



Somatic variation as an incidental finding in the pediatric next-generation sequencing era

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Abstract The methodologic approach used in next-generation sequencing (NGS) affords a high depth of coverage in genomic analysis. Inherent in the nature of genomic testing, there exists potential for identifying genomic findings that are incidental or secondary to the indication for clinical testing, with the frequency dependent on the breadth of analysis and the tissue sample under study. The interpretation and management of clinically meaningful incidental genomic findings is a pressing issue particularly in the pediatric population. Our study describes a 16-mo-old male who presented with profound global delays, brain abnormality, progressive microcephaly, and growth deficiency, as well as metopic craniosynostosis. Clinical exome sequencing (ES) trio analysis revealed the presence of two variants in the proband. The first was a de novo variant in the *PPP2R1A* gene (c.773G > A, p.Arg258His), which is associated with autosomal dominant (AD) intellectual disability, accounting for the proband's clinical phenotype. The second was a recurrent hotspot variant in the *CBL* gene (c.1111T > C, p.Tyr371His), which was present at a variant allele fraction of 11%, consistent with somatic variation in the peripheral blood sample. Germline pathogenic variants in *CBL* are associated with AD Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia. Molecular analyses using a different tissue source, buccal epithelial cells, suggest that the *CBL* alteration may represent a clonal population of cells restricted to leukocytes. This report highlights the laboratory methodologic and interpretative processes and clinical considerations in the setting of acquired variation detected during clinical ES in a pediatric patient.

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INTRODUCTION

Exome sequencing (ES) uses next-generation sequencing (NGS) as an unbiased approach to simultaneously evaluate the protein-coding regions of all genes in the human genome. In the pediatric setting, ES has shown clinical utility in patients with suspected genetic disorders (Meng et al. 2017; Clark et al. 2018). In addition to identifying disease-associated findings primary to the indication for testing, this broad approach increases the potential for revealing incidental genomic findings that are secondary to the indication for testing. The American College of Medical Genetics and Genomics (ACMG) provides recommendations for reporting secondary findings in 73 genes deemed medically actionable (Miller et al. 2021), based on their association with cancer predisposition, cardiovascular disease, and inborn errors of

metabolism. These secondary findings are typically germline and consequently may impact other at-risk family members.

The use of NGS has the potential to produce depths of sequencing read coverage suitable to identify somatic variation, as indicated by variants present at reduced allele frequency/fraction (VAF) from that expected in a heterozygous or hemizygous germline state. Mosaicism is defined as the presence of two or more cell populations, derived from a single fertilized egg (zygote), that are genotypically distinct (Gajecka 2016). Mosaicism is an important contributor to disease-causing variation that can be identified by ES in the pediatric setting (Cao et al. 2019; Miller et al. 2020a). Somatic mosaicism results from genomic alterations that occur postzygotically (Biesecker and Spinner 2013; Lupski 2013; Acuna-Hidalgo et al. 2015). Somatic variants may be present at relatively low VAFs, which can be challenging to detect depending upon the sequencing coverage achieved. Therefore, sequencing to higher depths can enhance the potential to identify somatic mosaicism.

Recently, ACMG published guidance for the detection of incidental acquired variants in germline genetic and genomic testing, specifically surrounding genes commonly altered in clonal hematopoiesis (Chao et al. 2021). This ACMG statement provides points for consideration surrounding test performance and data analysis, variant interpretation and reporting, and clinical interpretation and diagnosis. Genomic DNA derived from the leukocytes within peripheral blood is commonly utilized for germline genetic testing. In the setting of clinical testing, alternate sources for DNA can often include saliva or buccal cells. Notably, prior studies have demonstrated varying cellular composition among buccal and saliva samples, with variability in the percentages of leukocytes and epithelial cells related to sampling, age of individual, oral health, and other factors (Theda et al. 2018).

