Title: Splicing up the Diagnosis: Using Targeted RNA Studies to Reclassify Exome Sequencing Variants

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RNA analysis has emerged as a valuable tool for providing lines of evidence about the classification of putative splicing variants, and growing evidence suggest that transcriptome improves exome sequencing (ES) diagnostic yield. However, for a variety of reasons, whole transcriptome is not yet feasible for all exome patients. In the absence of transcriptome, targeted RNA studies can provide many of the same benefits. Given the broad clinical indications for ES cases and limitations to the types of variants which will benefit from RNA studies, it is important to carefully select which variants should undergo targeted RNA studies to maximize the efficiency and efficacy of such studies.

Here, we summarize our laboratory's criteria for assessing variant eligibility for RNA studies and describe a retrospective case series of ES probands that have benefited from RNA analysis over 5 years.

To maximize the yield of our targeted RNA testing, only variants in genes with characterized disease associations that have sufficient clinical overlap with the proband's phenotype are considered for RNA studies. We also require that variants are reasonably expected to have an impact on splicing based on *in silico* predictors and that the gene of interest is expressed well in the available sample type. Finally, the mechanism of disease (MOD) for the gene-disease relationship should be considered. Splicing impacts are typically more consistent with loss of function MODs, but there are rare exceptions.

The Ambry Translational Genomics (ATG) Lab used targeted RNA studies to generate evidence that led to the reclassification of nine ES cases between 2017 and 2021. Prior to RNA study completion, all nine patients remained undiagnosed after extensive testing, including karyotype (5/9), microarray (8/9), Fragile X (7/9), methylation studies (1/9), and multigene panel testing (5/9). In all nine cases, reclassifications based on RNA evidence resulted in an ES variant of uncertain significance (VUS) upgrade to a likely pathogenic/pathogenic. Most cases (6/9) had a neurodevelopmental phenotype, two had a neuromuscular phenotype, and one had multiple congenital anomalies. Impacted genes were as follows: *FOXP1* (x2), *ANKRD11, TNTT1, ADNP*, *FMR1, TRIP12, PTEN*, and *SPG11*.

Targeted RNA studies are a practical way to generate the evidence required for ES VUS resolution of carefully selected variants in the absence of transcriptome analysis. Data collected by targeted RNA studies will provide valuable insight for future validation and interpretation of transcriptome data.