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A Toolkit for Genetics Providers in Follow-up of Patients with Non-Diagnostic Exome Sequencing

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Abstract

There are approximately 7,000 rare diseases affecting 25–30 million Americans, with 80% estimated to have a genetic basis. This presents a challenge for genetics practitioners to determine appropriate testing, make accurate diagnoses, and conduct up-to-date patient management. Exome sequencing is a comprehensive diagnostic approach, but only 25–41% of patients receive a molecular diagnosis. The remaining three-fifths to three-quarters of patients undergoing exome

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Conflict of interest Diane B. Zastrow, Devon Bonner, Chloe Reuter, Jennefer N. Kohler, Liliana Fernandez, Megan E. Grove, Dianna G. Fisk, Yaping Yang, Christine M. Eng, Patricia A. Ward, David Bick, and Elizabeth A. Worthey declare that they have no conflict of interest. Euan A. Ashley is a founder and member of the scientific advisory board of Personalis and Deepcell. Euan A. Ashley is an advisor to Genome Medical and Sequencebio. Matthew T. Wheeler has a minor ownership interest in Personalis.

Human Studies and Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Animals Studies All institutional and national guidelines for the care and use of laboratory organisms were followed.

sequencing remain undiagnosed. The Stanford Center for Undiagnosed Diseases (CUD), a clinical site of the Undiagnosed Diseases Network, evaluates patients with undiagnosed and rare diseases using a combination of methods including exome sequencing. Frequently these patients have non-diagnostic exome sequencing results, but strategic follow up techniques identify diagnoses in a subset. We present techniques used at the CUD that can be adopted by genetics providers in clinical follow-up of cases where exome sequencing is non-diagnostic. Solved case examples illustrate different types of non-diagnostic results and the additional techniques that led to a diagnosis. Frequent approaches include segregation analysis, data reanalysis, genome sequencing, additional variant identification, careful phenotype-disease correlation, confirmatory testing, and case matching. We also discuss prioritization of cases for additional analyses.

Keywords

Exome sequencing; genome sequencing; undiagnosed diseases; sequencing reanalysis; rare diseases

Introduction

Rare disease is defined in the United States as any disease affecting less than 200,000 Americans (approximately 1 in 1,650 people; Orphan Drug Act 1983); the European Union defines rare disease as affecting fewer than 1 in 2,000 people (GARD 2017). It is estimated that there are 7,000 rare diseases affecting 25–30 million Americans (GARD 2017); a majority of these have a genetic cause. This presents a challenge for genetics practitioners to determine appropriate testing, diagnoses, and patient management. Next-generation sequencing (NGS) technology enabling clinical exome and genome sequencing has proved a powerful tool for genetics professionals in the assessment of the etiology of rare diseases. These tests allow simultaneous interrogation of nearly all genes, leading to possible diagnoses for individuals whose genetic diagnosis may be missed through targeted testing.

The clinical genetics community has embraced the power of genomic sequencing to deliver diagnoses for patients. Numerous groups have investigated the utility and diagnostic yield of exome sequencing (ES) across diverse cohorts. In cohorts with broad clinical indications, ES provides a molecular diagnosis for approximately 25–41% of patients (Lee et al. 2014; Meng et al. 2017; Retterer et al. 2016; Yang et al. 2014). Diagnoses are enriched in patients with neurologic phenotypes, younger age (Stark et al. 2016; Tan et al. 2017; Valencia et al. 2015), and if testing includes familial comparison samples (Zhu et al. 2015). Clinical correlation by the healthcare provider is recommended to assess patient phenotypic features that may provide support for or against candidate variants identified by ES or genome sequencing (GS) (Richards, et al. 2015).

While systematic approaches for variant interpretation have been well documented (Nykamp et al. 2017; Richards et al. 2015), there are aspects of both genomic sequencing analysis and interpretation that do not follow a standardized process (Gargis et al. 2015; Hedge et al. 2017; O'Daniel et al. 2017; Rehm et al. 2013; Worthey 2017). Variant filtering strategies (a.k.a. tertiary analysis) vary between labs, and filtering is often modified based on reported patient phenotype. Therefore, while a non-diagnostic report may indicate a limitation of

global genetic knowledge or of NGS technology, it could also reflect limitations of the variant filtering strategy or incomplete phenotypic information used for gene filtering or interpretation.

After the initial sequencing report, there is benefit in performing periodic reanalysis of genetic data in light of new gene-disease and variant-disease associations (Eldomery et al. 2017; Shashi et al. 2018; Wenger et al. 2016). Studies report that 10–36% of ES reanalyses may lead to a clinical diagnosis. Clinical GS, which differs from ES in its expanded coverage of non-coding regions and more uniform coverage in coding regions at the expense of average sequencing depth, has also been proposed as a reflex test for patients with uninformative ES or as an alternative to ES. The diagnostic potential of GS is anticipated to be greater than ES due to its ability to detect structural, regulatory, and deep intronic sequence variants (Bagnall et al. 2018; Lionel et al. 2018; Thiffault et al. 2018). However, the clinical integration of GS is complicated by the need for orthogonal methods to functionally validate candidate variants as well as more limited insurance reimbursement.

The Stanford Center for Undiagnosed Diseases (CUD), a member of the Undiagnosed Disease Network (UDN) (Ramoni et al. 2017; Splinter et al. 2018), approaches rare disease diagnosis with an emphasis on phenotype-driven ES and GS analysis (Reuter et al. 2018). Frequently, patients evaluated at the CUD have non-diagnostic ES results before and/or after enrollment. We developed a workflow for further evaluation of ES results that incorporates multiple investigations. We present a practical guide for additional diagnostic strategies after non-diagnostic ES, using tools accessible to genetics providers. These strategies are framed by solved CUD case examples after initial non-diagnostic ES results.

Methods

The Stanford CUD team includes genetic counselors, curators, board-certified clinical geneticists, clinical research coordinators, laboratory scientists, bioinformaticians, and other physicians across multiple specialties (cardiology, neurology, internal medicine, hematology, etc.). We work with board-certified molecular geneticists through both the UDN and the Clinical Genomics Program at Stanford. Patients undergo detailed phenotyping by expert clinicians, which incorporates standardized nomenclature [Human Phenotype Ontology (HPO) (Koehler, 2017)] to enable more precise data integration into genomic analysis and enhance data sharing. Reflecting each patient’s unique presentation, evaluations may comprise multiple components at the discretion of the study staff, as previously described (Reuter et al. 2018). The CUD curation team utilizes genetic counselors and physicians to review genetic test reports, perform clinical correlation of reported genetic variants, conduct tertiary genomic data analysis, and present results to the larger CUD team for discussion.

Genomic sequencing data generated prior to enrollment in the UDN are requested with patient consent and reanalyzed when available. If reanalysis is uninformative or the patient did not have prior sequencing, ES or GS is completed through the UDN sequencing cores. If a diagnosis is not reached with UDN sequencing, the team collaboratively decides on appropriate follow-up investigations. Genomic data are reanalyzed internally on an ongoing basis for all unsolved cases.

