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RESEARCH ARTICLE

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De novo mutation and skewed X-inactivation in girl with *BCAP31*-related syndrome

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Abstract

Full genome analysis of a young girl with deafness, dystonia, central hypomyelination, refractory seizure, and fluctuating liver function impairment revealed a heterozygous, de novo variant in the *BCAP31* gene on chromosome Xq28 (NM_001256447.2:c.92G>A), mutations of which caused the X-linked recessive severe neurologic disorder deafness, dystonia, and cerebral hypomyelination. Reverse transcription-polymerase chain reaction of the patient's white blood cells showed the absence of wild-type *BCAP31* messenger RNA (mRNA) but the presence of two novel *BCAP31* mRNAs. The major alternatively spliced mRNA is due to Exon 2 skipping and the utilization of a new initiation site in Exon 3 that leads to a frameshift and truncated transcript while the minor novel mRNA has a 110 nucleotide insertion to Exon 2. Phasing studies showed that the de novo variant arose in the paternal X chromosome. X chromosome inactivation assay was done and confirmed that the patient's maternal X chromosome was preferentially inactivated, providing evidence that the mutated *BCAP31* gene was the one predominantly expressed. According to the American College of Medical Genetics and Genomics guideline, this variant is deemed "pathogenic" (PS2, PS3, PM2, PP3, and PP4) and deleterious. This is the first reported female patient in *BCAP31*-related syndrome resulted from skewed X-inactivation and a de novo mutation in the active X chromosome.

KEYWORDS

BCAP31, DDCH, female with X-linked recessive disease, splicing variants, X-inactivation

1 | INTRODUCTION

Developmental delay and congenital neurologic disorder are rare conditions caused by gene mutations that are hard to identify due to phenotypic and genetic heterogeneity. While whole exome sequencing has been successful in identifying some causal mutations, most cases remain unsolved. Since 2012, multiple groups have reported that males with hemizygous defect in *BCAP31* gene on Xq28 involving splicing site mutations, nonsense, or small indels result in severe phenotypes such as deafness, dystonia, and cerebral hypomyelination (DDCH; MIM# 300475) or contiguous *ABCD1/DXS1375E* deletion syndrome (Albanyan, Al Teneiji, Monfared, & Mercimek-Mahmutoglu, 2017; Cacciagli et al., 2013; Rinaldi, Van Hoof, Corveleyn, Van Cauter, & de Ravel, 2020; Shimizu et al., 2020; Vittal, Hall, & Berry-Kravis, 2015; Vittal, Hall, Dames, Mao, & Berry-Kravis, 2016; Whalen et al., 2019). These phenotypes are also found with microdeletions between *SLC6A8* and *ABCD1* or partial deletion of *SLC6A8-BCAP31* (Calhoun & Raymond, 2014; van de Kamp et al., 2015; Osaka et al., 2012). *BCAP31* encodes B-cell receptor-associated protein 31, with ubiquitous expression and is abundant in endoplasmic reticulum (ER) membrane.

BCAP31 is found to be involved in the anterograde transport of ER-to-Golgi exchanges, in ER associated-degradation and in caspase 8-mediated apoptosis (Annaert, Becker, Kistner, Reth, & Jahn, 1997; Nguyen, Breckenridge, Ducret, & Shore, 2000; Wakana et al., 2008).

Among the reported cases involving *BCAP31* mutations, only one derived from a de novo mutation (Rinaldi et al., 2020). Most male patients inherited mutated chromosome X (chrX) from nonsymptomatic carrier mothers; however, in two of the reported cases, the patients' mothers presented mild features. In addition, one heterozygous mother has bilateral sensorineural hearing loss (Albanyan et al., 2017), and another heterozygous mother has similar facial features to her two affected sons, such as long face and ocular hypotelorism, but no neurological or cognitive symptoms (Vittal et al., 2016).