Although ES is useful for identifying Mendelian disorders, it is important to consider that identified variation may be unique to the sampled tissue type. Discerning the etiology of somatic variation has important implications for patient management, surveillance, diagnosis, and counseling. In this report, we discuss the interpretative, methodologic, and clinical considerations surrounding the detection of a pathogenic somatic *CBL* variant, as well as a heterozygous likely pathogenic *PPP2R1A* variant, identified by clinical ES in peripheral blood from a boy with profound global delays and growth deficiency without a clinical diagnosis of Noonan syndrome or juvenile myelomonocytic leukemia (JMML).

RESULTS

Clinical Presentation and Family History

The proband is a 16-mo-old full-term male, referred to medical genetics at 8 mo of age due to prenatally noted ventriculomegaly, progressive microcephaly, and global developmental delay. Head circumference at birth was 33 cm ($Z = -1.98$), whereas later measurements showed 41 cm (8 mo; $Z = -3.22$) and 41.8 cm (3 yr; $Z = -6.24$). Postnatal brain magnetic resonance imaging (MRI) showed supratentorial volume loss and lateral ventriculomegaly, mildly enlarged third ventricle, and retrocerebellar cerebrospinal fluid (CSF) space. Head computed tomography (CT) was ordered because of an abnormal head shape and revealed metopic craniosynostosis. Other anthropometric measurement showed severe progressive growth deficiency with weights of 2.93 kg at birth ($Z = -1.05$), 7.85 kg at 8 mo ($Z = -1.26$), and 9.2 kg at 2 yr 10 mo ($Z = -4.33$), and lengths of 54 cm at birth ($Z = 0.51$), 69 cm at 8 mo ($Z = -0.69$) and 84.5 cm at 2 yr 8 mo ($Z = -2.46$). The child was noted with normally shaped but low-set ears, hypotonia, joint hypermobility, and severe scoliosis. An ophthalmological evaluation showed axial hypermetropia, exotropia, and delayed visual maturation. Spinal MRI showed moderate to severe dextrorotary T9–T10 scoliosis, which was progressive on serial imaging and required casting. At 3 years of age, he continues to have profound

Table 1. Exome sequencing (ES) trio analysis variant characteristics and classification

Gene (transcript ID)	Genomic change (GRCh37)	HGVS coding reference	HGVS protein reference	Variant type	Predicted effect	dbSNP	Genotype/inheritance
PPP2R1A (NM_014225.5)	Chr 19:52716329 G > A	c.773G > A	p.R258H	Missense	Likely pathogenic	rs863225094	Heterozygous/de novo
CBL (NM_005188.3)	Chr 11:119148891 T > C	c.1111T > C	p.Y371H	Missense	Pathogenic	rs267606706	Somatic

global delays and swallowing issues. He is not able to crawl or sit unsupported and has no specific words, although he is able to follow some simple directions. A complete blood count with differentials has been completely normal without leukocytosis, anemia, cytopenia, or other signs of JMML. Neither parent has a history of developmental delay, learning issues, or short stature.

Genomic Analyses and Variant Interpretation

Clinical ES trio analysis was performed on genomic DNA isolated from peripheral blood of the proband and parents. This analysis identified a de novo missense variant in the *PPP2R1A* gene (c.773G > A), resulting in the replacement of an arginine with a histidine at amino acid 258 (p.Arg258His) (Table 1; Fig. 1) in the seventh HEAT (huntingtin-elongation-A subunit-TOR) repeat motif. This variant was classified as likely pathogenic in accordance with the standards and guidelines for variant interpretation set forth by ACMG and the Association for Molecular Pathology (AMP) (Richards et al. 2015). In brief, the *PPP2R1A* variant was verified as a de novo change, with both maternity and paternity confirmed via in silico analysis of trio ES data and end-point polymerase chain reaction (PCR) of loci with high population minor allele frequency used to compare inherited genotypes (Miller et al. 2020a). This variant was absent from large-scale population databases (Karczewski et al. 2020), has been