The Stanford CUD uses a structured framework for determining whether ES or GS results are causative of (or contributory to) the patient's phenotype. In order for any genetic test result to be considered diagnostic, there should be strong support in these three lines of evidence: (1) Variant-level evidence - is the variant affecting function of the gene; (2) Gene-level evidence - do the characteristics of disease and/or function of the gene correlate with the patient's phenotype; and (3) Segregation - do(es) the variant(s) segregate with disease in the family and in a pattern consistent with disease in the patient (Figure 1). Strong evidence in all three lines is highly supportive of a diagnosis. ES and GS reports often contain variants that meet some but not all of these criteria (e.g. variants of uncertain significance, variants in genes of uncertain significance, single heterozygous variants in genes associated with recessive conditions). We consider results with moderate to strong support in at least two of these three lines of evidence as candidate genetic diagnoses warranting additional investigation into the line of evidence that remains moderate-weak. For example, a de novo loss-of-function variant in a gene of uncertain significance has strong variant- and segregation-level support in a simplex case with multiple congenital anomalies; the gene-level evidence at initial identification, however, is weak.

While each genomic analysis is different, common approaches can be used to follow up a non-diagnostic report (Figure 2). For example, deletion/duplication studies are often recommended when one pathogenic variant is reported for an autosomal recessive condition. Other methods used at the Stanford CUD for variant identification include review of an expanded lab-provided sequencing report (a.k.a research variants) and internal tertiary analysis. Research variants can include: (1) those that were unable to be clinically validated due to technical or regulatory limitations; (2) variants in genes associated with disease but unrelated to the patient's reported phenotype; (3) de novo or biallelic variants in genes unrelated to the patient phenotype; and (4) predicted deleterious variants in genes with limited or no known disease association. For interesting candidate variants or genes, additional studies to interrogate gene function or efforts to match cases may provide the evidence needed to associate the gene with the patient phenotype or establish a variant as likely pathogenic. Confirmatory clinical testing (e.g. biochemical study) and family segregation analyses are also utilized for diagnostic and variant-level assessment, respectively.

Four Stanford CUD case examples illustrate some of the approaches that facilitated diagnoses following non-diagnostic ES. All patients were consented for participation in the UDN. ES was performed at Baylor Genetics Laboratory for cases 2 and 3. GS was performed at the HudsonAlpha Clinical Services Lab for cases 1 and 4.

Results

As of August 2018, 146 patients have been accepted for evaluation at the Stanford CUD, with 77 patients completing ES or GS, and 27 patients receiving a diagnosis. Accepted patients include children (n=95) and adults (n=51) spanning a wide range of disease types (e.g. neurologic, cardiovascular, musculoskeletal, immunologic). Following the approach described above, 9 of 27 patient diagnoses were reached based on additional analyses after non-diagnostic ES or GS. Additionally, 17 patients have candidate diagnoses identified using

this approach where validation is ongoing. The most common tools aiding additional diagnoses and candidate diagnoses are expanded reports (n=9), case matching (n=9), and segregation analyses (n=7). Table 1 summarizes the approaches used to yield a diagnosis for the four Stanford CUD case examples. Table 2 summarizes the strength of variant-, gene- and segregation-level evidence at the time of initial candidate assessment for each case and outlines which pieces of evidence were acquired to provide strong support for the candidate across the three evidence types.

Case Report 1 - Only one pathogenic variant in a gene linked to an autosomal recessive condition

Case Presentation—A 30-year-old male presented to the Stanford CUD with a history of childhood-onset spinocerebellar ataxia, myoclonus, oculomotor apraxia, dysarthria, and normal cognition. He had normal growth and development until 18 months of age when he developed spasticity and ataxia. These progressed throughout childhood, and by age 11 progressive nystagmus, dysarthria, and muscle fatigue were also noted. He became wheelchair-bound at age 16. Oculomotor apraxia with saccade abnormalities was noted at age 18. After age 18, however, disease progression plateaued, and his symptoms have since remained stable. Serial brain MRIs in childhood showed marked cerebellar atrophy. Electroencephalogram and electrocardiogram were unremarkable. Biochemical testing was normal. Extensive prior genetic testing was unrevealing, including: molecular studies for spinocerebellar atrophy (SCA) types 1–3, 5–8, 10, 12–14, 17, and 28; Fragile X; Friedreich ataxia; *SETX; ATTX; POLG1*; and trio ES in 2014.

Evaluation at the CUD, in addition to the above, demonstrated a mild sensory polyneuropathy. Trio GS identified a heterozygous pathogenic variant in *MRE11*: c.1726C>T (p.Arg576*). Heterozygous deleterious variants in *MRE11*, a member of the DNA double strand break repair pathway, have been linked to increased risk for female breast and ovarian cancer (Damiola et al. 2014; Couch et al. 2017). Biallelic pathogenic variants in *MRE11* are associated with Ataxia-telangiectasia-like disorder 1 (ATLD1; OMIM 604391), an autosomal recessive disorder characterized by progressive cerebellar ataxia, oculomotor apraxia, dysarthria, and the absence of telangiectasias (Federighi et al. 2017). The p.Arg576* variant was paternally inherited. A second *MRE11* variant *in trans* was not reported.

Gene-level Evidence: Clinical Correlation—Review of the primary literature describing ATLD1 revealed a strong correlation with the patient's phenotype and disease course (Table 2). Additional overlapping features included: myoclonus; action tremor; nystagmus with hypometric saccades; sensory neuropathy on EMG; cerebellar atrophy on brain MRI; and a plateau of disease progression in early adulthood (Regal et al. 2013; Federighi et al. 2017). Unlike Ataxia-Telangiectasia, individuals with ATLD1 lack immunodeficiency and have not yet been demonstrated to have a strong susceptibility to malignancy, despite showing cellular sensitivity to ionizing radiation on chromosomal breakage studies. Chromosomal breakage studies on patient fibroblasts were pursued for further clinical correlation.

Variant-level Evidence—To review pathogenicity of the p.Arg576* variant, *MRE11* was queried in the Human Gene Mutation Database (HGMD; Stenson et al. 2017) to assess the scope of disease-causing variants reported in *MRE11,* verify that nonsense variants are consistent with the known mechanism of disease for ATLD1, and determine whether the p.Arg576* variant had been reported in the literature to date. Upon review, it was confirmed that loss of protein or protein function resulting from nonsense variants is a described mechanism of disease in ATLD1. The p.Arg576* variant was listed as disease causing in association with cancer; it has been reported in the heterozygous state in a patient with a cancer predisposition syndrome. Beyond HGMD, further variant-level review included querying large control population databases, ClinVar, and the primary literature. Evidence from each of these databases supported pathogenicity of the p.Arg576* variant, and therefore this genetic result was considered as having strong variant-level evidence for the patient (Table 2).