Female carriers of X-linked disorders such as DDCH are normally not affected because they carry a normal copy of the gene. However, to compensate for the dosage effect of different X chromosome numbers in male (XY) and female (XX), X chromosome inactivation (XCI) happens in female cells (Minks, Robinson, & Brown, 2008). XCI is an epigenetic remodeling process through random methylation of each parental chromosome at the gastrulation or even as early as the 8–16 cell stage. (Amos-Landgraf et al., 2006; van den Berg et al., 2009; Morey & Avner, 2011; Shvetsova et al., 2019). Once XCI pattern is established in each cell, it is stably inherited in its somatic cell lineages. Individuals who have balanced inactivation between paternal and maternal X chromosomes present 50:50 XCI in cell pools. However, some individuals are found to have preferential inactivation of one parental X chromosome, resulting in skewed XCI, commonly defined as having an unbalanced XCI ratio (inactivation to activation) of 75–80%, and in extremely skewed XCI with an XCI ratio of 90–95% (Orstavik, 2009). Theoretically, a female carrying a mutation in gene associated with an X-linked recessive disorder will be symptomatic if she has skewed XCI in which the X chromosome with the wild-type allele is preferentially inactivated.

In this report, we present evidence that the molecular defect of a young girl with DDCH, refractory seizure, and fluctuating liver function abnormalities is due to a deleterious de novo *BCAP31* mutation in her paternally derived X chromosome and skewed X-inactivation of her maternally derived X chromosome. This is the first report of a female patient of *BCAP31*-related syndrome due to a pathogenic mutation in *BCAP31* in the context of skewed X-inactivation.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and full genome analysis pipeline

Fresh peripheral blood samples were collected from the patient at age 4 and her two biological parents. After removing the red blood cells (RBCs) by RBC lysis buffer (Qiagen), the high-molecular weight genomic DNA (HMW gDNA) was extracted from the white blood cells (WBCs) of each sample by a plug lysis process with the Bionano Prep™ Kit (Bionano Genomics). HMW gDNA from the trio then underwent 10× Genomics (10× G) Linked-Reads Chromium whole genome sequencing (WGS) on the NovaSeq 6000 Sequencing System (Illumina) to 40–60× read depth and optical mapping on the Bionano Genomics Saphyr System to 60× coverage. The FASTQ files were processed and aligned with GRCh38 (hg38; Genome Reference Consortium) via Long Ranger BASIC and ALIGN Pipelines (10× G) for linked-read alignment, variant calling, phasing, and structural variant calling. The de novo, phased genome assemblies of the patient and her two biological parents were compared to identify single nucleotide variants (SNVs) and structural variants (SVs). After filtering out synonymous SNVs and common SNVs/SVs based on data from the 1000 Genomes Project, Exome Aggregation Consortium, and Genome Aggregation Database (gnomAD), the genes affected by SNVs, SVs, or a combination of SNVs and SVs were examined in detail. In consultation with the clinicians, a thorough literature search was done on candidate genes with variants (both SNVs and SVs) predicted as deleterious by multiple tools in the web-based wANNOVAR suite (http://wannovar.wglab.org/) to look for genotype–phenotype correlation (Yang & Wang, 2015). All candidate variants were confirmed by Sanger sequencing of polymerase chain reaction (PCR) products amplified from the individuals. The following primers were used for the *BCAP31* mutation: forward 5′-AGCGAACGGA AGTTTGTG-3′ and reverse 5′-GGTGGGAGGGCATCATTT-3′.