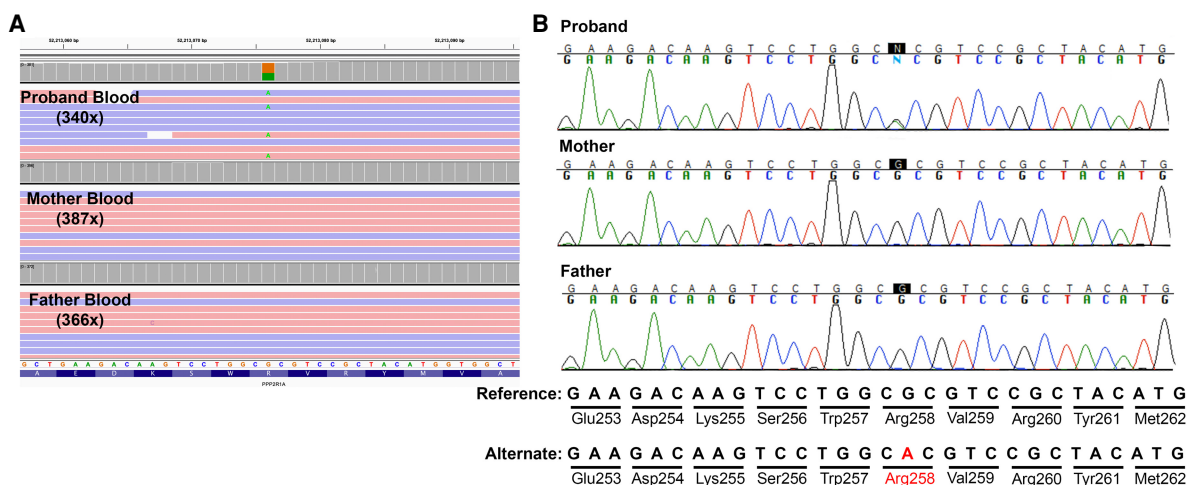


Figure 1. *PPP2R1A* variant identified by ES analysis and confirmation via Sanger sequencing. (A) De novo heterozygous *PPP2R1A* variant visualized by the Integrative Genomics Viewer (IGV) (reference base G in orange, variant base A in green) in the proband (top panel), with reference base detected at this position for samples from the mother (middle panel) and father (bottom panel). (B) Sanger sequencing analysis of the *PPP2R1A* variant visualized by Sequencher in the proband (top panel), mother (middle panel), and father (bottom panel) confirms the *PPP2R1A* variant is de novo in the proband.

reported in the literature with supporting in vitro functional studies suggesting a reduction in protein activity (Houge et al. 2015; Ji et al. 2019) hindering binding to the regulatory subunit B55 α encoded by *PPP2R2A* (Lenaerts et al. 2021), and is predicted to be damaging by multiple computational (in silico) functional prediction algorithms (ACMG/AMP: PS2, PM2, PM_PS3, PP3). The *PPP2R1A* variant was verified via orthogonal Sanger sequencing of proband and parental samples and observed only in the proband sample. This finding was considered to be diagnostic for the proband's clinical presentation.

The proband also harbored a somatic missense variant in the *CBL* gene (c.1111T > C) observed at a reduced VAF (11%) in ES resulting in the substitution of a tyrosine with a histidine at amino acid position 371 (p.Tyr371His) (Table 1; Fig. 2). Germline variants in the *CBL* gene are associated with autosomal dominant (AD) Noonan syndrome-like disorder with or without JMML (OMIM: 613563), a developmental disorder resembling Noonan syndrome with extensive phenotypic heterogeneity and variable expressivity (Martinelli et al. 2010). This variant was classified as pathogenic in accordance with ACMG/AMP guidelines (Richards et al. 2015). Through orthogonal studies, the *CBL* variant was visualized by Sanger sequencing at low level in the proband and was absent from both parents (ACMG/AMP: PS2), consistent with a somatic finding (Fig. 2). Multiple in vitro studies have associated this variant with up-regulation of critical genes in the RAS pathway and diminished E3 ligase activity

