Attacking a VUS from Multiple Angles: An Integrated Approach for Reclassifying Variants of Uncertain Significance

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BACKGROUND

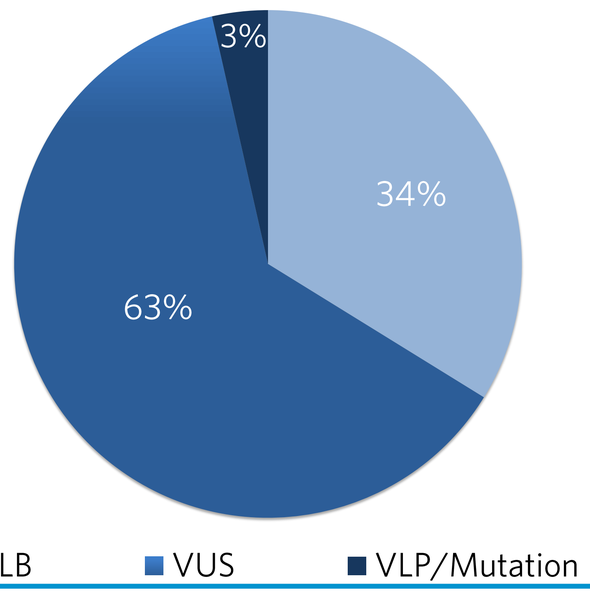
- Neurodevelopmental disorders (NDDs) involve a wide range of symptoms and severity. Individuals may present with multiple indications including epilepsy, autism, intellectual disability, developmental delay, dysmorphic features, and other congenital anomalies. The broad and nonspecific nature of NDDs can lead to significant diagnostic challenges.
- Multi-gene panel testing (MGPT) can help to stratify NDDs and identify causative variants, but 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 MGPT: parental co-segregation studies, protein structural analysis, and RNA functional splicing assays. Combining these with additional lines of evidence such as clinical data, *in silico* analysis, and population allele frequency, allows for an integrated and effective approach to variant classification.

TAKE-HOME POINTS

- An integrated approach combining parental co-segregation studies, protein structural analysis, and RNA functional splicing assays with additional strong lines of evidence resulted in a VUS reclassification rate of 42% (242/576).
- Parental co-segregation studies were performed on 423 VUS from Neurology panel genes. Parental samples were informative in 37% of variants tested.
- Of the 84 VUS receiving structural evidence, 92% contributed to reclassification.
- RNA functional splicing assays were performed on 7 VUS from Neurology panel genes. 1 (14%) variant was reclassified to VLP and 6 (86%) were downgraded to VLB.

Parental Co-Segregation Studies

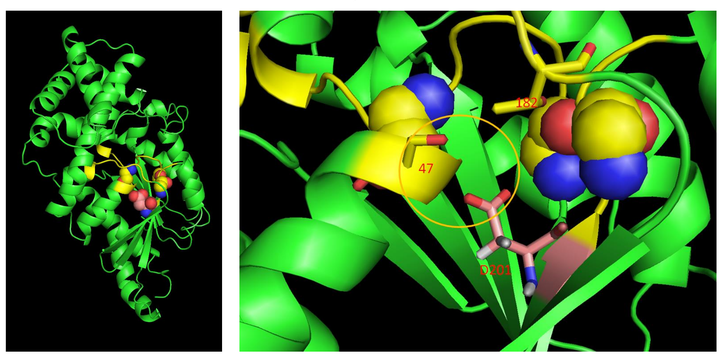
- Parental co-segregation studies were performed on 423 VUS from Neurology panel genes. Parental samples were informative in 37% of variants tested.
- Parental samples are tested for all VUS detected in proband Neurology MGPT in AD and X-linked genes. VUS in AR genes are tested when detected with second VLP/Mutation in same gene.
- Parental samples were informative in 37% of variants:
  - 15 (3%) variants upgraded to VLP/Mutation
  - 143 (34%) variants downgraded to VLB
  - 265 (63%) variants remained VUS
- 20/423 (5%) VUS were determined to be *de novo*. 13 (65%) were reclassified to VLP/Mutation and 7 (35%) remained VUS.