2.2 | X chromosome inactivation assay

The HUMARA (Human Androgen Receptor) assay (Allen, Zoghbi, Moseley, Rosenblatt, & Belmont, 1992; Bertelsen, Tumer, & Ravn, 2011; Echevarria et al., 2016) was used to determine the ratio of inactivated paternal versus maternal X chromosomes. Briefly, gDNA samples (0.25 μg) were incubated with 10 U *Hpa*II (New England Biolabs) at 37°C overnight followed by heat inactivation of the enzyme at 80°C for 20 min. The highly polymorphic androgen receptor promoter region of the *Hpa*II-treated samples and untreated control samples were amplified with 0.2 μM forward primer 5′-FAM - TCCAGAATCTGTTCCAGAGCGTGC-3′ and 0.2 μM reverse primer 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′ with 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.025 U AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific) by PCR amplification (initial denaturation at 95°C for 6 min followed by 35 cycles of 40 s at 95°C, 30 s at 60°C, and 1 min at 72°C and a final extension step at 72°C for 2 min). After adding the GeneScan 500 LIZ dye Size Standard (Thermo Fisher Scientific), the mixtures were analyzed on an ABI 3730 DNA Analyzer with GeneMapper software 3.7 (Applied Biosystems). XCI ratio was estimated by the area of the smaller base size (XC_1) and larger base size (XC_2) peaks, with formula as follows:

$$\text{peak area of } [(XC_1^{\text{digested}}/XC_1^{\text{nondigested}})/((XC_1^{\text{digested}}/XC_1^{\text{nondigested}}) + (XC_2^{\text{digested}}/XC_2^{\text{nondigested}}))]$$

2.3 | RNA extraction and RT-PCR

The WBCs were harvested in TRIzol® reagent (Thermo Fisher Scientific) and total RNA was isolated according to manufacturer's instructions. Complementary DNA was prepared from 1 μg total RNA, using the SuperScript® VI Reverse Transcriptase with random hexamers, following manufacturer's protocol (Thermo Fisher Scientific). Reverse-transcription polymerase chain reaction (RT-PCR) was done using the primer pair of *BCAP31*-ex1F 5′-CACGTTGACTGT GGGAAACTC-3′ and *BCAP31*-ex4R 5′-TTCCGTCACATCATCATACT TCC-3′. All RT-PCR products were gel extracted and sequenced to confirm the normal splicing, intron inclusion, and exon skipping forms.

2.4 | Ethical compliance

Institutional ethical review and approval was granted (IRB No. AS-IRB01-19041(N)), and informed consent was provided, for all data obtained from patients.

3 | RESULTS

3.1 | Clinical features of the patient

The female subject was the second child of the family born at 38 weeks with a birth body weight of 2,990 g. There were no remarkable perinatal events or maternal history. Bilateral deafness, hypothyroidism, and ventricular septal defects Type II were noted in the neonatal period. Episodic dyskinetic movements began at 6 months of age that progressed to general dystonia by 11 months. These dyskinetic/dystonia movements presented as eye deviation and head deviation to the left associated with tongue protrusion, tonic movements of bilateral upper, and lower extremities lasting 3–60 s per episode, with frequency of up to 40 episodes per hour. Brain magnetic resonance imaging (MRI) done on several occasions showed mild cerebral atrophy with bilateral ventricular dilatation, thinning of corpus callosum, decreased subcortical and periventricular white matter and delayed myelination (Figure 1). Electroencephalogram done on several occasions were remarkable for global cortical dysfunction and paroxysmal discharges mainly over left hemisphere. Concurrent problems included profound developmental delay, failure to thrive, intractable focal to bilateral tonic-clonic seizures, fluctuating liver function impairment (alanine transaminase/aspartate transaminase = 227/148 U/L; normal range 0–41/8–31 U/L), and recurrent aspiration pneumonia. There was no elevation of creatine kinase (296 U/L; normal range 30–223 U/L) or lactate (1.26 mM; normal range 0.5–2.2 mM). Muscle biopsy only showed neurogenic myopathy with normal electron transfer chain activity. *POLG* mutation analysis, tandem mass of metabolites, and dried blood spot 3-O-methyldopa level were negative. She had progressive deterioration with febrile episodes and passed away at 5 years old. Exome sequencing by an outside group performed previously using a candidate gene panel analysis approach for intellectual disability (that did not include the *BCAP31* gene), followed by whole exome analysis based on the phenotypes of intellectual disability, epilepsy, hypotonia, and MRI abnormalities, did not reveal any candidate variants.

