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

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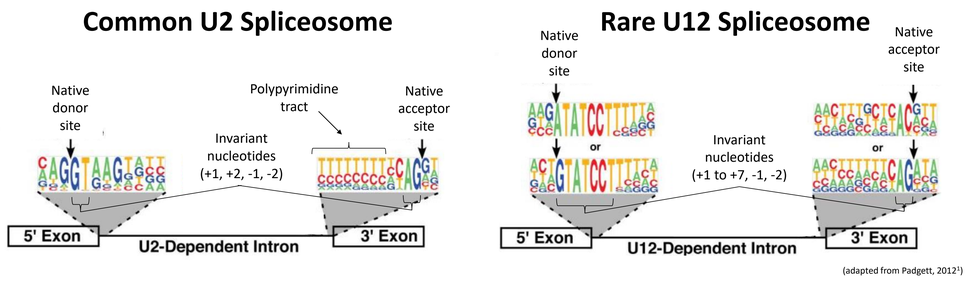
BACKGROUND

- The process of RNA splicing is characterized by a high degree of complexity and is executed by a sophisticated molecular apparatus.
- *In silico* splicing algorithms can effectively predict whether a variant weakens an original splice site or generates a novel one; however, the intricate nature of the RNA splicing means that these predictions are not *always* correct. Additionally, the ultimate consequence of splice site weakening or generation on the transcript (*i.e.*, the identity of the splice event) is even more difficult to predict.
- Analysis of canonical splice sites across many genes has led to the assumption of a high prior probability of pathogenicity for nucleotide changes at highly conserved canonical positions that does not always hold true.
- Unexpected splicing events can greatly affect the interpretation of genetic variants, particularly those that are commonly classified with a PVS1 line of evidence.
- We highlight 7 unanticipated RNA findings that were identified through the use of paired DNA and RNA testing (DGT-RGT) in a clinical diagnostic laboratory and demonstrate the importance of RNA functional data to variant interpretation.

Splicing 101



(adapted from Padgett, 2012[1])

TAKE-HOME POINTS

Our experience with DGT-RGT has demonstrated that variants can have unanticipated impacts on splicing such that in silico and functional predictions differ from observed RNA transcripts.

Paired DGT-RGT uncovers splice events that can drastically alter variant interpretation and lead to unexpected benign or pathogenic results

Canonical with No Abnormal Splicing

Alteration: *NF1* c.888+2T>C
Transcript: None above 2% PSI
Predicted protein: WT

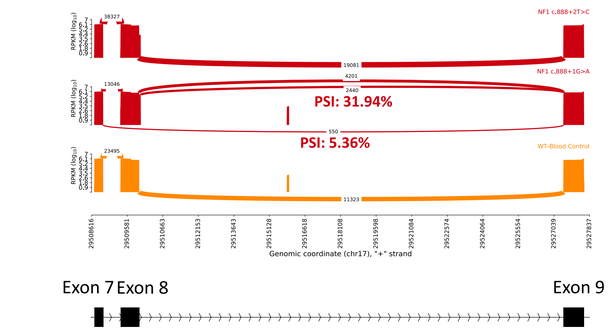


Figure 1: NF1 c.888+2T>C was identified in a woman with breast cancer diagnosed in her 40s. This individual did not report any personal or family history of neurofibromatosis. The DNA change impacts a nucleotide position within the donor site that is typically considered to be invariant. However, DGT-RGT did not detect any abnormal *NF1* transcripts above 2% PSI in the patient (top red plot) or control (bottom orange plot, although significant aberrant splicing was detected for a nearby variant (c.888+1G>A; middle red plot). Recent studies have also shown that T>C substitutions at the +2 position can generate wild-type transcripts in some genomic contexts[2]. Given the absence of detectable abnormal splicing (ACMG BS3_supporting), lack of reported neurofibromatosis phenotype in the proband, and the rarity of the variant (ACMG PM2), it was classified as a variant of uncertain significance rather than likely pathogenic.

Pseudo-intron Creation

Alteration: *BRIP1* c.727A>G (p.I243V)
Novel donor Ggtacccc
+ Cryptic acceptor 1 r.727_833del
Predicted protein: p.I243Qfs*9
+ Cryptic acceptor 2 r.727_836del
Predicted protein: p.I243Gfs*8
+ Native acceptor (exon 7) r.727_918del
PSI= 10.4% (not visible in Sashimi)
Predicted protein: p.I243_N306del

