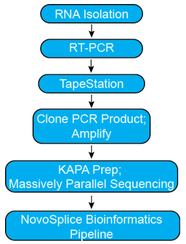


Figure 2. Splicing alterations observed in the benign variant BRCA2 9501+3A>T. LCLs with variant and controls were treated with puromycin (puro) or cycloheximide (CHX). TapeStation analysis (A) and Sanger sequencing of subcloned transcripts (B) identified very low levels of Δ25 in positive samples. (C) RT-PCR analysis was performed on patients that were positive for BRCA2 9501+3A>T (proband, mother, and LCL) and negative controls (father, LCL, normal breast RNA, and 4 normal blood controls). PCR products were analyzed using TapeStation. (D) CloneSeq results from RT-PCRs shown in (C). Samples that were positive for the variant had insignificant levels of the Δ25 transcript.

METHODS



The ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) consortium recommends capillary electrophoresis and Sanger sequencing of subcloned transcripts as the gold standard to identify the *BRCA1/2* transcripts that are present in patient samples[3]. We compared our capillary electrophoresis and Sanger sequencing results to our newly developed CloneSeq assay, which was designed to perform quantitative and qualitative characterization of splicing VUS. For this assay, we performed RT-PCR using primers designed at least 1 exon away from the variant of interest. PCR products were cloned into *E. coli*. Full plates with bacteria colonies were scraped and pooled together for plasmid DNA extraction. NGS libraries were constructed from the plasmid preps and run on the Illumina platform. Results were analyzed using our custom in-house bioinformatics pipeline to identify splicing events including exon skipping, novel intron insertion, and alternative 5′ donor and 3′ acceptor splicing sites.

RECLASSIFICATION TO VLP USING CLONESEQ

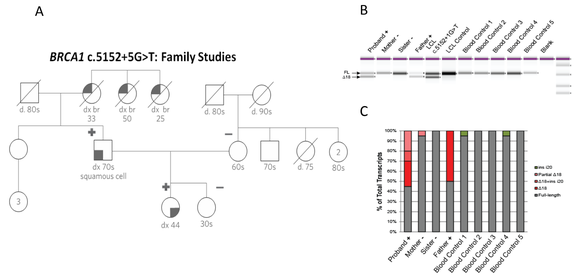


Figure 3. Data leading to reclassification of the previously uncharacterized VUS BRCA1 c.5152+5G>T. (A) Pedigree of HBOC family with variant. (B) TapeStation analysis identified a band corresponding to Δ18 in positive samples and not controls. (C) Sanger sequencing of subcloned transcripts identified high levels Δ18 in positive samples. (D) Using CloneSeq, we detected and quantified the Δ18 abnormal transcript in heterozygous individuals, in addition to identifying other alternatively spliced transcripts found both in the heterozygous and wild type controls. Median transcript levels calculated from triplicate experiments are displayed.

TAKE–HOME POINTS

- CloneSeq is a fast and reliable alternative to the gold-standard splicing assays, thereby reducing the rate of uncertain variants detected on clinical genomic tests.
- CloneSeq accurately classifies splicing variants as benign or pathogenic, allowing more patients to choose whether to undertake preventive measures, targeted treatment, or pre-symptomatic screening programs.

REFERENCES

1. Utilization of multigene panels in hereditary cancer predisposition testing: analysis of more than 2,000 patients. LaDuca H, et al. Genet Med. 2014 Nov;16(11):830-7.
2. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Richards S et al. Genet Med. 2015 May;17(5):405-24.
3. Comparison of mRNA splicing assay protocols across multiple laboratories: recommendations for best practice in standardize clinical testing. Whiley PJ, et al. Clinical Chemistry 2014; 60:(2) 341–352.

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