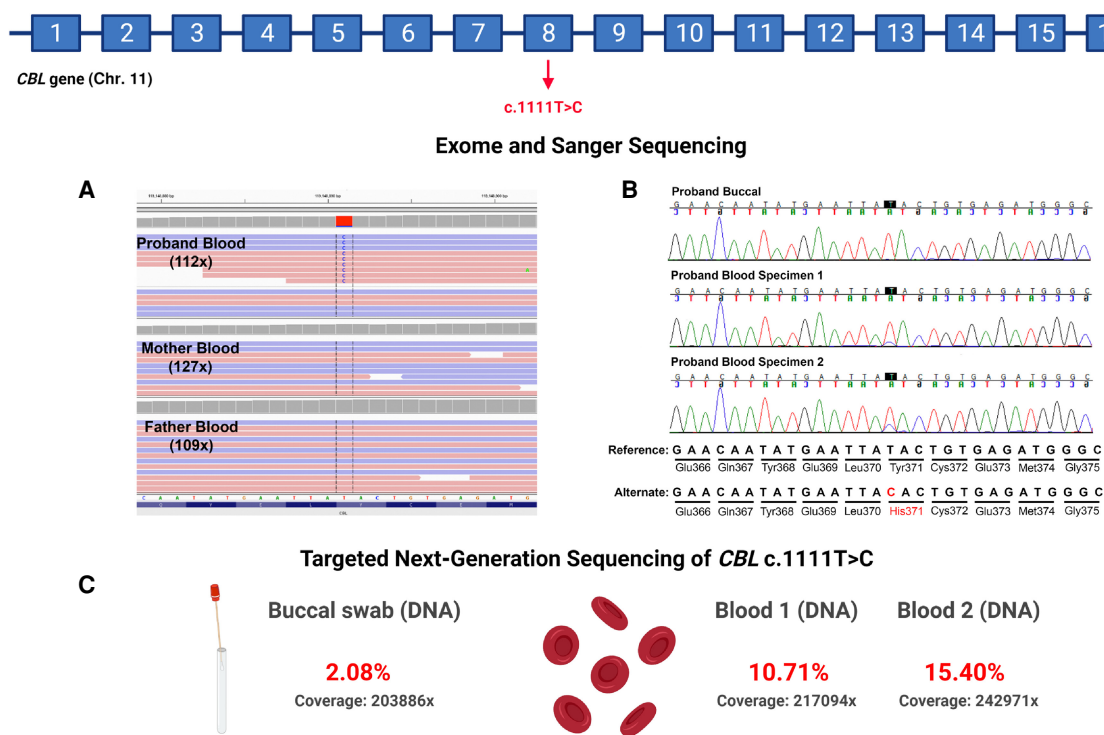


Figure 2. Identification of *CBL* variant. Diagram of the *CBL* gene, indicating the c.1111T > C variant in exon 8. (A) Somatic heterozygous *CBL* variant visualized by the Integrative Genomics Viewer (IGV) (reference base T in red, variant base C in blue) in the proband (top panel), with reference base detected at this position for samples from the mother (middle panel) and father (bottom panel). (B) Sanger sequencing analysis of the *CBL* variant visualized in Sequencher in the proband's buccal epithelium, exome sequencing (ES) peripheral blood sample (blood specimen 1), and post-ES peripheral blood sample (blood specimen 2). (C) Targeted next-generation sequencing (NGS) analysis for the *CBL* variant in buccal cells, ES peripheral blood sample (blood 1), and post-ES peripheral blood sample (blood 2). The percentage of the *CBL* variant and total coverage is indicated for each sample.

(ACMG/AMP: PS3) (Niemeyer et al. 2010; Javadi et al. 2013). This variant has been reported in multiple affected individuals with JMML and is a mutational hotspot, occurring at a conserved tyrosine kinase residue within the critical hotspot linker region of the protein (ACMG/AMP: PM1) (Kales et al. 2010; Loh et al. 2009). Last, this variant is predicted to be damaging by multiple computational (in silico) functional algorithms (ACMG/AMP: PP3).

To further discern the clinical significance of the *CBL* variant, subsequent sequencing analyses were performed on an alternate tissue source derived from the patient's buccal epithelium. Initial study by Sanger sequencing analysis on DNA obtained from the proband's buccal cells was negative for the *CBL* variant, suggesting this alteration may represent a clonal population confined to the peripheral blood (Fig. 2). This result was considered incidental or secondary to the primary indications for testing. The patient was referred to hematology/oncology for evaluation. Follow-up Sanger sequencing analysis was performed on peripheral blood obtained 12 mo post-ES analysis showed persistent presence of the *CBL* variant (Fig. 2).