Segregation: Looking for a Second Variant—ATLD1 is reported as an autosomal recessive disease, and therefore a second pathogenic variant in *trans* would be expected in order for ATLD1 to be considered diagnostic. The small possibility that the p.Arg576* variant was uniquely sufficient to cause disease was ruled out due to its presence in the patient's unaffected father as well as in large control databases (albeit at low frequency consistent with autosomal recessive rare disease - approximately ≤0.5%). Given strong gene-level and variant-level evidence for ATLD1 as a candidate diagnosis, the team pursued the possibility of a pathogenic, maternally inherited variant that may have been left off of the sequencing report for technological or bioinformatic reasons. The patient's GS data was queried for any rare, maternally inherited single nucleotide variants in *MRE11* that may have been filtered out for quality or due to lack of anticipated protein effect. None were identified. Next, the team queried output from two copy number variant (CNV) detection pipelines (one from the Stanford CUD and the other from the genetic testing laboratory) due to the possibility of a larger structural variant on the maternal copy of *MRE11.* Interestingly, both pipelines detected a 3kb likely deletion encompassing exon 13 of *MRE11* in the patient and his mother, which was absent from his father. To clinically confirm the deletion, we identified a laboratory offering deletion/duplication analysis of *MRE11* via targeted chromosomal microarray with a probe in exon 13. The patient's sample was sent and the deletion was confirmed. The demonstrated biallelic inheritance of deleterious *MRE11* variants, and phenotype-genotype correlation are consistent with a diagnosis of ATLD1.

Case Report 2 - The Variant of Uncertain Significance

Case Presentation—A 13-year-old Hispanic female presented to the Stanford CUD in 2015 with a history of regression, including loss of developmental milestones, head control, and speech at 18 months. Tremors began at 21 months, and seizures began at 22 months (occasional myoclonus). Her 5-year-old brother has a history of ataxia, autism, developmental delay, recurrent febrile seizures, and absent speech. Prior genetic testing was normal, including molecular analyses of *CSTB*, *EPM2A*, *NHLRC1*, *EFHC1*, *MECP2*, *POLG*, and an ataxia gene panel. Targeted testing for neuronal ceroid-lipofucinosis identified two variants in the *KCTD7* gene: c.280C>T (p.Arg94Trp), interpreted as pathogenic; and c.456G>A (p.Val152Val), interpreted as benign. Her brother had *KCTD7*

deletion/duplication analysis, which was negative. *KCTD7* mutations are associated with Epilepsy, progressive myoclonic 3 (EPM3, MIM:611726), an autosomal recessive condition characterized by myoclonic seizures, neurologic regression, truncal ataxia, limited expressive language, and visual loss. At the time, the *KCTD7* result was thought to be non-diagnostic and further testing proceeded. As part of her evaluation at the CUD, quad ES was performed on the two siblings and their parents in 2015. The initial ES report described 2 variants of unknown significance in disease genes related to clinical phenotype in both siblings: (1) a paternally inherited *KCTD7* p.Arg94Trp variant (previously identified in single gene sequencing); and (2) a paternally inherited intronic variant, c.6049–4A>T, in *CACNA1H*. Heterozygous *CACNA1H* pathogenic variants are associated with Epilepsy, childhood absence, susceptibility to, 6 [MIM:611942]. No other variants were reported. For the *KCTD7* variant the report stated: “A second pathogenic or unclassified variant in this autosomal recessive gene was not detected, however, the presence of a large or small deletion or duplication cannot be ruled out.” It was recommended that correlation of the findings with the patient’s phenotype be performed.

Gene-level Evidence: Clinical Correlation

The candidate genes were investigated to compare their related conditions with the phenotypes of our siblings. For *CACNA1H* (MIM:607904), OMIM (Hartz et al. 2016) lists two main reports linking childhood absence epilepsy (Chen et al. 2003) and idiopathic generalized epilepsy (Heron et al. 2007) with heterozygous variants in *CACNA1H*. There was not clear segregation of the *CACNA1H* variants with disease in these reports (e.g. inherited from an unaffected parent). Both groups suggested that *CACNA1H* variants may be susceptibility alleles for epilepsy, but other unknown factors would need to be involved. A more recent gene-disease association is reported between *CACNA1H* and Hyperaldosteronism, familial, type IV (MIM:617027) in five unrelated patients (Scholl et al. 2015) with a heterozygous missense variant (p.Met1549Val) that is predicted to cause gain of function. Since *CACNA1H* has a weak gene-disease association, it did not seem sufficient to be the main contribution to the patients’ phenotype.

Descriptions of EPM3 better overlap with our patients’ presentations. OMIM describes reports of 9 unrelated families with EPM3 and *KCTD7* mutations (Kousi et al. 2012; Staropoli et al. 2012; Van Bogaert et al. 2007). The lab report also referenced a case report of a patient with progressive myoclonic epilepsy and homozygosity for the same missense mutation as our patient, p.Arg94Trp (Krabichler et al. 2012). A PubMed search identified two additional affected families with other *KCTD7* variants (Blumkin et al. 2012; Farhan et al. 2014). EPM3 leads to onset of myoclonic epilepsy often before 2 years and developmental regression. Truncal ataxia and dysarthria are commonly present, which overlaps with our siblings’ phenotypes. Affected siblings in one of the families showed variability in severity, which is also similar to our family. Due to the lack of another candidate after ES and strong gene-level evidence (Table 2), we focused on *KCTD7* as a candidate gene.

Segregation: Looking for a Second Variant—Since EPM3 is an autosomal recessive disease, we needed another *KCTD7* variant *in trans* to establish a molecular diagnosis. The

proband's single gene testing previously identified a predicted benign *KCTD7* variant (p.Val152Val). Our internal tertiary analysis of the quad ES data did not identify additional *KCTD7* variants. Therefore, the synonymous variant was prioritized for reassessment.

Variant-level Evidence—We next curated the *KCTD7* synonymous variant to re-assess possible pathogenicity (Supplemental Table 1). This consisted of using in silico predictions, and querying allele frequency. Since the *KCTD7* synonymous variant was interpreted as benign in 2014, the landscape of variant interpretation has changed dramatically. The ACMG-AMP Guidelines for Sequence Variant Interpretation were published in order to support clinical labs in making more consistent interpretations (Richards et al. 2015). Large databases have become publicly available that help to assess variants in apparently healthy populations [Exome Aggregation Consortium in 10/2014 (Lek et al. 2016); and Genome Aggregation Database in 2/2017 (http://gnomad.broadinstitute.org)]. We used MutationTaster (Schwarz et al. 2014) to assess pathogenicity of the *KCTD7* p.Val152Val variant, which incorporates splicing predictions from *NNSplice* (Reese et al. 1997). MutationTaster predicted this variant to be disease causing, due to splice site changes (donor increased, Supplemental Figure 1), however other splicing prediction tools did not concur. This evidence supported potential for a disruptive molecular consequence of the second, synonymous variant. Despite the conflicting interpretations of pathogenicity for the missense variant by two clinical laboratories, the presence of compound heterozygous variants in both siblings and the strong gene-disease correlation with our patients' phenotypes supported additional analyses (Table 2).

