Reflex RNA Sequencing for Enhanced Variant Classification on Exome Sequencing/Genome Sequencing Improves Patient Outcomes

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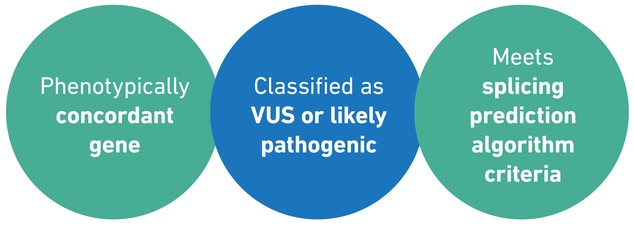
P645

INTRODUCTION

Splice variants represent a significant portion of variants of uncertain significance (VUS) in clinical exome and genome sequencing (ES/GS). However, it is challenging to confirm the impact of these variants by DNA analysis alone due to a lack of functional evidence in RNA splicing. This impacts patients with rare disease, where novel variants are more likely to be classified as VUS. To address this concern, we integrated clinically validated RNA sequencing (RNAseq) into the clinical ES/GS workflow as a reflex test to enhance classification of variants with potential impact on splicing.

METHODS

- DNA and RNA extracted from peripheral blood for clinical ES/GS cases with additional consent obtained for reflex RNAseq
- **Inclusion criteria:** variants classified as VUS or likely pathogenic, associated with patient phenotype, and predicted to affect splicing
- Reflex RNAseq performed on NovaSeq; splicing events detected via manual IGV review using Sashimi plots
- Variant reclassification based on RNAseq functional evidence using internal guidelines and ACMG criteria



RESULTS

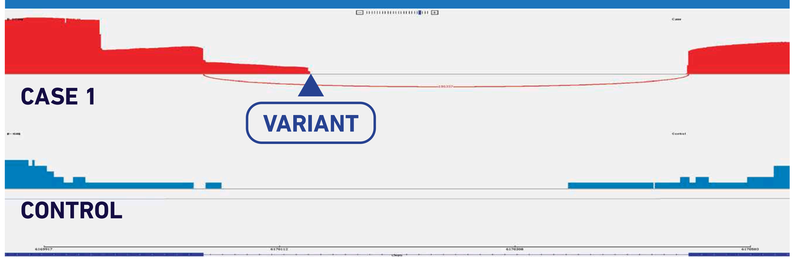
Table 1. Ten cases subjected to reflex RNAseq. 5 out of 10 variants were upgraded from VUS to likely pathogenic (5/10, 50%)

Cases	DNA Variant	TPM*	Impact on RNA	ACMG reclassification
Case 1	*CHD5* NM_015557.3:c.5383-90G>A	0.0466	Intron retention	VUS → LP
Case 2	*FOXP4* NM_001012426.2:c.1357+2T>G	2.901	Exon skipping	VUS → LP
Case 3	*PHF6* NM_032458.3:c.156G>A, p.L52=	0.9892	Exon deletion	VUS → LP
Case 4	*CRELD1* NM_015513.6:c.637+3A>T	3.486	Exon skipping	VUS → LP
Case 5	*CHD3* NM_001005271.3:c.3547+5G>C	29.11	Intron retention	VUS → LP
Case 6	*TTN* NM_133378.4:c.54812-1G>T	0.2851	n/a	VUS → VUS
Case 7	*ATP1A1* NM_000701.8:c.12+173G>T	25.94	n/a	VUS → VUS
Case 8	*TSC1* NM_000368.5:c.1264-10_1264-6del	4.246	n/a	VUS → VUS
Case 9	*OPHN1* NM_002547.3:c.385-4787A>G	0. 0765	n/a	VUS → VUS
Case 10	*TRIP12* NM_004238.3:c.3917-21A>G	25.93	n/a	VUS → VUS

*Transcripts Per Million (TPM) in whole blood

Case 1: 4-YEAR-OLD MALE

- ADHD, autistic behavior, delayed speech and language development
- Large arachnoid cyst located in the brain requiring a shunt, mild facial dysmorphism, failure to thrive
- Presented at 18 months with no prior genetic testing