Given the limitation in the detection of low VAF alterations by Sanger sequencing, targeted NGS analysis was performed on all available proband DNA samples including buccal epithelium and the initial and 12-mo post-ES blood. The *CBL* variant was present at reduced VAF in both the ES and 12-mo post-ES peripheral blood samples, at 11% and 15%, respectively. The *CBL* was also present in buccal cells at ~2%, a VAF unresolvable by the original initial Sanger analysis.

DISCUSSION

Trio exome analysis revealed two variants of interest within this proband. The de novo likely pathogenic *PPP2R1A* variant in the germline was considered clinically significant and felt to account for the proband's clinical features. The *PPP2R1A* gene encodes the α isoform of the scaffolding subunit of the serine/threonine protein phosphatase type 2A (PP2A) implicated in the negative control of cell growth and division (Janssens and Goris 2001). The subunit consists of 15 tandem repeat loops of HEAT motif (Xu et al. 2006). Alterations in HEAT loops of the scaffolding subunit are associated with AD neurodevelopmental disorder, along with other genes encoding PP2A subunits *PPP2R5D*, *PPP2R5C*, *PPP2R5B*, *PPP2CA*, and *PPP2R2C*. *PPP2R1A*-related disease (OMIM:616362) is characterized by mild to severe intellectual disability, abnormal head size (microcephaly or macrocephaly), hypotonia, severe motor delay, severe speech delay, and brain malformations (corpus callosum hypoplasia, ventriculomegaly, periventricular leukomalacia) (Houge et al. 2015; Lenaerts et al. 2021). Ptosis, hearing loss, ear-shape abnormality, seizures, scoliosis, joint hypermobility, and short stature have also been reported. Previously reported cases with the *PPP2R1A* variant affecting p.Arg258 showed significant microcephaly, developmental delay, and brain abnormality (hypoplastic corpus callosum and ventriculomegaly), but scoliosis or growth deficiency was not reported (Lenaerts et al. 2021). Craniosynostosis has not been previously reported with *PPP2R1A*-related disease. Craniosynostosis, progressive scoliosis, and severe growth deficiency seen in our proband may suggest that serine/threonine phosphatase is also involved in osteogenic differentiation (Karkache et al. 2021).

The second variant, a previously described pathogenic somatic variant within the *CBL* gene, was of uncertain significance in relation to the proband's clinical presentation and of unknown origin. The *CBL* gene is a proto-oncogene that encodes a RING finger E3 ubiquitin ligase and acts as both a negative regulator of several receptor protein tyrosine kinase signaling pathways by targeting receptors for degradation and as an adaptor protein in tyrosine phosphorylation-dependent signaling (Thien et al. 2001; Fu et al. 2003). The domain structure of the CBL protein comprises an amino-terminal tyrosine kinase binding (TKB) domain followed by a linker region, RING domain, proline-rich region (PRR), carboxy-

terminal phosphorylation sites, and ubiquitin association domain (Thien et al. 2001). Pathogenic variation in *CBL* is described in association with Noonan syndrome–like disorder with or without JMML, characterized by facial dysmorphism, a wide spectrum of cardiac defects, reduced growth, variable cognitive deficits, and musculoskeletal anomalies. Individuals with germline and somatic alterations in the *CBL* gene have an increased risk of developing JMML (OMIM: 607785), an aggressive myeloproliferative and myelodysplastic neoplasm of early childhood characterized by excessive macrophage/monocyte proliferation (Loh et al. 2009; Niemeyer et al. 2010; Pérez et al. 2010; Pathak et al. 2015).

VAFs < 30% detected in the setting of germline NGS analysis warrant further evaluation including examination of local sequence context, mapping quality, variant quality, and strand bias, in addition to orthogonal methodologic verification. Variants meeting the aforementioned quality verification may be attributed to varying etiologies including somatic mosaicism, a hematologic disease process, or, commonly, an acquired clonal change in hematopoietic precursor cells (Miller et al. 2020a; Chao et al. 2021). Distinguishing variant etiology is important and has ramifications in relation to diagnosis, surveillance and management, and counseling as related to recurrence risk (such as in the setting of gonadal or gonosomal mosaicism). Clonal hematopoiesis of indeterminate potential (CHIP) is characterized by the detection of acquired alterations in hematopoietic-associated genes in individuals without evidence of hematological malignancy (Heuser et al. 2016; Karner et al. 2019; Steensma and Ebert 2020).

