Utility of Targeted RNA Analysis in Neurological Disorders

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Introduction: Clinical genetic testing for neurological disorders is a powerful diagnostic tool, and exome sequencing is recommended as a first-tier genetic test for many neurodevelopmental indications. However, its clinical utility is diminished by the large number of variants of unknown significance (VUS) detected in patients. In particular, interpreting variants with potential splicing impact poses a significant challenge. While *in silico* predictive tools, like SpliceAI, have improved over the years, accurate classification of potential splicing variants still requires a demonstrated effect on splicing. While RNA studies have proven successful in clarifying VUS in oncology diagnostics by characterizing aberrant splicing patterns, their applicability in neurological disorders remains uncertain. One major challenge for RNA studies for neurological conditions is the lack of accessibility to disease-relevant tissues for analysis. In this study, we sought to determine whether targeted RNA analysis can effectively reclassify variants in genes associated with neurological disorders.

Methods: Variants that might affect splicing based on location and/or *in silico* predictions (30 VUS and 2 likely pathogenic) were identified in patients who underwent neurology genetic testing (single gene, multi-gene panel, or exome). Variants detected in genes expressed in blood or bone marrow were selected for RNA analysis. RNA extracted from whole blood of the patients and healthy controls underwent RT-PCR (deep) sequencing. To quantify the potential impact of RNA studies in genes with established neurological disease associations, we reviewed the whole blood expression levels, based on the GTEx Portal, for 207 genes commonly ordered for neurodevelopmental clinical genetic testing. A value of >1 transcript per million (TPM) in bulk tissue gene expression data for whole blood was used to determine sufficient expression for RNA studies eligibility.

Results: Based on splicing events and impacts observed in RNA, 91% (29/32) of the variants changed classification. Sixteen VUS were upgraded to pathogenic or likely pathogenic and one likely pathogenic was upgraded to pathogenic, while twelve were downgraded to benign or likely benign. Those variants that became clinically actionable (i.e., VUS to pathogenic or likely pathogenic) included:

- Seven single nucleotide substitutions in an intron (ATRX c.4957-3A>G, DEPDC5 c.3155+5G>A, FOXP1 c.1652+5G>C, PTEN c.210-12C>G, SPG11 c.5866+5G>C, TNNT1 c.32+5G>A, and TRIP12 c.4190+5G>A)
- Four alterations affecting guanine at the last nucleotide of an exon (ADNP c.201G>C, ANKRD11 c.226G>A, NF1 c.586G>A, and TSC1 c.2041G>A)
- Two small deletions in an intron (FMR1 c.104+3_104+6delAAGT and WDR45 c.976+5_976+10delGTGGGA)
- One duplication encompassing exon-intron boundary (FOXP1 c.1531-9_1534dup13)
- One first nucleotide of an exon (NF1 c.6757G>T)
- One mid-exonic alteration (NF1 c.6280C>A)

Of the three variants that did not change classification after RNA analysis, two were missense VUS alterations that had no splicing impact but may still affect protein function, and one likely pathogenic alteration remained the same classification despite a demonstrated splicing impact due to atypical clinical presentation in the patient.

Based on gene expression data from GTEx, 71% (147/207) of commonly ordered neurodevelopmental genes were above the cut-off for expression in whole blood and would be eligible for RNA studies if an appropriate variant was detected as part of clinical testing.

Conclusion: In summary, RNA analysis provided molecular evidence supporting pathogenicity or benignity for all variants and resulted in the reclassification of over 90% of the variants. Half of the variants included in this study had clinically actionable reclassifications. Equally important were downgrades of the other variants that would have remained VUS without RNA analysis. Although RNA-based studies are limited to genes expressed in the blood or other readily obtainable tissues, our data indicates that RNA analysis can be used to clarify the pathogenicity of potential splicing variants identified in genes associated with neurological disorders.