Follow-up: Family Segregation Analysis—The quad ES detected the biallelic *KCTD7* variants in both siblings. The p.Arg94Trp variant was found to be paternally inherited, while the p.Val152Val variant was maternally inherited, consistent with autosomal recessive inheritance. The family has three unaffected siblings, two of whom were available for testing. We requested testing for the *KCTD7* variants from the ES lab for the available siblings. They both were confirmed to be heterozygous carriers of one of the *KCTD7* variants. Therefore, segregation analysis provides additional evidence for the candidate disease diagnosis (Table 2).

Functional Studies—After the suggestion of a splicing impact for the synonymous variant, we performed Sanger sequencing on the affected siblings' cDNA, confirming a novel splice site leading to exon truncation (Fresard et al. 2018). RNA sequencing was also performed, and aberrant splicing was confirmed in *KCTD7* at the site of the p.Val152Val variant (Fresard et al. 2018). These studies helped to confirm suspicions that the previously interpreted benign variant has a deleterious impact on the KCTD7 protein.

Communication with the Clinical Laboratory—We shared our internal curation and functional analysis with the ES laboratory. In the intervening period, research was published describing affected siblings and functional analysis of *KCTD7* mutants, including the p.Arg94Trp variant (Moen et al. 2016). The laboratory reassessed these variants, adding the p.Val152Val variant to the report as a VUS, and upgrading the p.Arg94Trp variant from a VUS to likely pathogenic.

Case Report 3 – Reanalysis and a Gene of Uncertain Significance

Case Presentation—A 10-year-old Hispanic female presented to the Stanford CUD with a history of episodic metabolic decompensation with lactic acidosis and muscle breakdown, requiring hospital admission. Her onset of symptoms began at 2 days of age with hypoglycemia, severe lactic acidosis, and hyperammonemia. She also has mild developmental delay and short stature. Between 1 and 4 years she developed dilated cardiomyopathy, with subsequent normalization of resting systolic function. A mitochondrial disorder was suspected, but appropriate testing had been normal (including a mitochondrial nuclear gene panel, metabolic labs, electron transport chain complexes I-IV, pyruvate carboxylase and pyruvate dehydrogenase complex). Singleton ES, including the mitochondrial genome, was performed for the patient and identified eight variants of uncertain significance. Her clinical team felt they were not consistent with her phenotype. She was subsequently accepted at the Stanford CUD, where reanalysis of her clinical ES data was performed in 2015.

Collecting Additional Information from the Family—As part of the patient's informed consent visit, an updated family history/pedigree was obtained and consanguinity was disclosed. This new information helped to focus the ES reanalysis to prioritize homozygous variants. When combined with the suspected mitochondrial disease etiology, six homozygous variants in six genes were reviewed. A candidate variant was detected in the *ATP5F1D* [MIM: 603150] gene (c.245C>T, p.Pro82Leu), which had no known disease association at the time (Olahova et al. 2018).

Variant-level Evidence—The homozygous *ATP5F1D* variant was curated to assess pathogenicity. Multiple in silico models predicted a damaging effect for this variant (SIFT, PolyPhen-2, Mutation Taster, CADD). The variant had not been reported in large population databases at the time of assessment (ExAC, NHLBI ESP, 1000genomes). However, there was low coverage in ExAC (8353 individuals). Ideally, assessment of allele frequency should include ethnic-specific data; in this case the absence of the variant could also reflect underrepresentation of the Hispanic population. Novel variants (not previously reported) are often prioritized for additional assessment, as they could reflect the rarity of the disease and a potential for pathogenicity. When there is low coverage or underrepresentation a novel variant should not be discarded, but efforts should be made to re-query the data. For instance, the variant is now present in gnomAD v2.1 on 1/148,408 alleles overall and 1/23,912 Latino alleles with no homozygotes reported. This variant had strong variant level evidence due to the predicted deleterious effect and novel nature (Table 2).

Gene-level Evidence—The *ATP5F1D* gene encodes the F_1 delta subunit of complex V mitochondrial ATP synthase, which participates in the last step of oxidative phosphorylation. Pathogenic variants in six mitochondrial and nuclear genes had been reported to cause complex V mitochondrial disease, two of which are ATP subunits (*ATP5F1E,* MIM:606153*; ATP5F1A*, MIM:164360). Complex V oxidative phosphorylation disorders have a variable phenotype typically including neonatal-onset hypotonia, hypertrophic cardiomyopathy, lactic acidosis, and 3-methylglutaconic aciduria (Ghezzi et al. 2018), which is consistent with our patient's phenotype. Also, a reported patient with *ATP5F1E* deficiency had a

homozygous missense mutation in the F_1 epsilon subunit of complex V. She presented with neonatal onset lactic acidosis and 3-methylglutaconic aciduria, but also had mild intellectual disability and peripheral neuropathy at 22 years of age (Mayr et al. 2010). Therefore, gene curation showed that pathogenic variants in similar ATPase subunits have been associated with mitochondrial disease similar to our patient's phenotype.

Case Matching/Data Sharing—While segregation and variant-level evidence supporting this candidate seemed consistent (Table 2), additional evidence was needed to support a gene-disease association. An abstract was presented as a poster at a conference with details of the patient and clinical correlation (Kohler et al. 2016). A group with a similar patient contacted us after seeing the abstract. This patient also had a homozygous suspected pathogenic variant (Olahova et al. 2018) in the *ATP5F1D* gene. Collaboration followed to assess gene-disease association and confirm pathogenicity of both variants.

Confirmatory and Functional Analysis—Sanger sequencing confirmed homozygosity of the candidate variant in the proband and heterozygosity in each of her parents. Skin fibroblasts from our patient were sent for clinical analysis of respiratory chain complex V activity, which had not been previously assessed (only complex I-IV were ruled out). This analysis showed qualitatively decreased activity of complex V. The UDN has access to a model organism lab, and a fly model was made to knockdown the fly homolog of *ATP5F1D* (Olahova et al. 2018). This led to a severe phenotype, which was rescued by wild type human *ATP5F1D*, but only partially rescued by the patient's p.Pro82Leu variant *ATP5F1D*. This clinical and model organism data helped support the novel diagnosis of *ATP5F1D* deficiency.

Case Report 4 – The Negative Report and the Research Variant

Case presentation—A 2-year-old male presented to the Stanford CUD with global developmental delay, short stature, failure to thrive (FTT) and dysmorphic features. The patient had an increased nuchal fold noted at 18 weeks gestation and mega cisterna magna (11mm) noted on fetal MRI at 32 weeks gestation. Non-invasive prenatal testing was negative. He had an uncomplicated term delivery, however, he remained hospitalized for a week due to jaundice, prolonged hyperbilirubinemia and poor feeding. At 2 months of age he developed FTT, gastroesophageal reflux disease, progressive eczema and consistent mildly elevated calcium (11.2 mg/dL). At 8 months of age he was noted to be hypotonic with poor head control and globally delayed. He took his first independent steps at 2 years old. At the time of evaluation, he could babble and point to indicate his needs, but spoke no words. On physical exam at age 2 years his weight was 10.2 kg (6th percentile), his height was 81.0 cm (2nd percentile) and his head circumference was 50 cm (74th percentile). His exam was also notable for mild dysmorphic features including posteriorly rotated ears, prominent nasal tip and forehead, 2,3 toe syndactyly and short hands and feet. Prior to his enrollment he had an 8 × 60k oligonucleotide whole genome microarray, CGG repeat expansion analysis for Fragile X syndrome and trio ES, which were negative. As part of his CUD evaluation trio GS was performed and was initially negative. However, parallel copy number variant (CNV) bioinformatics pipelines developed at Stanford CUD and the genetic testing laboratory identified a *de novo* 5.75kb heterozygous deletion in the *ARID1B* gene in

the patient's GS sample. Heterozygous pathogenic variants in *ARID1B* cause Coffin-Siris syndrome (MIM:135900).