De Novo Variants

Gene	Alteration c.	p.	Classification
OFD1	c.655-2A>G		Mutation
ATP1A2	c.2810G>A	p.R937H	VLP
CHD2	c.3221G>A	p.R1074Q	VLP
CREBBP	c.4493G>A	p.R1498Q	VLP
DDX3X	c.1538T>C	p.V513A	VLP
DYNC1H1	c.10051A>T	p.I3351F	VLP
EHMT1	c.2426C>G	p.P809R	VLP
KCNQ2	c.839A>G	p.Y280C	VLP
KCNQ2	c.994A>G	p.R332G	VLP
KCNQ2	c.1627G>A	p.V543M	VLP
KCNT1	c.1429G>A	p.A477T	VLP
PCDH19	c.593G>C	p.R198P	VLP
SMARCA4	c.2441C>T	p.T814M	VLP
ANKRD11	c.295G>A	p.G99S	VUS
DYNC1H1	c.568G>A	p.E190K	VUS
GRIN2B	EX2dup		VUS
KCNQ2	c.704C>T	p.A235V	VUS
PHF8	c.2405A>C	p.Q802P	VUS
PRRT2	c.640G>C	p.A214P	VUS
WDR45	c.976+5_976+10delGTGGGA		VUS

GNAO1 c.601G>T p.D201Y VUS → VLP



- D201Y is on the GNAO1 DNA binding domain shown in yellow; spheres represent nearby pathogenic variants
- The D to Y change increases local energy
- D201Y interacts metal binding residues 47 & 182
- Several nearby pathogenic variants (G40R, G45R, D174G, R209C)
- Based on the position of D210Y and energy increase, it will likely disrupt the local motif and reduce binding ability to DNA

Pathogenic Variant	
Variant	ΔΔG
D201Y	4.31
G45R (Mut)	6.98

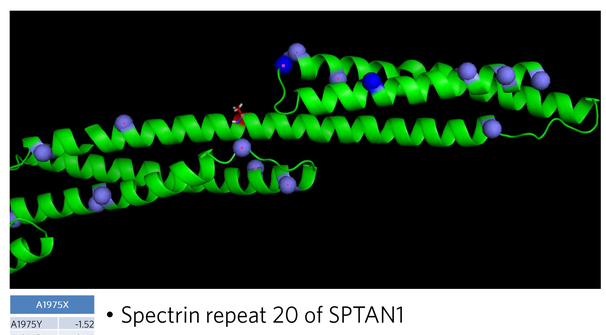
Structural Modeling Assessment

- Structural scientists leverage state-of-the art tools for protein modeling and analysis, with internal clinical data, against the vast information available in the protein databank (PDB).
- The impact of a variant on protein structure, such as structural destabilization or impaired cofactor binding is analyzed in the context of other well-established pathogenic alterations. If the variant is more destabilizing or blocks the same interaction site as other similar adjacent pathogenic variants, this can be used as evidence of pathogenicity. Similarly, comparison with the impact of adjacent benign alterations can provide evidence towards neutrality.
- 146 VUS were assessed for structural impact. 84 (58%) received structural evidence and 62 (42%) were inconclusive.
- Of the 84 VUS with structural evidence, 92% contributed to reclassification.
  - 64 (76%) variants upgraded to VLP/Mutation
  - 13 (16%) variants downgraded to VLB
  - 7 (8%) variants remained VUS

SPTAN1 c.5924C>G p.A1975G VUS → VLB

VLB	
AMBRY	
A1851V	0.66
A2051T	1.39
A2064T	2.02
ExAC	
K2045E	-1.50
G1961R	-1.26
G1793S	-0.77
G1749R	-0.75
R1849W	-0.64
S1732I	-0.56
N1883S	-0.48
T1831A	-0.32
A2050T	-0.22
A2079T	-0.17
K2005R	-0.07
S1724I	-0.06
G1842R	-0.03
E1901K	0.15
A1975V	0.21
N1951D	0.23
K2083Q	0.46
R1849Q	0.52
A1909T	0.62
A1851V	0.66
A2075T	0.72
A1851G	0.85
E1885G	0.94
R1770C	0.99
E1872G	1.02
I1747V	1.22
A2051T	1.39
R1750C	1.53

