BRIEF COMMUNICATION

Reanalysis of clinical exome identifies the second variant in two individuals with recessive disorders

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Clinical exome/genome sequencing is increasingly being utilized by clinicians to diagnose various likely genetic conditions, but many cases remain undiagnosed. In a subset of those undiagnosed cases, a single heterozygous variant in an autosomal recessive (AR) condition with consistent phenotype may be identified, raising the question if a second variant is missing. Here, we report two cases of recessive conditions in which only one heterozygous variant was initially reported by clinical exome sequencing, and on research reanalysis a second heterozygous variant in *trans* was identified. We performed a review of the existing exome reanalysis literature and found that this aspect is often not emphasized. These findings highlight the importance of data reanalysis in undiagnosed cases where only a single disease-associated variant is identified in an AR condition with a strong link to presenting phenotype.

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INTRODUCTION

Exome/genome sequencing is increasingly being utilized in clinical settings. This approach has improved the diagnostic yield [1, 2], but despite this the number of undiagnosed cases remains high. Periodic reanalysis of genomic data can increase the diagnostic rates due to availability of better bioinformatic tools and accumulation of genetic knowledge over time [2, 3]. The American College of Medical Genetics/Association for Molecular Pathology (ACMG/AMP) recommends variant- and case-level reanalysis every 2 years [4]. However, identification and interpretation of disease-causing variants continues to be challenging, with variants missed or not prioritized due to incomplete phenotypic information, limited bioinformatics analysis, or insufficient disease association [5]. We have previously completed a pilot study wherein we reanalyzed 102 cases that remained undiagnosed following clinical exome sequencing (CES). Using an in-house Variant Explorer Pipeline (VExP) that integrates a suite of analytical tools with genotype-phenotype data and probabilistic models to optimize variant assessment, this analyses identified a confirmed or potential genetic diagnosis in 24 of 75 CES-negative/reclassified cases [3]. Most of those new diagnoses resulted from identification of *de novo* variants in known genes or *de novo*/recessive variants in novel candidate genes.

Pathogenic variants in individuals with autosomal recessive (AR) disorders can be either homozygous or compound heterozygous. In some compound heterozygous cases, a single heterozygous variant may be clinically identified in a gene associated with an autosomal recessive condition that is a strong fit for the phenotype, implying that a second variant (in *trans*) may be present but undetected. Here, we describe two such cases in which only a single heterozygous genetic variant in *RYR1* and *BBS1* genes were identified by CES. We reanalyzed the CES data with high clinical suspicion for a second variant and found that both individuals indeed carried a second heterozygous variant in *trans*. This report emphasizes the importance of reanalysis of CES data in undiagnosed cases in which only a single disease-associated variant is identified in an AR condition of interest.

METHODS

Subjects

Participant written informed consent and genomic data were obtained in accordance with the IRB-approved research protocol at the Manton Center for Orphan Disease Research of Boston Children's Hospital. Clinical records were reviewed.

Exome data reanalysis

The probands and parents were enrolled in the Gene Discovery Core of the Manton Center for Orphan Disease Research, under IRB-approved research protocols (03-08-128 R and 10-02-0053) at Boston Children's Hospital. Exome sequencing data were processed through the VExP [3] by using the BWA aligner (version 0.7.17) for mapping reads to the human genome (hg19) and Picard Tools (version 2.23.3) to mark/delete duplicate reads. Single nucleotide variants and small insertions/deletions were jointly called across all samples by using both GATK (multi sample variant calling, version 4.1) and SAMTools (version 1.10). Furthermore, VExP was performed to annotate 21 relevant genetic databases (from allele

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Table 1. List of recessive cases with known disease-causing genes identified by reanalysis.

Individual ID	Gene	HGVS_g (GRCh37)	Variants	Segregation	Variant identification	ACMG/ AMP Codes	Pathogenicity	Diagnosis (#OMIM)
1 (BEG_1599-01)	*RYR1*	NC_000019.9:g.38943477_38943478insCGGGAAGCCA	NM_000540.3:c.1263_1264insCGGGAAGCCA, (p.Gly422Argfs*86)	Maternal	Initial finding	PVS1, PM2, PM3	P	Minicore myopathy with external ophthalmoplegia (#255320)
		NC_000019.9:g.38985003_38985004insGAGCTGGTGTCCAC ATGGTGGTGCGCTGGGCCCAAGAGGACTTCGTGCAGAGCCCC	NM_000540.3: c.6286_6287ins57, (p.Glu2096delins20)	Paternal	Reanalysis	PM2, PM3, PM4	LP	
2 (MAN_2163-01)	*BBS1*	NC_000011.9:g.66293650_66293652del	NM_024649.5:c.1167_1169del, (p.Ile389del)	Maternal	Initial finding	PM2, PM3, PM4	LP	Bardet-Biedl syndrome (#209900)
		NC_000011.9:g.66293652 T > G	NM_024649.5:c.1169 T > G, (p.Met390Arg)	Paternal	Reanalysis	PS3, PS4, PM2, PP3	P	

