A Plot Twist: When RNA Evidence Challenges Our Expectations Of DNA Results

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Background:
Paired DNA and RNA testing (DGT-RGT) improves accuracy of DNA results by detecting spliceogenic variants that reside outside of standard next generation sequence (NGS) capture and by providing a functional line of evidence in variant classification. An additional benefit of DGT-RGT is the identification of variants that cause unanticipated or unconventional splicing events. Here we present a variant-level case series highlighting unexpected RNA findings identified at one clinical diagnostic laboratory through DGT-RGT.

Variant Presentation:
Variant 1-NF1 c.888+2T>C impacts a canonical position within a splice donor site and thereby would be classified as likely pathogenic (LP) per current ACMG guidelines. Recent studies have shown that T>C substitutions at the +2 position are capable of generating wild-type transcripts in some genomic contexts. DGT-RGT did not identify significant abnormal splicing associated with this variant, consistent with a lack of neurofibromatosis in the carrier.

Variant 2- BRIP1 c.727A>G (p.I243V) is a mid-exonic missense change for which *in silico* splice site algorithms predicted the creation of a strong *de novo* donor site. RNA studies confirmed the use of this novel donor site, but unexpectedly showed that an existing cryptic acceptor site within the exon was simultaneously activated, effectively creating a pseudo-intron within the exon.

Variants 3 & 4 NF1 c.5750-184_5750-178dupTTTCTTC and *ATM* c.3480G>T (p.V1160V) are deep intronic and synonymous mid-exonic changes, respectively. RNA testing identified abnormal transcripts arising from the use of a distant cryptic acceptor site. Both variants increase the pyrimidine content within a cryptic polypyrimidine tract upstream of the cryptic acceptor. Poly-pyrimidine tracts are important components in acceptor splice site recognition, yet to our knowledge, cryptic poly-pyrimidine tract activation has not been reported as a mechanism for abnormal splicing.

Variants 5 & 6-BRCA2 [c.6816_6841+1534del1560; c.6762delT] and *APC* c.1042C>T (p.R3248*) are anticipated to result in nonsense-mediated decay (NMD) due to premature termination codons (PTCs) and therefore would be classified as pathogenic according to ACMG guidelines. However, RNA testing showed that these variants caused in-frame abnormal splicing events that removed the PTCs, a finding that was consistent with the absence of the associated gene-disease phenotype in the carriers.

Variant 7- LZTR1 c.2232G>A (p.A744A) is a high-frequency synonymous exonic variant located downstream of an intron that is spliced via the uncommon U12 spliceosome. *In silico* splicing algorithms predict the creation of a novel U2 acceptor site. RNA testing indicated that the novel U2 acceptor site was frequently utilized in conjunction with an existing, upstream, cryptic U2 donor site but only in some individuals. Other probands with low-level abnormal splicing were homozygous for a common polymorphism that weakens the cryptic U2 donor site. To our knowledge, this is the first example of a single nucleotide change influencing the U2/U12-identity of an intron and it also exemplifies the individual variability within the transcriptome.

Conclusions:

Our variant series demonstrates the utility of paired DGT-RGT in the discovery of variants that have unanticipated outcomes. Several examples demonstrate how, in the absence of RNA data, *a priori* classifications using ACMG rules can result in the misinterpretation of variants as clinically actionable potentially leading patients to seek unnecessary clinical interventions. Furthermore, this series underscores the intricacies of RNA splicing and how *in silico* splicing algorithms cannot always accurately predict the transcripts that arise from spliceogenic variants. Taken together, this study demonstrates that paired DGT-RGT provides the most complete representation of a variant's splicing impact irrespective of variant type, leading to more accurate variant interpretation and underscoring the importance of paired DGT-RGT testing.