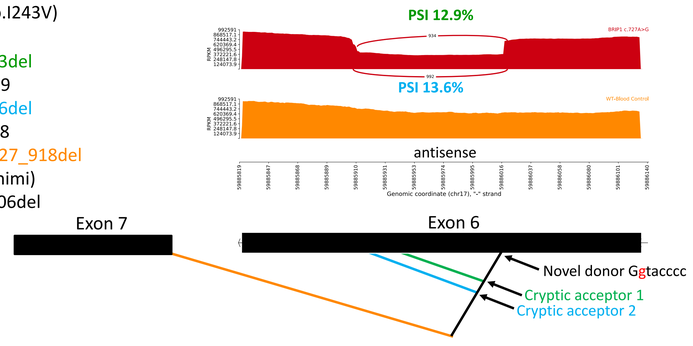


Figure 2: *BRIP1* c.727A>G (p.I243V) was identified in a woman with a personal and family history of ovarian cancer who was also heterozygous for a nonsense mutation in *BRCA1*. The c.727A>G variant creates a novel donor site in exon 6 that is paired with the native acceptor site for exon 7 or one of two cryptic acceptor sites within exon 6 to generate three different partial exon skipping events. Pairing with either of the cryptic acceptor sites creates a pseudo-intron within exon 6 (red plot). Given the substantial splice defect (ACMG Code PS3) and the rarity of the variant (ACMG PM2), the variant was classified as likely pathogenic.

Variable Spliceosome Impact

A.
Alteration: *LZTR1* c.2232G>A p.A744A
Transcript: *LZTR1* r.2220_2233delins2219+1_2219+61
Predicted protein: p.Y741H*42

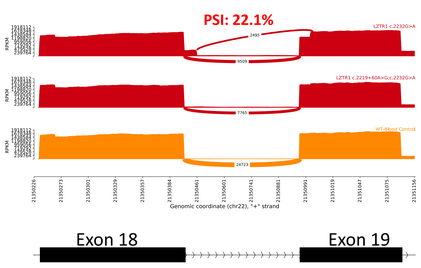
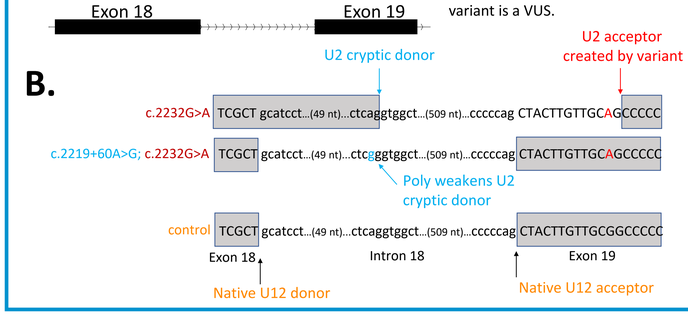


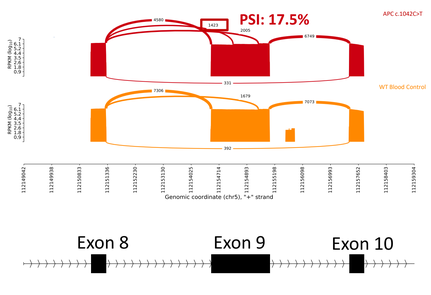
Figure 3. *LZTR1* intron 18 is spliced via the uncommon U12 spliceosome. **A)** The c.2232G>A variant creates a novel U2 acceptor site in exon 19 that is unexpectedly utilized in some carriers in conjunction with an upstream cryptic U2 donor site (top red plot). In other carriers, utilization of the novel U2 acceptor site is exceedingly rare (middle red plot). **B)** The difference is due to an *in cis* polymorphism (c.2219+60A>G) present in some carriers that is predicted to weaken the cryptic U2 donor site. Given the incomplete nature of the splice impact and lack of informative clinical data, this variant is a VUS.

B.



Nonsense-Mediated mRNA Decay Rescue

A.
Alteration: *APC* c.1042C>T, p.R348*
Transcript: *APC* r.934_1074del141
Predicted protein: p.V312_Q358del47



A) *APC* c.1042C>T, p.E348* has been identified in multiple probands without histories of polyposis or other APC-associated phenotypes. In addition to generating a premature termination codon (PTC), this variant was predicted to disrupt splicing through the strengthening of a cryptic acceptor site. DGT-RGT revealed the underrepresentation of the variant allele in normally spliced reads and the presence of an in-frame transcript that skipped the PTC in the patient sample (top red plot) that was absent in the control (bottom orange plot). Two naturally occurring, alternative splice isoforms are expressed at similar levels in both samples. As a result of the observed potential rescue mechanism, this alteration was deemed ineligible for PVS1 weight and is classified as a variant of uncertain significance.

B.
Alterations: *BRCA2* c.6762delT; c.6816_6841+1534del1560
Transcript: *BRCA2* r.6762delU; r.6816_6841del26 (27 deleted nt total)
Predicted protein: p.F2254_V2280delinsLHVPKMRKWFCQIQELEK

Native Exon 10/11 Sequence:

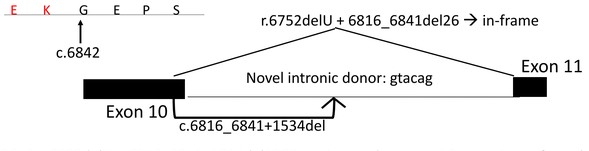
c.6762delT
ACA CAT TCT CTT TTT ACA TGT CCC GAA AAT GAG GAA ATG GTT TTG TCA AAT TCA AGA ATT
T H S L F T C P E N E E M V L S N S R I