A1975X	
A1975Y	-1.52
A1975R	-0.67
A1975F	-0.58
A1975L	-0.51
A1975I	-0.45
A1975W	-0.40
A1975M	-0.36
A1975K	-0.33
A1975Q	-0.05
A1975A	0.00
A1975E	0.16
A1975N	0.17
A1975H	0.20
A1975V	0.21
A1975D	0.63
A1975C	0.66
A1975T	0.74
A1975G	0.80
A1975S	0.89
A1975P	3.87

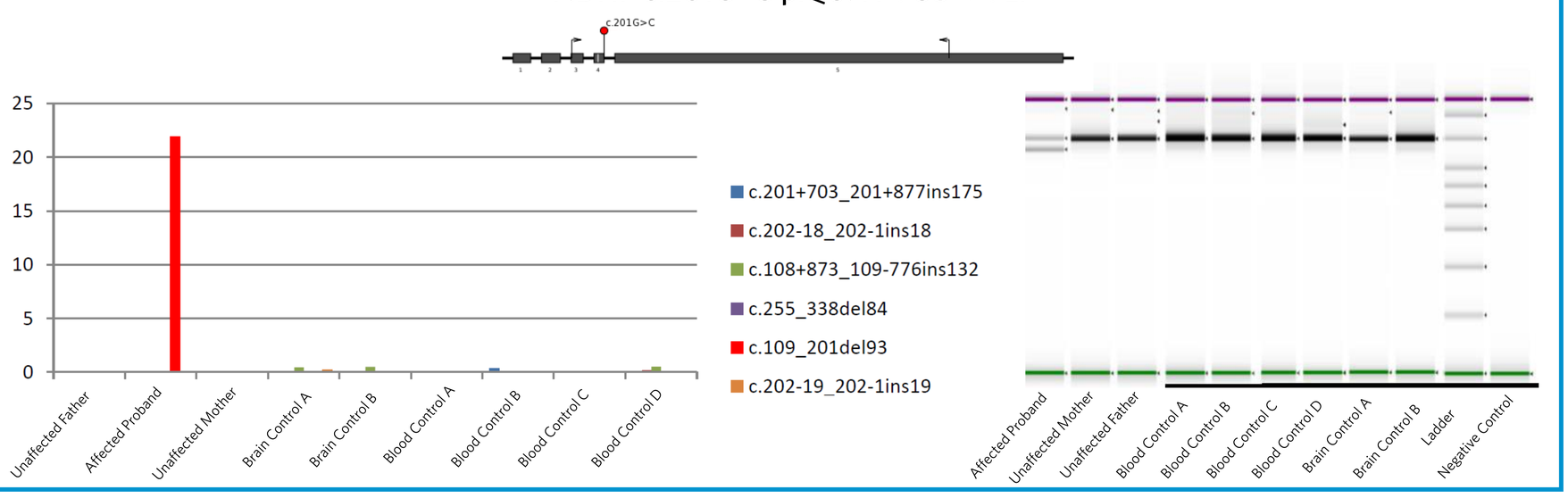


- Spectrin repeat 20 of SPTAN1
- A1975G shown in red, known VLBs in blue, high frequency ExAC variants shown in purple
- Variant lies on the surface, does not interact with other parts of repeat, is not at an interface
- Energy of destabilization is significantly less than two VLB's and numerous ExAc variants; no pathogenic variants in the region
- Not predicted by ELM to disrupt any motifs, does not appear in any motifs in PhosphoSite

RNA Functional Studies

- Ambry's Translational Genomics (ATG) laboratory performs gold-standard mRNA splicing assays, including capillary electrophoresis, sanger sequencing of subcloned transcripts, and high-throughput RNA-seq.
- RNA functional splicing assays were performed on 7 VUS from Neurology panel genes. All were reclassified.
  - 1 (14%) variant upgraded to VLP
  - 6 (86%) variants downgraded to VLB
- Interestingly, in 5/7 (71%) variants, *in silico* programs did not accurately predict splicing effects, leading to false positives/negatives.
- The *ADNP* c.201G>C variant in the adjacent example is the last nucleotide of coding exon 2 (CDS2) and found *de novo*. RNA-seq (left panel) and gel analysis (right panel) demonstrate that it leads to in-frame skipping of CDS2 in the proband (c.109_201del93).

ADNP c.201G>C p.Q67H VUS → VLP



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