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Title: Attacking a VUS from multiple angles: An integrated and functional approach for reclassifying variants of uncertain significance

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Abstract: Neurodevelopmental (ND) disorders involve a wide range of symptoms and severity. Individuals may present with multiple indications including epilepsy, autism, intellectual disability, and developmental delay. The nonspecific nature of ND disorders can lead to significant diagnostic challenges. Multi-gene panel testing (MGPT) can help to stratify ND disorders and identify causative variants. Though large MGPTs allow for the molecular diagnosis of many possible rare diseases, they can also result in numerous variants of uncertain significance (VUS). High VUS rates can be confusing and discouraging to both patients and clinicians. Here we describe three effective approaches for reducing VUS rates in neurology MGPTs. Combining parental co-segregation studies, protein structural analysis, and RNA functional splicing assays with additional strong lines of evidence, lead to a significant decrease in VUS rates. Parental co-segregation studies were performed following initial results of the proband's neurology MGPT. Parental samples were tested for all VUS detected in proband MGPT in autosomal dominant (AD) and X-linked (XL) genes. Of the 200 VUS submitted for parental co-segregation studies, approximately 5% (10/200) were upgraded to variant likely pathogenic (VLP), 35% (70/200) were downgraded to variant likely benign VLB, and 60% (120/200) remained VUS. These data show that testing of parental samples is informative in ~40% of VUS. Protein structural analysis can be leveraged to identify and explain the effects of variants on protein function. The structure based assessment strategy utilized for classifying variants consists of several complementary tools (Rosetta, FoldX, ELM, etc.) to address the distinct structural effects of variants. Of the 49 VUS with a structural assessment, 96% contributed to reclassification. Approximately 78% (38/49) were upgraded to likely pathogenic VLP, 18% (9/49) were downgraded to likely benign VLB, and 4% (2/49) remained VUS. RNA functional assays designed to identify alternative splicing were most impactful in downgrading VUS. 100% of splicing VUS (3/3) submitted for RNA functional studies resulted in reclassification from VUS to VLB. These data show that a comprehensive approach to variant reclassification is an effective method for reducing VUS rates in neurology MGPT.