GGA AAA AGA GGA GAG CCC CTT ATC TTA GTG GGA GAA CCC TCA . . . .
G K R R G E P L I L V G E P S
c.6816_6841+1534del

Exon 10/11 Sequence with frameshift and deletion:

ACA CAT TCT CTT TTA CAT GTC CCG AAA ATG AGG AAA TGG TTT TGT CAA ATT CAA GAA TTG
T H S L L H V P K M R K W F C Q I Q E L

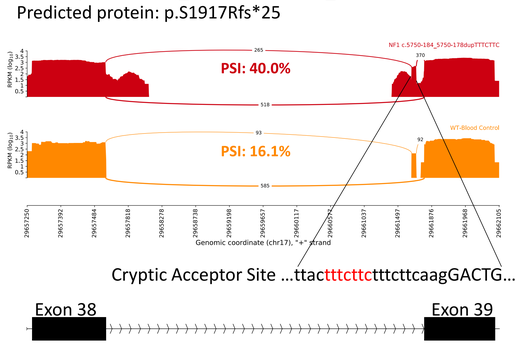
GAA AAA GGA GAA CCC TCA . . .
E K G E P S
c.6842



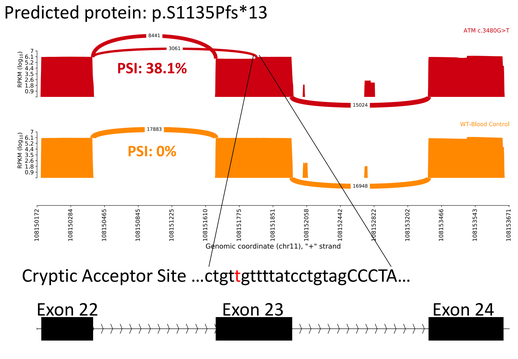
B) *BRCA2* c.6762delT; c.6816_6841+1534del1560 are two coding exon 10 variants confirmed to be in *cis* in two female probands. The first proband was unaffected in her 50s. The second proband was unaffected in her 30s; her sister (30s) and mother (60s) were also reported to be positive and unaffected. The deletion (blue text) removes the canonical donor site of exon 10 and creates a novel intronic donor at the deletion breakpoint (c.6841+1535). The novel donor, in combination with c.6762delT (red text), results in a transcript with a restored reading frame at c.6842 (green text). Based on DGT-RGT, when *in cis*, these alterations are not eligible for application of PVS1 and are classified as a variant of uncertain significance.

Cryptic Pyrimidine Tract Modifications

A.
Alteration: *NF1* c.5750-184_5750-178dupTTTCTTC
Transcript: *NF1* r.574_5750ins5750-174_5750-108
Predicted protein: p.S1917Rfs*25



B.
Alteration: *ATM* c.3480G>T (p.V1160V)
Transcript: *ATM* r.3403_3494del92
Predicted protein: p.S1135Pfs*13



A) *NF1* c.5750-184_5750-178dupTTTCTTC was identified in a child with multiple Café au Lait spots, suspected subcutaneous neurofibromas, axillary freckles and macrocephaly who was negative for alterations in *NF1* and *SPRED1* on previous DNA-only genetic testing. DGT-RGT revealed the presence of an NMD-prone transcript at higher levels in the case (40.0% PSI, top red plot) than in the control (16.1% PSI, bottom orange plot). The corresponding DNA change duplicates seven nucleotides (red text), likely improving the pyrimidine content of the poly-pyrimidine tract associated with a cryptic donor site. Given the substantial splice defect (ACMG PS3), the rarity of the variant (ACMG PM2) and corresponding clinical features of the patient (ACMG PS4), this variant was classified as a pathogenic mutation. **B)** The *ATM* c.3480G>T (p.V1160V) was detected in two individuals, one with a personal history of endometrial cancer and a paternal family history of ovarian cancer; and the other with a personal history of ovarian cancer with no family history provided. RNA analysis identified the presence of an NMD-prone transcript at 30% PSI in the proband (top red plot) that was absent in the control (bottom orange plot). The variant altered a G to a T residue (red text) within the putative poly-pyrimidine tract of a cryptic acceptor site, activating that acceptor site in the proband. Although there is a substantial splice defect (ACMG PS3) and the variant is rare (ACMG PM2), due to the lack of additional evidence and the incomplete nature of the splice defect, it remains a variant of uncertain significance.

REFERENCES

1. Padgett RA. *Trends Genet*. 2012 28(4):147-54
2. Lin JH et al. *Hum Mutat.* 2019 10;40:1856-1873
3. Richards S et al. *Genet Med*. 2015 May;17(5):405-24

METHODS

- RNA results are prospectively curated in cases with paired DGT-RGT and unique or challenging cases are marked for additional technical and clinical review.
- Follow-up studies are performed as necessary and variant classification is based on ACMG-AMP guidelines[3].