Challenges in Interpretation of RNA-Seq Data Limit Variant Reclassification

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Introduction

- RNA sequence analysis (RNA-seq) provides a powerful companion approach to DNA sequencing test menu in molecular diagnosis of rare inherited disorders.
- With case series that illustrate success and challenges, we will discuss utility of RNA-seq towards reclassification of variants of uncertain significance (VOUSs).

Methods

- RNA-seq testing was offered for genes with sufficient expression in one of three validated tissue types: blood, skeletal muscle or skin fibroblasts.
- Next Generation Sequencing (NGS) was performed on an Illumina instrument using TruSeq® Stranded Total RNA library (Illumina).
- The sequencing data were aligned with HISAT2® and quantified using HTseq®.
- Using the counts associated with each gene we compared the test sample with the set of tissue-specific reference samples to determine changes in expression levels or changes in splicing patterns.

Figure 1. Bioinformatics pipeline to create files for the assessment of splice junction patterns and cumulative gene expression levels.

Intended Use

Detection of significant aberrant splicing patterns and/or expression level changes relative to tissue-matched controls.

- providing supporting evidence that:
  - can be helpful in variant reassessment/reclassification with an updated DNA test result.
  - may suggest the presence of a possible deep intronic cryptic variant (to be confirmed by DNA sequencing).
  - implicate the gene and its associated disease for the patient.
- RNA-Seq tests are NOT intended to
  - identify sequence variants.
  - reclassify variants within RNA-Seq report.

Case 1

- Aberrant splicing in approximately half of sequenced transcripts attributed to a heterozygous variant in *TSC2*.
  - Approximately half of transcripts show inclusion of part of intron 15 with a novel aberrant splice donor site, predicted to insert 10 aberrant amino acids with a premature stop codon leading to a loss of distal 25 exons.

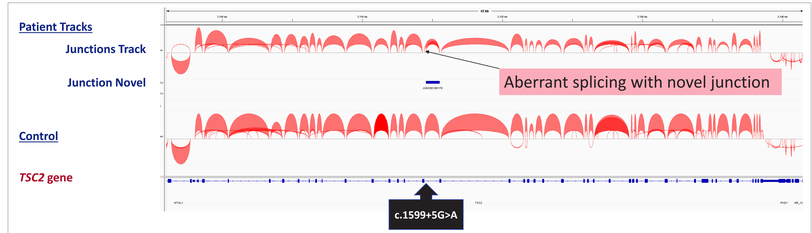

Figure 2. RNA-Seq data showed significant (~50%) aberrant splicing due to the heterozygous c.1599+5G>A variant near exon 15/intron 15 boundary of *TSC2*.

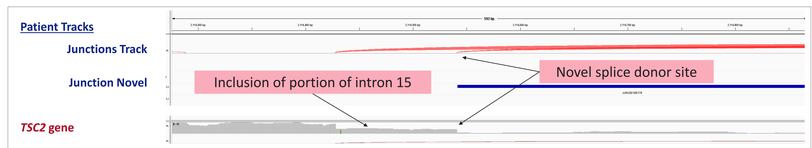

Figure 3. Zoomed-in view showing significant aberrant splice junctions, attributed to the heterozygous c.1599+5G>A variant in *TSC2*.

Case 2

- Both normal (canonical) and abnormal (aberrant) splicing due to a hemizygous variant.
  - intron 2 inclusion indicating a partially leaky splice site.
  - skipping of exon 2 that is predicted to lead to an in-frame deletion of 33 amino acids (p.Pro8_Gln40del).
  - creation of a novel GT-donor site at the site of the c.120+5G>T variant that is predicted to lead to an in-frame insertion of a Valine (p.Gln40_Leu41insVal).
  - canonical junctions for exons 1-2 and exons 2-3 were observed, comprising approximately half of the total.

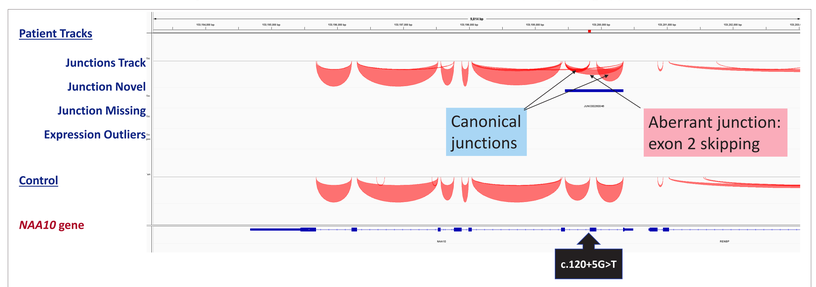

Figure 4. RNA sequencing data showed partial aberrant splicing due to the hemizygous c.120+5G>T variant near exon2/intron 2 boundary of *NAA10*.

Case 3

- Approximately 7% of reads show aberrant splicing at exons near heterozygous variant in *COL5A1*.
  - Junctions skipping exon 56.
  - Aberrant splice acceptor site within exon 56.
  - Majority of junctions are normal (canonical).

Figure 5. RNA-Seq analysis did not make any calls for aberrant splicing at or around heterozygous c.4339-3C>G variant at the intron 55/exon 56 boundary of *COL5A1*.

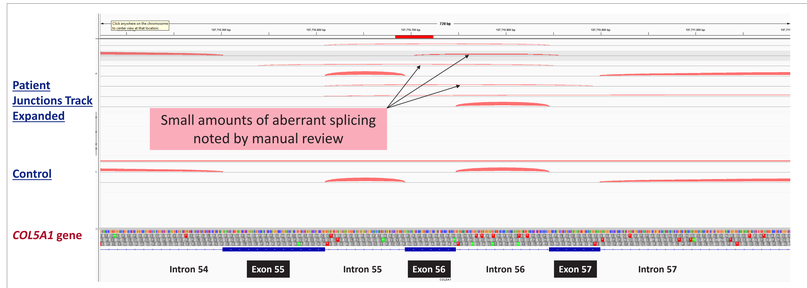

Figure 6. Zoomed-in view showing minor amounts of aberrant splice junctions, noted upon manual review, attributed to the heterozygous c.4339-3C>G variant in *COL5A1*.

Conclusions

- Detection of significant aberrant splicing patterns and/or cumulative expression level changes relative to tissue matched controls can lead to reclassification of VOUSs.
- The best utility of RNA-Seq tests is when there is adequate expression of the gene/s of interest in a validated tissue type (blood, skeletal muscle or skin fibroblasts) and the results from prior DNA sequence analysis are provided.
- If the variant-of-interest is hemizygous or homozygous, it allows easier interpretation of the results due to the absence of interference from a normal allele. Typical positive results show aberrant splicing in approximately half of the reads at or across the region of a heterozygous variant-of-interest.
- In approximately 20% of tested samples, RNA-Seq provided a positive result showing significant aberrant splice pattern or altered cumulative expression level compared with tissue-matched controls.
- Due to the complexity of the data, indeterminate results are equally common (approximately 20% of tested samples) and consistent with partial defects. The clinical significance of those variants in question remains uncertain.

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