Gene-level Evidence—We started by assessing whether our patient fit within the Coffin-Siris syndrome (CSS) phenotypic spectrum. To gain a general understanding of the phenotype and underlying disease mechanism we reviewed CSS summaries provided by GeneReviews (https://www.ncbi.nlm.nih.gov/books/NBK1116/), GARD (https://rarediseases.info.nih.gov/) and Genetics Home Reference (https://ghr.nlm.nih.gov/). The classic description of CSS correlated with some aspects of our patient's phenotype such as developmental delay and hypotonia. However, we did not appreciate other cardinal features including 5th digit hypoplasia, coarse facies or hirsutism. Moreover, several of our patient's clinical features were not typically reported in association with CSS including eczema and mega cisterna magna. The diagnosis of CSS is typically established by identification of a heterozygous pathogenic variant in several genes involved in the formation and function of the SWI/SNF complex. As more individuals with CSS are identified through molecular testing the phenotypic spectrum and genotype-phenotype associations have continued to evolve. To determine whether all of our patient's features fit within the CSS phenotypic spectrum we pursued a deeper review of the phenotype associated specifically with *ARID1B* haploinsufficiency. We queried publicly available databases which provide patient phenotypic and genotypic information including OMIM, HGMD, DECIPHER and PubMed. Only through performing a primary literature review did we identify additional reported cases that correlated strongly with our patient's phenotype including less commonly reported features that overlapped with our patient (Table 2).

Variant-level Evidence—At the time of discovery of our patient's deletion, there were no other CNVs less than 10kb in *ARID1B* reported in ClinVar, HGMD, DECIPHER nor PubMed. To provide evidence of variant pathogenicity, we investigated the predicted impact of the 5.75kb deletion on *ARID1B* function. We manually reviewed the patient's GS data in IGV and identified that the deletion encompasses exons 12 and 13 (NM_020732.3) of the *ARID1B* gene, which encode for the AT-rich interactive functional domain (ARID) responsible for binding the SWI/SNF complex to DNA. Furthermore, we predicted that deletion of exons 12 and 13 would result in a frameshift by reviewing that the last codon on exon 11 is a complete triplet (i.e. AAG) while the first codon of exon 14 is not (i.e. only A). Structural variants resulting in a frameshift is an established mechanism of disease in CSS. Taken together this evidence strongly supports that the deletion identified in our patient would impair the function of *ARID1B* and result in haploinsufficiency (Table 2).

Segregation—CSS is an autosomal dominant condition that primarily results from a *de novo* pathogenic variant. To assess whether the patient's deletion was inherited from a parent we manually reviewed the parents' GS data in IGV. We did not observe the same deletion in either parent's sample. Furthermore, the bioinformatics pipeline did not identify the same deletion in any other UDN GS sample. Given the strength of gene- and variant-level evidence we pursued clinical confirmation of the deletion (Table 2). We contacted clinical laboratories offering dosage analysis of the *ARID1B* gene that included probes in this

region. Orthogonal confirmation was obtained via a gene-targeted high-density array CGH with a median probe spacing of 18 base pairs within coding regions of *ARID1B*.

Discussion

After receiving an ES or GS report, the Stanford CUD uses a three-part framework to both assess sequencing report variants in the context of the patient, and to determine which variants are significant enough for further assessment. While this may seem intuitive to genetic clinicians who regularly interpret single-gene or gene panel results, the utility of the structured framework becomes apparent when reviewing ES or GS reports with a few to dozens of variants. Candidate diagnoses suggested by the variants may be readily excluded by clinical correlation with patient phenotype. Even pathogenic variants may not be indicative of a clinical diagnosis (Biesecker et al. 2018). Further assessment of non-diagnostic ES and GS results can continue using approaches that lead to diagnoses, exemplified by our cases and summarized in Table 1 and Figure 2.

Variant Identification

Correlation with a patient's phenotype should help to prioritize variants for additional assessment. Cases 1 and 2 had sequencing reports containing a single heterozygous variant in a gene associated with autosomal recessive disease, which is not sufficient to be diagnostic. Careful variant review and clinical correlation resulted in strong variant- and gene-level evidence towards candidate diagnoses. While most clinicians do not have access or time to reanalyze raw sequencing data, the clinical laboratory may perform an additional variant search if requested. When we applied the three-part evidence framework as a guide, resources were directed towards identifying a second variant in *trans* to establish the last line of evidence needed before considering two compound heterozygous variants diagnostic.

While recent research has suggested the efficacy of genomic reanalysis following non-diagnostic ES (Bagnall et al. 2018; Lionel et al. 2018), another more immediate step that clinicians can take is to request additional variants from the laboratory. Many clinical laboratories offer the ability to request an expanded report of variants that may be of interest but did not meet criteria to be included on the clinical report. Some labs may provide additional variants as a spreadsheet table or in report form, while other labs may provide intermediate files from their analysis pipeline. These variants often will require orthogonal confirmation, which may be the reason for not being included on the clinical report. Both of our cases using GS identified likely pathogenic CNVs that had not previously been detected. However, neither of these CNVs was reported on the sequencing reports. This highlights the importance of requesting expanded reports from testing laboratories, and also communicating suspicion of candidate variants (e.g. Case 1). As CNV pipelines advance, laboratories may be able to detect and report these findings on a clinical report. By requesting additional variants and applying the three-part evidence framework to guide prioritization, clinicians may identify candidate diagnoses missed by limitations due to knowledge, technology, or reported phenotype.

Reanalysis of ES data and/or previously identified variants can be beneficial. The ACMG/AMP guidelines suggest reassessment of VUS and likely pathogenic variants

(Richards, et al. 2015), which could be done in preparation for patient visits. Case 2 involved reviewing a variant that was previously interpreted as benign, but an in silico model and population databases suggested potential for pathogenicity. The strong clinical suspicion of a mitochondrial etiology in Case 3 allowed elimination of variants of uncertain significance from the ES report, and drove ES reanalysis for plausible new gene candidates. An emerging alternative to requesting reanalysis through the clinical lab is to use publicly available software [e.g. AMELIE (Birgmeier et al. 2017), Exomiser (Smedly et al. 2015)] that uses sequencing files, variant call files, candidate gene lists, or candidate variant lists and patient phenotype terms (e.g. HPO terms) to rank patient variants by suspicion for disease/diagnosis. Due to advances in knowledge and publicly available large genetic databases, reassessment of variants and genetic data can identify candidates for additional analysis.