CHIP is associated with an increased risk of development of hematologic neoplasms and generally increases in prevalence with advancing age. The prevalence of CHIP in healthy children is thought to be extremely rare and has not been extensively studied to date in this cohort. CHIP variants have recently been reported to occur at leukemic hotspots in <1% of peripheral blood samples from a noncancer pediatric cohort (Feusier et al. 2021). Specific genes have been identified at high risk for acquiring somatic alterations, including *TET2*, *ASXL1*, *DNMT3A*, *TP53*, *SETD2*, *CHEK2*, *ATM*, *NF1*, and *CBL* (Chao et al. 2021).

Of note, the *CBL* p.Tyr371His variant was not absent from large population databases (gnomAD; Karczewski et al. 2020). Within gnomAD v2.1.1 this variant was present in samples derived from three individuals with ages ranging from 50 to 80 yr. It was detected among two exome analyses, and one genome, with retention even among the gnomAD v2.1.1 noncancer data set. Although the genome analysis flagged this variant as failing the random forest filter in gnomAD v2.1.1, gnomAD v3.1.1 displays this as a passing variant. Representative Integrative Genomics Viewer (IGV) images in gnomAD demonstrate that two of the three variants occurred at reduced VAF, thus highlighting the occurrence of somatic variation in population databases.

The importance of *CBL* to myeloid differentiation and cancer is likely due to its role in numerous signaling pathways and biological functions (Lyle et al. 2019). Heterozygous germline variants in the *CBL* gene have been detected in unrelated patients with a Noonan syndrome–like disorder with JMML (Loh et al. 2009; Martinelli et al. 2010; Niemeyer et al. 2010; Pérez et al. 2010; Hecht et al. 2020). Within the COSMIC database there are 30 entries for the p.Tyr371His variant (<https://cancer.sanger.ac.uk/cosmic>; last accessed October 4, 2021). The histology subtype most frequently reported to harbor the p.Tyr371His variant is JMML (20), followed by acute myeloid leukemia (3), chronic myelomonocytic leukemia (3), acute leukemia of ambiguous lineage (1), chronic myeloid leukemia (1), myelodysplastic syndrome (1), and an astrocytoma Grade IV (1). The majority of oncogenic alterations in *CBL* are missense substitutions which inactivate the ubiquitin ligase activity of *CBL*, while retaining its downstream signaling functions, resulting in oncogenic activation of signaling pathways (Loh et al. 2009; Makishima et al. 2009). Notably, within JMML the p.Tyr371 is a “hotspot” that it is rarely altered in other myeloid malignancies. *CBL* variants in JMML and myeloid malignancies are associated with somatically acquired isodisomy, resulting in loss of the wild-type allele (Loh et al. 2009).

The breadth and depth associated with NGS-based ES methodologies enable the potential for detecting somatic variation, and increasingly such variation is recognized in relation to acquired clonal variants from precursor cells (Chao et al. 2021). When a variant is suspected to be somatic in origin, analysis of an additional tissue source is recommended to discern whether the variant is acquired with restriction to a hematopoietic lineage or somatic mosaic in nature. In our study, an additional tissue source was requested (buccal epithelium) to aid in resolving the etiology and clinical significance of the *CBL* variant. Targeted NGS analysis showed the presence of the *CBL* variant at 2% amid the buccal epithelium derived DNA. Although this may represent low-level mosaicism, studies have shown buccal samples contain varying levels of hematopoietic cells, specifically leukocytes (Theda et al. 2018; Chao et al. 2021). We hypothesize that admixed leukocytes amid the collected buccal sample are a likely contributor to the observed VAF, with the most likely explanation that the *CBL* variant is acquired and restricted to a clonal population of hematopoietic origin. Targeted NGS analysis also showed an increase in the VAF between the blood sample obtained for ES and the sample obtained 12 mo post-ES, at 11% and 15%, respectively. More data points are needed to discern if this patient exhibits clonal expansion over time.