P pathogenic; *LP* likely pathogenic.

frequency and gene-phenotype consortiums) and 23 coding/noncoding variant pathogenicity predictors into the output of the system. Variants were classified based on ACMG/AMP guidelines [6].

Literature analysis

We used "exome reanalysis" as key words to perform literature review in PubMed and identified 190 studies (accessed on September 21, 2022). We then manually reviewed them to identify 36 studies that fulfilled our criteria (exome reanalysis; cohort study; germline). Table S2 lists the exome data reanalysis articles and outcome from those 36 studies.

RESULTS

Individual 1

The proband is an 18-month-old female who was born at 39-week gestation via vaginal birth with a birth weight of 3.27 kg. She presented at birth with significant hypotonia and difficulty feeding due to poor latch requiring nasogastric tube feeding. No findings to suggest hypoxic-ischemic injury or seizure activity were identified. Chromosomal microarray revealed a microdeletion, arr[hg19] 15q24.2q24.3(76,529,086-76,611,347)x1. This interval contains part of the *ETFA* gene, which is associated with glutaric acidemia IIA, an autosomal recessive condition. The individual's presentation and metabolic testing did not match with the glutaric acidemia IIA phenotype, thus suggesting that she is a carrier for the condition. Genetic testing for myotonic dystrophy type 1, Prader-Willi syndrome and spinal muscular atrophy were all negative. Electromyogram and muscle biopsy were not performed.

Following discharge, her feeding concerns and failure to thrive improved, although hypotonia, delayed motor milestones and restricted extraocular movements continue to remain a significant concern. A CES was performed in the summer of 2021 and a heterozygous frameshift variant in the *RYR1* gene was identified and found to be inherited from her asymptomatic mother (NM_000540.3:c.1263_1264insCGGGAAGCCA, (p.Gly422Argfs*86)). Due to significant phenotypic overlap with RYR1-related myopathy, the family was enrolled in the IRB-approved research study in late 2021 and reanalysis was performed utilizing the VExP pipeline [3]. The research reanalysis revealed a second heterozygous variant in *RYR1*, which was inherited from the father (NM_000540.3: c.6286_6287ins57, (p.Glu2096delins20)) (Table 1). This newly identified variant was confirmed by Sanger sequencing and was classified as "likely pathogenic" based on ACMG criteria, thereby confirming the diagnosis of autosomal recessive *RYR1*-related congenital myopathy.

Individual 2

The proband is a 9-year-old male who was born by normal vaginal delivery at 42-weeks of gestation with a birth weight of 3.5 kg. A fracture of the right clavicle was noted soon after birth. At 2 years of age, he was noted to be obese. In addition, he had poor night vision and was nearsighted. He subsequently fractured his foot twice, once while playing in a bouncy house, and again while stepping down the stairs. He has a history of delayed speech, attention problems, and low energy. Pertinent family history includes macular degeneration in distant relatives. He continues to be in the 90-95 percentile in weight and BMI, around the 75th percentile in height, and has small genitalia.

An extensive evaluation was performed by several specialists. His ophthalmologic evaluation revealed retinal dystrophy, refractive amblyopia, high myopia, and esotropia of the right eye. On electroretinogram, there was marked attenuation of retinal responses, more so in scotopic than photopic conditions. An optical coherence tomography revealed a thin to absent outer lamina, which represents the photoreceptor layer. Renal evaluation showed mild nephrocalcinosis.