Additional Analyses and Approaches

The diagnostic power of ES and GS increases with the availability of parental and sibling samples. A benefit of family-based ES/GS is segregation of variants and identification of de novo variants. If there is a candidate gene or variant from a singleton ES or GS, segregation analysis of the candidate variant(s) in additional family members can confirm the suspected inheritance, but also provide evidence for pathogenicity of a variant (ACMG-AMP PP1 criteria). For example, the quad ES in Case 2 included the parents and two affected siblings, and found two variants *in trans* in both affected siblings. Subsequently, unaffected siblings were included for variant analysis and segregation was consistent with autosomal recessive inheritance.

Additional methods of confirmatory analysis of candidate variants or diagnoses are available to help assess pathogenicity. In Case 2, investigation of the possible splicing effect from the synonymous variant was crucial to providing pathogenic evidence. There are some labs that offer targeted RNA analysis and transcriptome-wide RNA sequencing clinically. As RNA sequencing becomes more widely adopted (Cummings et al. 2017; Fresard et al. 2018; Kremer et al. 2017), this may become more readily available in the clinical setting as a tool to assess and/or identify candidate variants identified by ES or GS. For Case 3, additional clinical testing (e.g. respiratory chain complex V activity) and functional analyses (e.g. model organisms and cellular models) helped to confirm variant pathogenicity and gene-disease association. The Alliance of Genome Resources (www.alliancegenome.org) or MatchMaker Exchange (Philippakis et al. 2015) may be able to connect a clinician with a model organism research partner. When additional studies are not readily available, directly contacting experts in that gene or pathway may facilitate genetic diagnosis. Discussion of any identified variants with physicians who have expertise in the disease state can help to assess a genotype and phenotype match.

Targeted deletion/duplication analyses typically use single-exon resolution detection methods in genes for which intragenic copy number variants (CNVs) are an established mechanism of disease. However, single-exon resolution is less consistently offered for genes with limited evidence on the prevalence of pathogenic CNVs (Truty et al. 2018). This is notable given a growing body of evidence demonstrating 23–25% of clinically relevant pathogenic CNVs involve only a single exon (Gambin et al. 2017; Truty et al. 2018). Cases 1

and 4 emphasize the importance of single-exon resolution CNV detection for patient diagnostics, especially in a candidate gene of interest. The 3kb deletion identified in the patient from case 1 would likely have been missed or withheld from reporting by standard microarray and targeted deletion/duplication analyses without single-exon resolution (Gambin et al. 2017; Nowakowska 2017). For case 4, the 5.75kb deletion in *ARID1B* was missed on initial microarray. Orthogonal confirmation was obtained with gene-targeted high-density array CGH with single-exon resolution. Querying clinical laboratories to ensure their assay includes at least one probe in each exon of a gene or genes of interest before sending a sample is key to maximizing diagnostic potential for a patient.

Data sharing increases the chances of identifying other patients with rare or new diseases. Case 3 underlines the importance of case matching. Even with quite compelling variant- and gene-level evidence (a novel homozygous variant in a gene closely related to other mitochondrial disease genes), additional evidence was still required to establish a new gene-disease relationship. Thus, identifying another unrelated patient with recessive variants in the same gene was pivotal. While our data was shared via an abstract at a conference, there are many online matching services such as Matchmaker Exchange to facilitate phenotype and genotype matchmaking (Philippakis et al. 2015). Another method is to contact gene/disease experts for potential internal cases to compare similar genotype or phenotype. For laboratories and/or patients, reporting identified variants via large variant databases (e.g. ClinVar, GenomeConnect) allows sharing of novel variants and their interpretations. A clinician could contact laboratories that have submitted candidate variants to ClinVar for possible matching with their patients.

Tools and Resources

It is important to consider what may have been missed in prior analysis due to assumptions of patient phenotype. Genetics resources such as OMIM and GeneReviews are helpful in getting a broad phenotypic overview and often provide useful links to additional resources and publications. However, their phenotypic information can be limited in scope. A primary literature search (e.g. PubMed) should be conducted to include more recent publications and case reports. Also, more recently described genes and diseases may still have an evolving phenotype due to the small number of patients and limited phenotypes reported. As exemplified in Case 4, patient features of CSS were not the classic phenotype but did match other patients with CSS due to *ARID1B* haploinsufficiency. Frequent literature searches for newly published reports involving candidate genes may be helpful. Search terms (e.g. genes or diseases) can be saved and automatically run in PubMed at set frequencies with e-mail updates (i.e. Saved Searches feature). Similarly, clinicians can set up a Google Scholar alert for a gene or disease of interest (https://scholar.google.com/intl/en/scholar/help.html#alerts).

Several tools are publicly available to help build and/or aggregate gene- and variant-level evidence (Table 3). For structural variants of unknown significance, a genetic clinician without access to raw genomic data may investigate the same variant level evidence by querying the region within the reported CNV break points in UCSC Genome Browser or NCBI Genome Data Viewer. ClinVar and HGMD provide variant-level data for use in clinical assessment. Cases 1 and 4 describe using HGMD to query variant spectrums and

similar deletions. While the public version of HGMD is not as robust as the professional version, an advantage is the variant-level references it provides. A PubMed search can be difficult for identification of literature specific to a variant. A recently available tool to query literature by gene and variant is Mastermind Genomic Search Engine. Another helpful tool is the variant data aggregator, Varsome, which allows efficient query of variant-level and gene-level data. Our toolkit updates and complements the databases and computational predictive programs listed in the ACMGG/AMP standards and guidelines for variant interpretation (Richards et al. 2015).

Ongoing Collaboration

Collaboration with the patient, laboratory, and physician are of intrinsic importance to gather more information and correlate genotype with a possible diagnosis. Communication aids the assessment and integration of the proposed framework and three lines of evidence. The patient and family may provide a more detailed family history that could help with possible inheritance patterns, identifying symptomatic relatives, and/or an evolving patient phenotype (as in Case 3). Communication between the reanalysis team for Case 3 and the clinical care team, specialists in mitochondrial disease, was critical for prioritization of variants and eventual candidate diagnosis. Reporting additional information after these discussions back to the laboratory can help further identify causative variants and result in amendment of the sequencing report (Cases 1, 2, and 4). Often the genetic counselor is already situated as the main communicator between these entities. All the cases presented here required collaboration between the clinical team, the patient/family, and the laboratory to achieve the diagnosis.

Limitations

We are sharing our experience in a selected group of patients who have remained undiagnosed after initial, sometimes exhaustive, work-up. The Stanford CUD has unique resources that span clinical and research realms that may not translate to practicing clinicians. Genome and RNA sequencing are clinically available, but may not be covered by health insurance. Functional studies and model organisms are research options with more limited availability. Our patient volume allows us to spend more time on each patient's data analysis. This approach may not be an efficient use of clinicians' time if applied to each patient seen in a general genetics clinic. However, our approach can be useful for specific patients depending on their sequencing results, length of time undiagnosed, clinician suspicion for a genetic etiology, and lack of other options for obtaining a diagnosis.