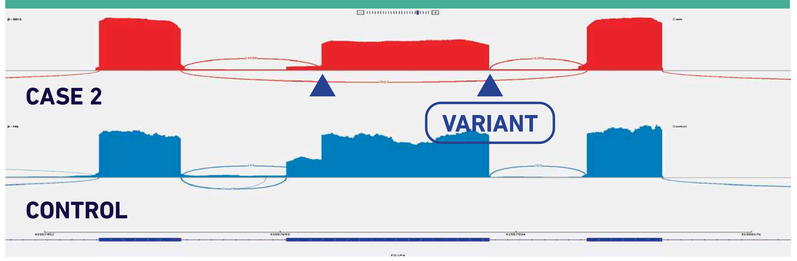
- Duo WGS: *CHD5* c.5383-90G>A, unknown inheritance. Predicted to cause acceptor gain and alter splicing.
- RNAseq: 37 nucleotide intron retention, *CHD5* r.5382_5383ins88 (p.Leu1795Glufs*38)

CLINICAL OUTCOMES

- *CHD5* is associated with autosomal dominant Parenti-Mignot neurodevelopmental syndrome (PMNDS), which includes intellectual disability, speech/motor delay, epilepsy, hypotonia, craniosynostosis, and dysmorphic features
  - The clinical team confirmed that the patient appears to have no history of seizures
- **With VUS -> LP reclassification, the patient has a molecular diagnosis and can be monitored for possible development of seizures and other PMNDS symptoms over time**
- **RNAseq provided answers within weeks that standard of care testing approaches may have taken months or years to identify**

Case 2: 5-YEAR-OLD MALE

- Delayed speech and language development, motor delay, autistic behavior, hypotonia, EEG abnormality
- Abnormal carnitine level, inflammation of the large intestine
- Multiyear diagnostic odyssey with negative CMA and NGS panel testing



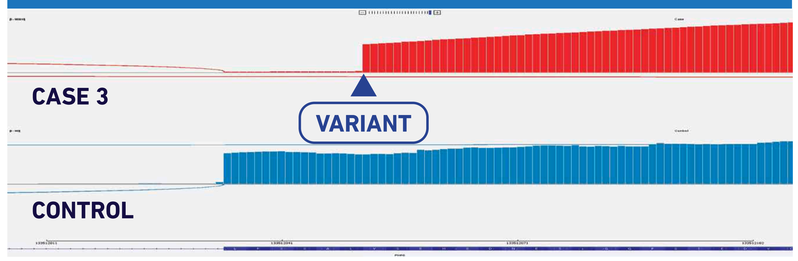
- Trio WGS: *FOXP4* c.1357+2T>G, maternally inherited. Predicted to disrupt splicing donor site.
- RNAseq: skipping of exon 11, *FOXP4*: r. 1150_1357del (p.L384Sfs*36)

CLINICAL OUTCOMES

- Several probands with an inherited *FOXP4* variant identified in the literature
  - Heterozygous *FOXP4* variants have been reported in patients with developmental disorder with language delay and congenital abnormalities
  - Evidence for incomplete penetrance/variable expressivity as parents with a variant have a mild or subclinical phenotype
- Additional information obtained from the clinical team:
  - The patient's mother had a degree of developmental and learning delay in childhood which resolved
- **With VUS -> LP reclassification, the patient can be enrolled in antisense oligonucleotide therapy**
- **RNAseq allowed for resolution of the patient's several year-long diagnostic odyssey**

Case 3: 12-YEAR-OLD MALE

- Global developmental delay, intellectual disability, hypotonia, seizures, abnormal hippocampus morphology, dysmorphic features, and more
- Family history is positive for mother with borderline intellectual disability, dysplastic moles, and hypodontia



- Duo WGS: *PHF6* c.1357+2T>G (p.L52=), maternally inherited. Synonymous variant predicted to alter splicing.
- RNAseq: partial exon 3 deletion, *PHF6*: r.139_156del (p.L47_L52del)

CLINICAL OUTCOMES

- *PHF6* is associated with X-linked Borjeson-Forssman-Lehmann (BFL) syndrome, characterized by intellectual disability, hypotonia, truncal obesity, and underdeveloped genitalia
- Some carrier females have mild symptoms of BFL syndrome as well as hypothyroidism
- **RNAseq revealed a molecular diagnosis for the patient and his mother**

Conclusions: The addition of reflex RNAseq to ES/GS enabled the robust detection of splicing variants in genes with very low-expression RNA in whole blood. This additional testing significantly enhanced the reclassification of qualified VUS and improved treatment management for the patients. These results highlight the utility of RNAseq in the rare disease setting.

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References: Truty, R., Ouyang, K., Rojahn, S., Garcia, S., Colavin, A., Hamlington, B., Freivogel, M., Nussbaum, R. L., Nykamp, K., & Aradhya, S. (2021). Spectrum of splicing variants in disease genes and the ability of RNA analysis to reduce uncertainty in clinical interpretation. *American Journal of Human Genetics, 108*(4), 696–708. https://doi.org/10.1016/j.ajhg.2021.03.006