

RNA FUNCTIONAL STUDIES FOR THE CLASSIFICATION OF GERMLINE VARIANTS OF UNCERTAIN SIGNIFICANCE THAT MAY IMPAIR SPLICING

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BACKGROUND

- ▶ RNA splicing is the process by which noncoding intronic regions of a gene are removed after transcription.
- ▶ Genetic variants within introns may impair splicing, resulting in an RNA molecule that does not encode a functional protein.
- ▶ Depending on the sequence context, certain positions within an intron or exon cannot tolerate changes without functional splicing consequences, while other positions can tolerate a variety of genetic variation without impairing splicing.
- ▶ In these studies, we established an IRB approved research process to enroll and collect samples from selected patients after clinical testing has been performed.
- ▶ Using the collected sample, we generated and interpreted functional RNA splicing data to aid in the reclassification of the variant.

METHODS

- ▶ RNA is extracted from blood samples acquired from a patient(s) carrying a variant of interest.
- ▶ cDNA is synthesized and RT-PCR is performed, amplifying regions within the gene to be assessed.
- ▶ Splicing patterns are visualized on an agarose gel, and splicing products are identified via sequencing.
- ▶ The wild-type (WT) splicing pattern is confirmed in age/gender matched blood derived control samples, and preferably also within RNA extracted from relevant normal tissue (i.e. breast, ovarian, colon, etc.).
- ▶ Further analysis is performed to determine whether the allele carrying the variants of interest can produce any wild type splice product.

RESULTS

▶ We analyzed multiple variants within genes in which pathogenic variants cause an increased cancer risk.

CASE STUDY 1. *MLH1* c.306G>T

Figure 1. RNA Analysis. (A) Gel and (B) sequence evidence of *MLH1* c.306G>T splicing defect. The red line denotes the end of exon 3.

- One patient carried two variants in *MLH1*, c.303T>G and c.306G>T, which were classified as Benign and Uncertain, respectively.
- c.306G>T is the last base of exon 3 and is predicted to impair splicing.
- We observed an aberrant splicing product skipping exon 3 only in the patient sample, but not in RNA from normal colon tissue or RNA from blood from 5 age/gender matched controls (Figure 1A).
- We observed that the WT splice product from the patient sample was only produced from a single allele with the c.303T>G informative polymorphism. Sanger sequencing confirmed that the allele containing c.306G>T variant allele did not produce any WT splice product (Figure 1B), and that the c.303T>G and c.306G>T variants are located *in trans*.
- c.306G>T was reclassified as Likely Pathogenic based on this functional data as well as splicing prediction (Sahashi et al, *Nucleic Acids Res.* 2007; 35:5995).

A

B

CASE STUDY 2. *CDH1* c.715G>A

Figure 2. RNA Analysis (A) Gel and (B) sequence evidence of *CDH1* c.715G>A splicing defect.

- One patient carried the variant *CDH1* c.715G>A, which was classified as Uncertain.
- c.715G>A is 28 bases into exon 6 and is predicted to create a cryptic splice acceptor, which would create an out-of-frame mRNA if used.
- We observed an aberrant splicing product showing usage of the cryptic acceptor only in the patient sample, but not in RNA from blood in 5 age/gender matched controls (Figure 2A).
- However, the WT splice product from the patient sample was observed to be produced by both the WT allele, and the allele containing the c.715G>A variant (see Figure 2B for representative digital PCR sequencing traces).
- Because the mutant allele can produce a WT splice product, c.715G>A remained classified as Uncertain.

A

B

DISCUSSION

- ▶ These studies demonstrate that precise and specific functional RNA studies are helpful to differentiate variants that fully impair splicing and those that only partially impair splicing.
- ▶ Variants that fully impair splicing without contributing to the WT splice product can be reclassified, while variants with intermediate splicing defects would be considered variants of uncertain significance. Such variants may require additional data to determine if the splicing defect is sufficient to cause increased cancer risk.