Conclusions and Practice Implications

After a negative ES report, additional approaches such as those used at the Stanford CUD can lead to patient diagnoses. The three-part evidence framework helps to prioritize sequencing results for additional diagnostic assessment. Genetics providers can use the toolkit of resources to gather variant- and/or gene-level evidence. Based on our cohort, undiagnosed patients with strong suspicion for a genetic etiology may be chosen to apply the framework in a clinical genetics setting. Clinically available approaches such as segregation analysis, additional testing (e.g. array, biochemical), and case matching can be performed to further interrogate a candidate diagnosis. While these concepts are not novel, the three-part

framework and tools allow a structured approach for dissemination, education, and integration into clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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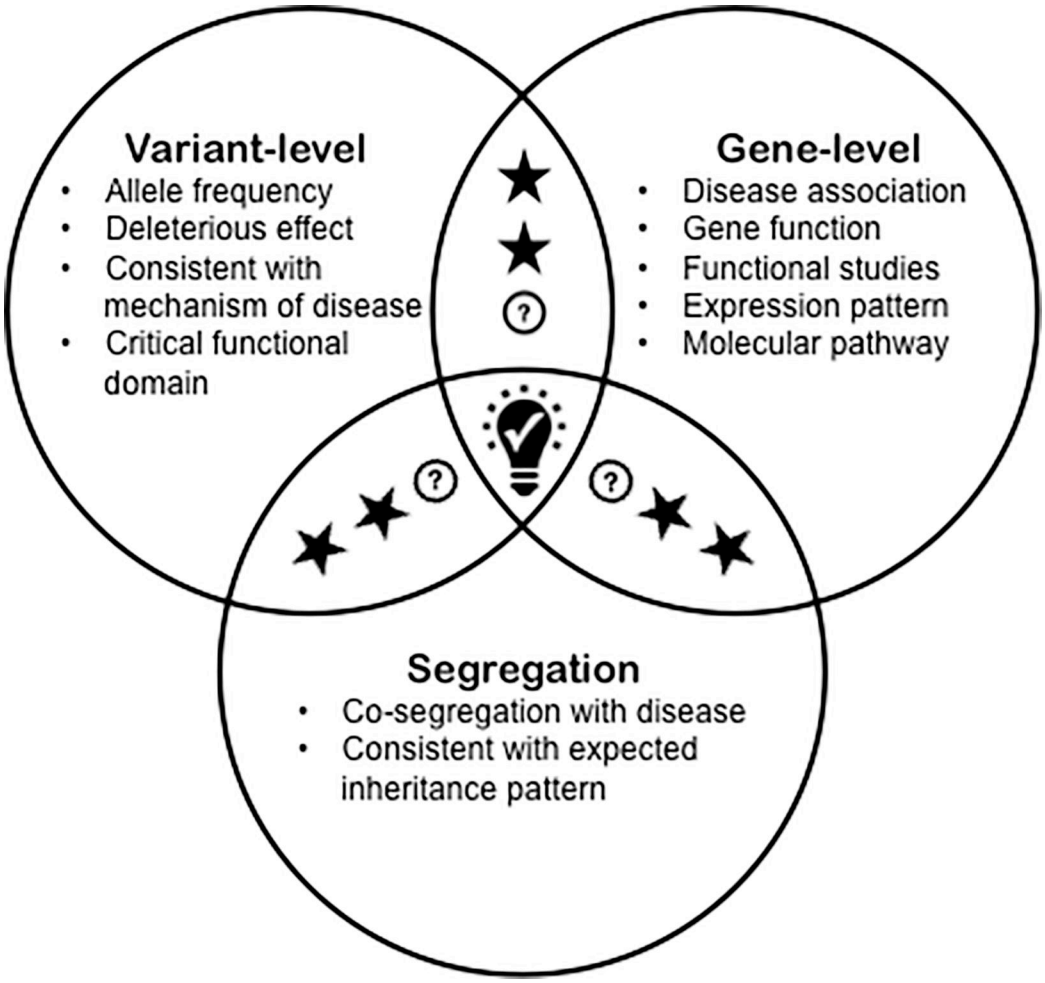

Figure 1. Lines of evidence used in evaluation of genomic findings.

Example characteristics contributing to variant-level, gene-level, and segregation evidence are listed. Each line of evidence (circle) is evaluated independently. Strong evidence in all three lines is indicative of a genomic diagnosis (center, light bulb). Strong evidence in two of three lines of evidence is of sufficient significance to pursue follow-up studies contributing to the third line of evidence (A star per line of evidence indicates strong evidence; question mark indicates a weak line of evidence).

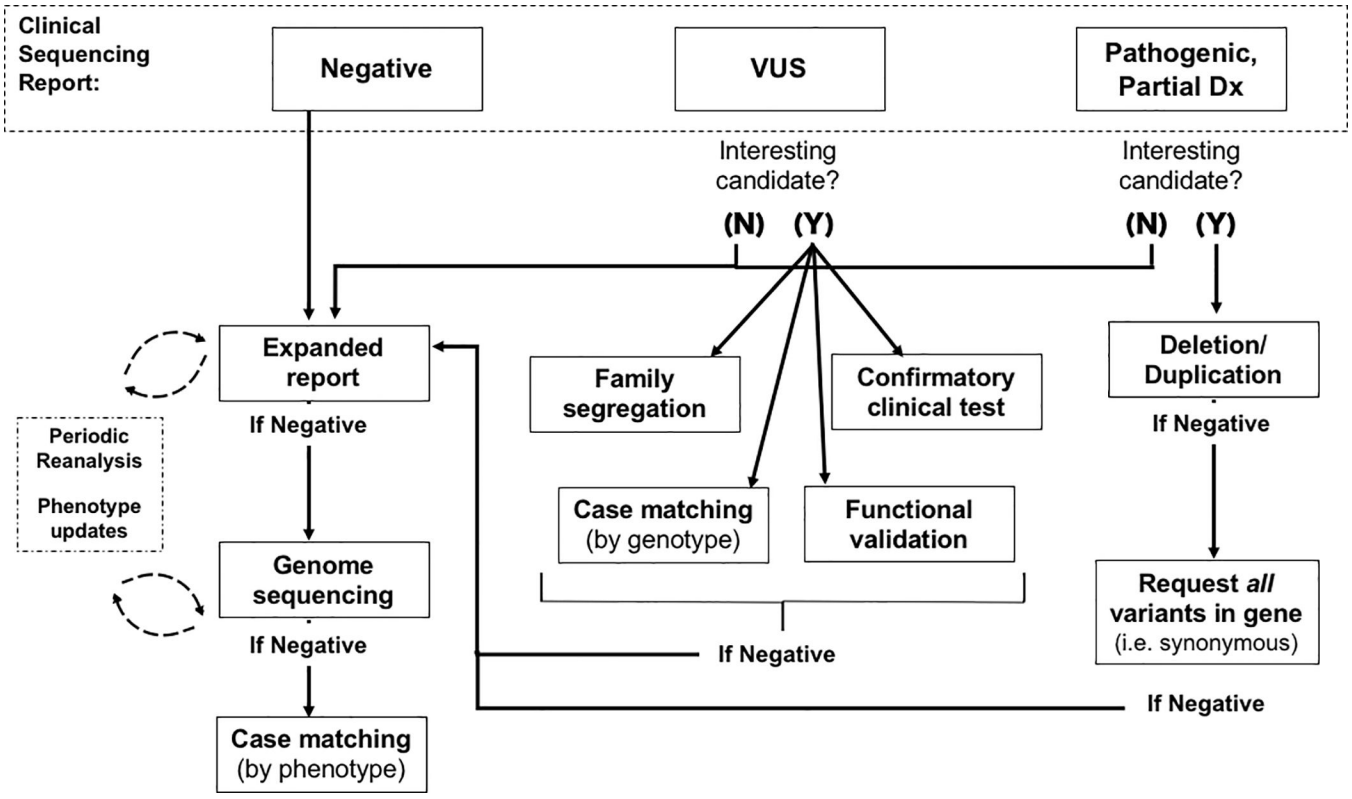

Figure 2. Suggested workflow for non-diagnostic exome sequencing follow-up.