3.2 | Identification of a de novo variant in *BCAP31* by full genome analysis

Since exome sequencing did not reveal candidate variants in the primary screen, we performed Chromium (10× G) Linked-Reads WGS on the patient and her biological parents. All variants were called and phased with Long Ranger BASIC and ALIGN Pipelines and followed by filters of allelic frequency with threshold set at 0.01 for missense, indels, and splice site mutations. Then, the remaining variants after filtering were evaluated according to three possible genetic models: autosomal recessive, autosomal dominant, and X-linked recessive. As the parents were without the disorder, only de novo variants were considered in the autosomal dominant and X-linked recessive models. After determining that the sequence quality was high by expert inspection, and literature review that showed good correlation with the associated clinical phenotype, we identified a de novo heterozygous

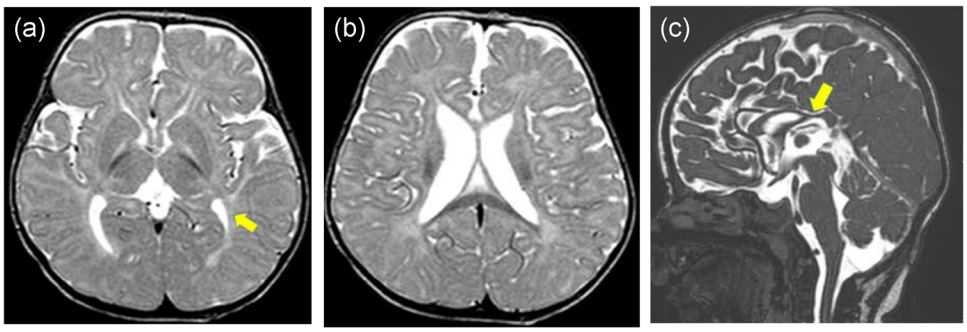

FIGURE 1 Brain MRI of the patient at 7 months old showing (a) reduced subcortical and periventricular white matter volume and hypomyelination with absence of myelination at optic radiation (arrow), (b) global cerebral atrophy and bilateral ventricular dilatation, and (c) thin corpus callosum (arrow)

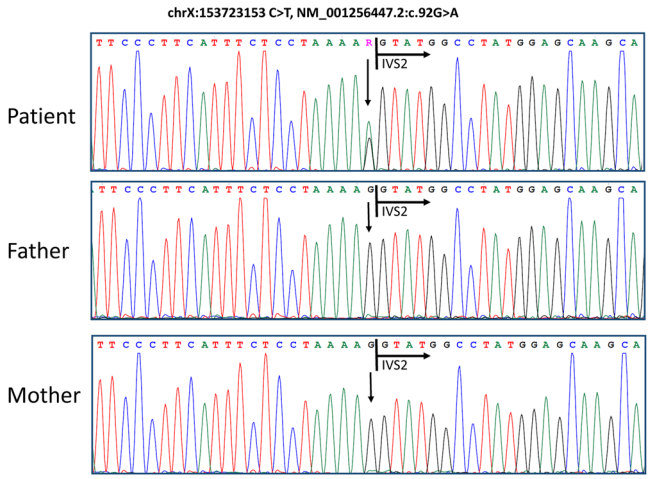

FIGURE 2 Sanger sequencing confirmed a de novo mutation in *BCAP31* gene from linked-reads WGS analysis pipeline for the trio. Patient carried a de novo T allele (arrow, shown as A in minus strand) on chrX:153723153, in *BCAP31* Exon 2. The de novo mutation located at the end of Exon 2. WGS, whole genome sequencing

variant in the *BCAP31* gene as a prime candidate for the patient's condition. This mutation is confirmed by Sanger DNA sequencing and is found on the plus strand of hg38 chrX:153723153C>T that predicts to cause NM_001256447.2:c.92G>A, p.Arg31Lys change (Figure 2). This novel variant has not been reported in Taiwan BioBank (https://taiwanview.twbiobank.org.tw/browse38) or any other database. Even with careful analysis of the sequencing and Bionano optical mapping data, no other disease-causing variant (including SNVs, small indels, and structural variations) could be identified in the patient's *BCAP31* gene (data not shown).

