

# TITLE: RNA and Reclassification: Assessing RNA Data in Rare Disease

## AUTHORS

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## INTRODUCTION

The diagnostic yield of clinical exome sequencing (ES) for patients with rare disease continues to improve due to the addition of complementary methods, including RNA analysis. When paired with ES, RNA analysis of putative spliceogenic variants provides insight into their functional impact. While demonstration of aberrant splicing is a crucial component of RNA analysis, the interpretation of RNA data is complex and requires expertise to appropriately weigh this line of evidence as part of overall variant classification. Here we present two cases that underwent ES and RNA analysis, both with significant aberrant splicing demonstrated, but with different variant classification outcomes.

## METHODS

ES identified variants with predicted splice impacts for two patients with rare syndromic neurodevelopmental disorders: homozygous *ATP6V0A2* c.2055+4A>C in case 1 and heterozygous *CNOT3* c.387+5G>A in case 2. Biallelic variants in *ATP6V0A2* are associated with autosomal recessive cutis laxa type II, characterized by redundant skin, developmental delay, abnormal growth, and dysmorphic features. *De novo* heterozygous variants in *CNOT3* are associated with a syndromic intellectual disability disorder, including speech delay, autism, and dysmorphic features. Both variants were classified as variants of unknown significance (VUS) according to ACMG criteria in the absence of RNA studies. Targeted RT-PCRseq was performed on whole blood to determine the splicing impact of the variants. The resulting RNA data was evaluated for the magnitude, specificity, and reproducibility of the splice impact as well as the presumed impact on the clinically relevant protein function, based on the recommendations from the ClinGen SVI splicing subgroup.

## RESULTS

RT-PCRseq demonstrated an aberrant splicing impact for the variants in both cases that was absent in controls. In case 1, skipping of *ATP6V0A2* exon 16 was observed in 99.03% (10,069/10,168) of transcripts. In case 2, skipping of *CNOT3* exon 5 was observed in 49.82% (18,399/36,931) of transcripts. In both cases, the aberrant splice effect generated in-frame transcripts that are expected to escape nonsense-mediated decay. After accounting for the zygosity

of the variants, the magnitude, specificity, and reproducibility of the splicing impacts were evaluated as similarly significant in both cases. However, the interpretation of the resulting protein impact differed for the two cases. The in-frame transcript associated with the *ATP6V0A2* variant in case 1 is expected to be deleterious because germline deletion of exon 16 is a recurrent pathogenic variant, adding additional evidence of pathogenicity. Due to uncertainty about the effect of exon 5 skipping on *CNOT3* protein function, the aberrant splice effect in this case did not provide further evidence for pathogenicity. The interpretation of *CNOT3* exon 5 skipping may change as we learn more about its protein structure and function. When the RNA data is evaluated alongside other relevant lines of evidence for variant classification, there is enough support to classify the *ATP6V0A2* variant as likely pathogenic, while the *CNOT3* variant remains classified as a VUS.

## CONCLUSIONS

Due to the complexity of RNA data, careful consideration of the splicing impact on the protein is essential for accurate variant classification. While confirming aberrant splicing can clarify variant impact and often leads to an upgraded classification, it represents only one line of evidence. Splicing impacts may not always be pathogenic, and confirmation of a splice impact may not always be sufficient to reclassify a variant in the absence of other lines of evidence. However, even in such cases, adding supportive functional evidence enhances the potential for future variant reclassification as additional evidence emerges. Overall, RNA analysis in combination with ES offers remarkable potential for resolving rare disease VUS and increasing diagnostic yield.