A CES was sent in consideration of his clinical findings during the summer of 2020. He was identified to carry one heterozygous

likely pathogenic variant in the *BBS1* gene, inherited from the mother (NM_024649.5:c.1167_1169del, (p.Ile389del)). The family was enrolled in the research study and reanalysis was performed in early 2021. A second heterozygous missense variant (NM_024649.5:c.1169 T > G, (p.Met390Arg)) (Table 1) inherited from the father was identified. The newly identified *BBS1* variant was also confirmed by Sanger sequencing, and the clinical report was reissued to the clinician. Based on ACMG criteria, both *BBS1* variants were classified as likely pathogenic, which confirmed the diagnosis of Bardet-Biedl Syndrome.

DISCUSSION

Here, we describe two cases of *RYR1*- and *BBS1*-related disorders whose genetic diagnoses were only confirmed and established following reanalysis of preexisting CES data. In both cases, the individuals were initially identified to carry only one pathogenic variant in a recessive gene that matched the observed phenotype. In such situations, careful reanalysis of the CES/clinical genome sequencing (CGS) data is imperative to ensure that a second *trans* variant is not missed. We reviewed the VCF files obtained from the sequencing facility to evaluate the reason for missing those variants. The second *trans* variant in *RYR1* was labeled as "potential false positive", likely due to the size of the non-frameshift indel (Table S1) while the second *trans* variant in *BBS1* failed to pass the quality metrics as it involved the adjacent nucleotide, and therefore was rejected by the sequencing variant caller. While both the missed variants were detected by our reanalysis pipeline [3], other more elusive variants such as small copy number variants (CNV) or transposon insertions [7] may be missed by CES, which may need bioinformatic pipeline optimization, CGS, or other approaches including RNA sequencing.

RYR1 encodes for the skeletal muscle-specific ryanodine receptor that serves as a Ca^{2+} release channel located at the terminal cisternae of the sarcoplasmic reticulum (SR) connecting the SR and transverse tubule required for excitation–contraction coupling [8]. Variants in *RYR1* are associated with a wide range of clinical phenotypes, including autosomal recessive *RYR1*-related myopathies (including central core disease and multiminicore myopathy), as well as autosomal dominant myopathies, exercise-induced myalgias, heat stroke, and malignant hyperthermia [9, 10]. The identification of a second variant in combination with the consistent presenting phenotype confirmed the diagnosis of recessive *RYR1*-related myopathy in Individual 1.

BBS is a genetically heterogeneous ciliopathy characterized by retinal degeneration/dystrophy, obesity, polydactyly, hypogonadism and genital abnormalities, intellectual disability, renal abnormalities, and behavioral dysfunction [11]. The *BBS1* gene mutated in Case 2 encodes Bardet-Biedl syndrome-1 protein, a component of the BBSome complex (BBS1–9) that is thought to be involved in ciliogenesis [12]. Recessive *BBS1* variants are associated with Bardet-Biedl syndrome (BBS) and non-syndromic retinitis pigmentosa [13]. *BBS1* is the most frequently affected gene in BBS, accounting for ~23% of reported cases in Europe and North America [14].

We reviewed the literature to evaluate the diagnostic outcome of CES reanalysis and identified 36 CES studies that reported new genetic diagnoses (1188/9489; 12.52%) and new recessive diagnoses (181/1188) (Table S2). Only three individuals from three (one each) of the 36 studies reported identification of a second variant when one variant was previously known (Table S3) [2, 15, 16]. This number represents a tiny fraction of the total number of new diagnoses made (3/1188; 0.25%) and new recessive diagnoses made (3/181; 1.65%) which suggests that either we are missing several of those diagnoses or such situations are truly rare. This needs to be further evaluated in large-scale reanalysis studies.

In conclusion, we identified a second *trans* heterozygous variant in two undiagnosed cases in which only one heterozygous variant in a condition of phenotypic interest was initially identified by clinical report. Similar cases have been reported in only a tiny fraction of reanalysis diagnoses, suggesting that this aspect of reanalysis needs further evaluation. Our work highlights the utility of CES reanalysis in undiagnosed AR cases when only a single variant in a disease-associated gene has been identified.

DATA AVAILABILITY

The genetic tests analyzed in this paper were ordered as clinical tests and thus the data are not able to be made available for privacy reasons, but these confirmed variants were submitted to ClinVar (#SUB12473625).

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AUTHOR CONTRIBUTIONS

QL, RA, KS, CAG, JAM, and PBA drafted the paper. All authors edited the paper and approved the final version.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

The study was conducted in accordance with the ethical standards of the participating Institutional Review Board at Boston Children's Hospital (IRB number 10-02-0053, approval date 20 May 2021).

ADDITIONAL INFORMATION

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