3.3 | Novel *BCAP31* alternative splicing transcripts in patient

The novel variant (NM_001256447.2:c.92G>A) sits at the second anticodon that encodes the 31th amino acid of *BCAP31* which is highly conserved in vertebrates. Moreover, the variant is part of a consensus 5′ donor splice site sequence at the end of the exon. We evaluated the wild-type and variant allele sequence by the Human Splicing Finder (HSF; http://umd.be/Redirect.html; Desmet et al., 2009) to see whether the variant would cause any changes to the splice site. The HSF predicts that the wild-type G allele position is a donor splice recognition site (underlined G in CTCCTAAAAG gtatggccta), but that the mutant A allele will alter the donor site recognition, thereby disrupting proper splicing.

To confirm the hypothesis that the A variant in the patient's *BCAP31* gene would lead to improper splicing, we examined the transcript pattern of WBCs in the patient and her parents by RT-PCR followed by Sanger sequencing (Figure 3). The results showed that there were no wild-type transcript in the patient's WBCs, but that

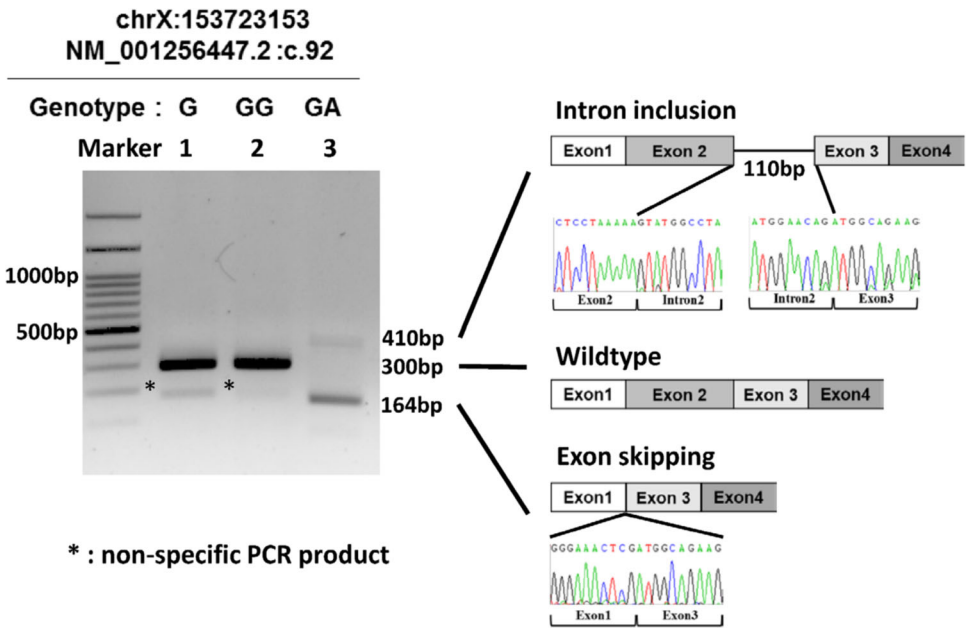

FIGURE 3 Novel *BCAP31* alternative splicing transcripts in WBCs in the patient. RT-PCR was performed with cDNA from WBCs carrying different genotype at chrX:153723153 (Lane 1: Father [G], 2: Mother [GG], and 3: Patient [GA]) using *BCAP31*ex1F forward primer and *BCAP31*ex4R reverse primer. The mother expressed the wild-type transcript (300 bp) exclusively. The father also expressed the wild-type transcript exclusively (*: the faint band at 184 bp is a nonspecific PCR product). The patient does not express the wild-type transcript. Instead, she expressed two novel alternative spliced transcripts: a major transcript (164 bp) that is devoid of the entire Exon 2 and a minor form (410 bp) with a partial (110 bp) insertion of Intron 2. Both transcripts are predicted to result in early terminated immature translation. cDNA, complementary DNA; RT-PCR, reverse-transcription polymerase chain reaction; WBCs, white blood cells