Our established exome workflow incorporates phenotypic overlap between clinician reported clinical features and disease-association among genes harboring rare variation. Additionally, ClinVar reported pathogenic or likely pathogenic variants are prioritized for review in our pipeline. The *CBL* variant was present in the proband's exome sequencing results because of phenotypic overlap for global developmental delay and its presence within ClinVar as a pathogenic variant (VCV000013811.5). This variant was further investigated because of reduced VAF. The presence of the de novo likely pathogenic variant in *PPP2R1A* was thought to account for the proband's clinical presentation as discussed with the clinical geneticist following this patient. Thus, the *CBL* variant was reported as an incidental finding and targeted sequencing in another tissue source was recommended. Our ES consent includes an option to receive medically actionable variants unrelated to the patient's medical condition, which includes the ACMG 3.0 recommendations (Miller et al. 2021) and any additional genes the laboratory has determined to be likely medically actionable. Although the ACMG 3.0 secondary findings list does not currently include *CBL*, the recent ACMG recommendations surrounding incidental detection of acquired variants does include *CBL* as one of the genes commonly altered in clonal hematopoiesis (Chao et al. 2021). Notably within our internal ES cohort ($n = 780$) this is the first and only somatic *CBL* variant to be identified and reported.

Although there are currently no established National Comprehensive Cancer Network (NCCN) screening guidelines surrounding findings within the *CBL* gene, there are monitoring options for management and surveillance. Regular physical examinations and complete blood counts can be performed in children with CBL syndrome (Villani et al. 2017). Our proband is currently 3 years of age; he exhibits no signs of JMML including hepatosplenomegaly, cytopenias, and absolute monocytosis. At the time of this report, the proband's complete blood count (CBC) remains within normal range, measurement of which was recommended to be obtained every 3 mo per the clinical care team. This finding illustrates that incidental acquired findings may result in additional clinical management and interventions. Our findings highlight the challenges associated with the interpretation of somatic variation and the methodologic and clinical considerations in such instances.

METHODS

Sequencing, Bioinformatics, and Quality Control

Proband and parental peripheral blood samples were submitted for clinical ES to The Steve and Cindy Rasmussen Institute for Genomic Medicine at Nationwide Children's Hospital,

Table 2. Metrics for ES coverage

Gene (transcript ID)	Target mean coverage	Target coverage 20× breadth (±20 bp padding around each exonic region)
<i>PPP2R1A</i> (NM_014225.5)	189×	100%
<i>CBL</i> (NM_005188.3)	118×	100%

Columbus, Ohio. Genomic DNA extraction from peripheral blood and genotyping assay using a custom Agena MassArray panel (Agena) to ensure sample provenance and verify familial relationship was performed as described by Miller et al. (2020a). Libraries were subject to target capture using SureSelect Human All Exon V6 (Agilent) followed by paired-end 151-bp sequencing to 137× mean depth on a HiSeq 4000 (Illumina), with 96.5% of targeted bases at 20× or greater. Metrics for ES coverage specific to *PPP2R1A* and *CBL* are shown in Table 2.

Exome sequencing data were demultiplexed using Illumina bcl2fastq Conversion Software. Secondary analysis was performed using Churchill 2.0, which utilizes BWA-MEM to align sequence reads to the reference sequence (GRCh37/hg19 Feb 2009), Picard Tools for deduplication, and GATK Unified Genotyper for single-sample variant calling (Kelly et al. 2015). Mitogen Genetics (Sunquest) was used for annotation and tertiary analysis filtering informed by clinician-provided phenotypes, which were converted into Human Phenotype Ontology (HPO) terms (Köhler et al. 2017).