Based on type of results from exome sequencing, further steps for additional interrogation are displayed. These queries include additional testing, requesting additional data from sequencing laboratory, case matching, and phenotypic updates. VUS=variant(s) of unknown significance; Dx=diagnosis; N=no; Y=yes.

Table 1

Approaches for Additional Analysis of Sequencing Results

	Case 1	Case 2	Case 3	Case 4
ES report	No causative variants identified	VUS in *KCTD7*	No causative variants identified	No causative variants identified
Reanalysis			Homozygous variant in *ATP5F1D*	
GS report	Pathogenic variant in *MRE11*			Negative
Expanded report	Deletion in *MRE11*			Deletion in *ARID1B*
Internal variant curation	Deletion in *MRE11*	Compound heterozygous *KCTD7* variant, splicing affect predicted	Homozygous variant in *ATP5F1D*	Deletion in *ARID1B*
Segregation studies		Performed	Performed	
Case matching			Yes	
Updated pedigree			Yes	
Additional clinical studies	Deletion/duplication analysis with single exon resolution		Respiratory chain complex V analysis	Deletion/duplication analysis with single exon resolution
Functional studies		RNA sequencing, splicing assay	Model organism	

ES=exome sequencing, GS=genome sequencing, VUS=variant of uncertain significance

Table 2

Summary of Sequencing Results Assessment

	Evidence Type	Evidence Description	Initial Candidate Assessment	Final Candidate Assessment
Case 1	Gene-level (*MRE11*)	• Associated with ATLD1 • Overlap with patient phenotype	Strong	Strong
	Variant-level	• p.Arg576* identified on genome report (Pathogenic) • Previously reported in affected heterozygote • Consistent with disease mechanism	Strong	Strong
	Segregation-level	• Autosomal recessive inheritance expected • No variant in trans • **Expanded report identified *MRE11* deletion (in *trans*)**	Weak	**Strong**
Case 2	Gene-level (*KCTD7*)	• Associated with EPM3 • Overlap with patient phenotype	Strong	Strong
	Variant-level	• p.Arg94Trp identified on exome report (VUS) ○ Previously classified as pathogenic • p.Val152Val from *KCTD7* sequencing report ○ Previously classified as benign ○ **Predicted to affect splicing** • **Functional studies show aberrant splicing**	Weak	**Strong**
	Segregation-level	• Autosomal recessive inheritance expected • Compound heterozygous variants • Co-segregation with disease in affected siblings	Strong	Strong
Case 3	Gene-level (*ATP5F1D*)	• No known disease association • Other ATP subunits associated with complex V mitochondrial disease • Complex V disease overlaps with patient phenotype • **Case matching** • **Clinical testing showed decreased complex V activity**	Moderate	**Strong**
	Variant-level	• p.Pro82Leu • Novel • Deleterious effect predicted • **Model organism hypomorph**	Moderate	**Strong**
	Segregation-level	• Homozygous variants prioritized	Strong	Strong
Case 4	Gene-level (*AR1D1B*)	• Associated with CSS • Partial overlap with patient phenotype • **Primary literature review supported phenotypic match**	Moderate	**Strong**
	Variant-level	• 5.75kb heterozygous deletion identified on expanded report • Encompasses ARID functional domain • Predicted frameshift (established disease mechanism) • **Orthogonal validation**	Moderate	**Strong**
	Segregation-level	• *de novo* CNV • Consistent with expected inheritance for CSS	Strong	Strong

ATLD1= Ataxia-telangiectasia-like disorder 1; VUS= variant of uncertain significance; EPM3= Epilepsy, progressive myoclonic 3; CSS= Coffin-Sirus syndrome

Table 3

Exome and/or Genome Sequencing Toolkit

Sequence Databases	NCBI Genome Data Viewer	https://www.ncbi.nlm.nih.gov/genome/gdv/
	UCSC Genome Browser	https://genome.ucsc.edu/
	MITOMAP	https://www.mitomap.org/MITOMAP
Population Databases	Exome Aggregation Consortium (ExAC)	http://exac.broadinstitute.org/
	Genome Aggregation Database (gnomAD)	http://gnomad.broadinstitute.org/
Computational Models/ in silico prediction	Transcript inferred Pathogenicity Score (TraP)	http://trap-score.org
	Mendelian Clinically Applicable Pathogenicity (M-CAP) Score	http://bejerano.stanford.edu/mcap/
	MutationTaster	http://www.mutationtaster.org/
	Human Splicing Finder	http://www.umd.be/HSF3/
	NNsplice	http://www.fruitfly.org/seq_tools/splice.html
Variant and Disease Databases	OMIM	https://www.omim.org/
	ClinVar	http://www.ncbi.nlm.nih.gov/clinvar/
	Human Gene Mutation Database	http://www.hgmd.cf.ac.uk/ac/index.php
	Leiden Open Variation Database	http://www.lovd.nl/3.0/home
	DECIPHER	https://decipher.sanger.ac.uk/
	Database of Genomic Variants	http://dgv.tcag.ca/dgv/app/home
Literature Databases	PubMed	http://www.ncbi.nlm.nih.gov/pubmed
	Mastermind Genomic Search Engine	https://www.genomenon.com/mastermind
	Google Scholar	https://scholar.google.com
ClinGen Tools	Structural Variation Database Search	http://dbsearch.clinicalgenome.org/search/
	Dosage Sensitivity Map	https://www.ncbi.nlm.nih.gov/projects/dbvar/clingen/
Genomics Data Visualizer	Integrative Genomics Viewer	http://www.broadinstitute.org/igv
Variant Data Aggregator	Varsome	https://varsome.com
Case Matching	Matchmaker Exchange	http://www.matchmakerexchange.org
	GenomeConnect	https://www.genomeconnect.org
Model Organisms	Alliance of Genome Resources	https://www.alliancegenome.org
Standardized Phenotype Terms	Human Phenotype Ontology	https://hpo.jax.org/app/
Lab-specific Sequencing Coverage by Gene	Baylor Genetics Exome Coverage	https://www.bcm.edu/research/medical-genetics-labs/exome.cfm