two novel transcripts were found. The major novel transcript is devoid of Exon 2 (NM_001256447.2:r.54_190del, p.Ser2AlafsTer42) while the minor transcript has a 110nt insertion in Intron 2 (NM_001256447.2:r.190_191ins[g>a;190+1_190+110], p.Arg31LysfsTer11). The major transcript leads to a frameshift transcript utilizing a new start codon site in Exon 3.

In summary, a de novo heterozygous variant in the patient results in a novel frameshift transcript that replaces the wild-type form of *BCAP31*. This variant is deemed pathogenic (ACMG criteria PS2, PS3, PM2, PP3, and PP4), according to de novo variant (PS2), RT-PCR study (PS3), absent from controls (PM2), deleterious effect on the gene product (PP3), and clinical phenotype (PP4) based on the criteria of ACMG/AMP 2015 guideline (Li & Wang, 2017).

3.4 | Skewed XCI in the patient

Because the patient was a girl, we expected that she would express both wild-type and mutant transcripts in her tissues. However, she only expressed the defective frameshift *BCAP31* transcript and her clinical features resembled those of X-linked recessive male patients carrying *BCAP31* mutations. Haplotype phasing with linked-read sequencing data around the *BCAP31* gene showed that the de novo allele arose from the paternal X chromosome while the wild-type allele came from the maternal X chromosome (Figure 4). To explain the patient's *BCAP31* expression pattern and clinical features, we hypothesized that uneven XCI occurred in the patient. We performed the HUMARA assay to assess the methylation ratio of maternal and paternal X chromosome in WBCs, with the knowledge that XCI patterns between blood and other analyzed tissues were highly correlated (Echevarria et al., 2016). The androgen receptor allele profile of triplicate experiments showed skewed XCI, with the maternal X chromosome (carrying the wild-type *BCAP31* allele) inactivated in 88% ± 2% of the WBCs (Figure 5). The HUMARA study confirms that the paternal X chromosome (carrying the de novo mutation) was active in the majority of the cells and explained the patient's "X-linked recessive" phenotype.

4 | DISCUSSION

The molecular diagnosis of rare congenital disorders has changed from family studies by linkage analysis followed by positional cloning to direct whole exome or whole genome next generation sequencing.