As standard practice in our exome workflow, BAM files are reviewed for all assessed variants using the IGV (Broad) (Robinson et al. 2011) to manually examine the read count and strandedness, location of the variant within the read, VAF, homology, mapping, and read quality. Genomic regions with known homology as defined by Mandelker et al. (2016) have been incorporated through BED file track into IGV to visually flag the level of homology of the local region. Identity-confirmed parental sequences were also reviewed along with the proband data for side-by-side comparison and the IGV alignment preferences were set to allow for display of coverage allele-fraction threshold at 2% and retention of soft-clipped reads. Sanger sequencing of de novo and suspected somatic variants was performed on the proband and available parental samples. PCR amplification followed by Sanger sequencing and data analysis was performed as described by Miller et al. (2020a).

The genomic DNA template derived from peripheral blood was added to Platinum SuperFi II PCR Master Mix (Invitrogen) along with *CBL* primers (forward (5'-GGA AACAAGTCTTCACTTTT-3') and reverse (5'-GCTCAATCTTTACATCCTTATC-3')) and amplified by PCR as previously described (Cottrell et al. 2021). Because of limited residual volume associated with the buccal samples, DNA was incorporated into a 50-μL solution of 1× Platinum SuperFi II PCR Master Mix (Invitrogen) and 20 nM *CBL* primers in the sample tube for 10 min at 37°C. Following incubation, we performed two 25-μL PCR reactions, as outlined above. Finally, products were purified using 1.8× SPRIselect and used as DNA template in a second round of PCR. Amplified products were purified using 1.5× SPRIselect and processed as described (Miller et al. 2020b; Cottrell et al. 2021). Sequencing of the pooled libraries (amplicon libraries; Miller et al. 2020b) occurred on an Illumina iSeq 100. Metrics for targeted NGS coverage specific to *CBL* are shown in Table 3.

Raw sequencing data were converted to unaligned BAMs by Picard Tools IlluminaBasecallsToSam (v2.19.0) to preserve the unique molecular identifier (UMI) information. Alignment was performed to the GRCh38 reference using BWA-MEM (v0.7.15) and deduplicated using GATK's MarkDuplicates (v4.1.9), considering both the alignment positions and the UMIs to identify duplicates. Base counts of the reads at the variant site were calculated using BAM-read count (v0.8.0) and visually inspected using IGV.

Table 3. Metrics for targeted next-generation sequencing (NGS)

Sample	Total reads	Unique mapping reads	Target mean coverage
Blood specimen 1	1,818,744	583,440	210,143×
Blood specimen 2	1,942,102	545,189	235,867×
Buccal	1,589,282	564,599	195,879×

Variant Interpretation

Custom scripting allowed for enrichment of variant attribute data including disease association and phenotype overlap. For each proband, the annotated filtered variant list was evaluated at a consensus case conference attended by clinical laboratory directors, genetic counselors, variant scientists, residents, fellows, and ordering providers when available. Variants that met group consensus were assessed according to the ACMG/AMP guidelines (Richards et al. 2015). Following assessment, variants are reported at the discretion of the clinical laboratory director as either likely causal for proband phenotype or as findings of undetermined clinical relevance to the proband phenotype based upon strength of phenotype overlap with the associated disease.

ADDITIONAL INFORMATION

Database Deposition and Access

Variant data have been deposited into the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) under accession numbers VCV000013811.5 and VCV001738402.1.

Ethics Statement

Oral consent was obtained from the family for publication. The work presented herein was approved by the institutional review boards (IRBs) designated to oversee and monitor biomedical human subject research at Nationwide Children's Hospital (IRB18-0062).

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Author Contributions

M.Me., M.T.M., C.E.C., and K.L. conceptualized the study; V.J., M.T.M., C.E.C., and K.L. performed the formal analysis; V.M., C.E.C., and K.L. established the methodology; V.J., S.A.W., and B.J.K. performed the investigation; C.M., M.Mo., and A.E.J.-K. provided resources; M.Me., V.J., and K.L. visualized the project; M.Me., M.T.M., and K.L. wrote the original draft; M.Me., M.T.M., M.Mo., A.E.J.-K., V.M., E.R.M., C.E.C., and K.L. reviewed and edited the manuscript; and M.T.M., C.E.C., and K.L. supervised the project.

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Competing Interest Statement

The authors have declared no competing interest.

Referees

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