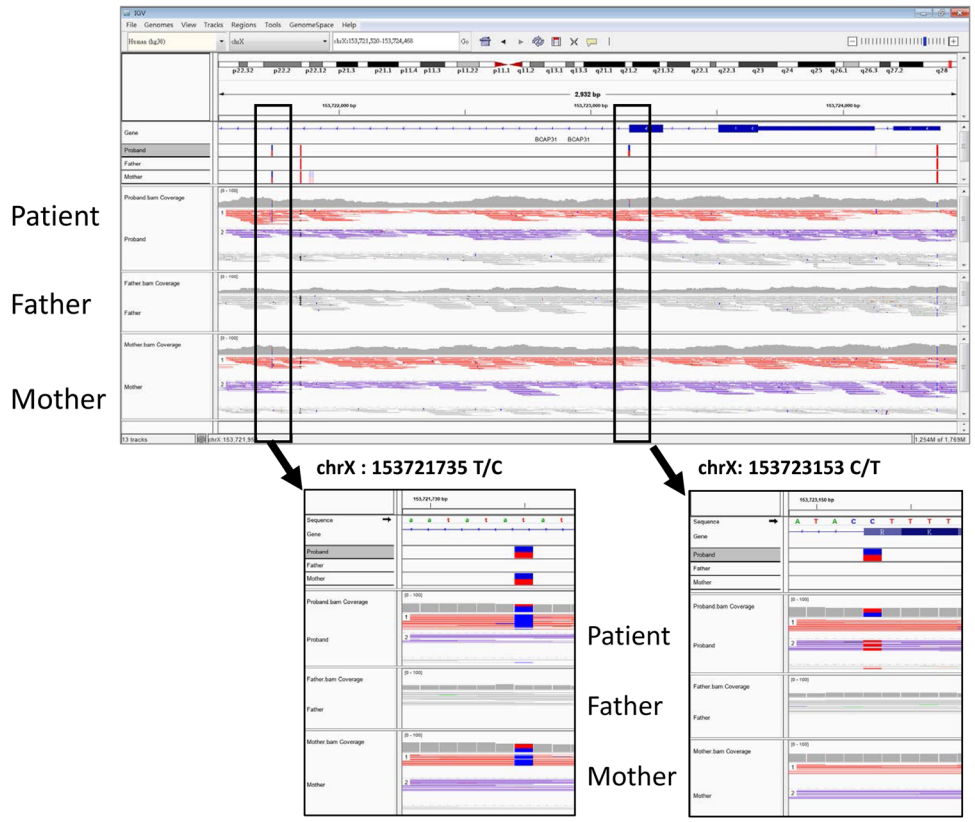

FIGURE 4 Phasing the haplotypes in trio. The trio's phased Variant Call Format and BAM Format performed with the Integrative Genomics Viewer (IGV) sorted and colored with 10× G linked-read haplotype (HP) tag (haplotype of the molecule that generated the read). The region of *BCAP31* gene in the patient and mother can be clearly phased into two strands with HP tag. Patient's Phase I (red) carried maternal chrX:153721735+C and Phase II (purple) carried the de novo chrX:153723153+T, indicating the de novo mutation in Phase II was derived from the paternal X chromosome

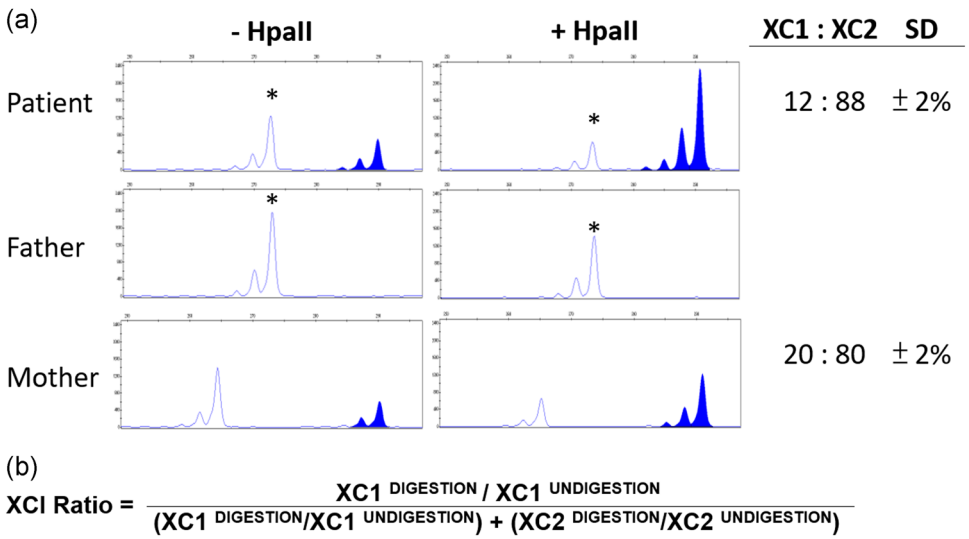

FIGURE 5 Skewed X chromosome inactivation found in the patient. (a) The allelic profiling of HUMARA (Human Androgen Receptor) PCR assay were performed with *Hpa*II digested (+*Hpa*II) or mock digested (−*Hpa*II) DNAs with 5FAM-labeled forward primer, and quantified by the Applied Biosystems 3730 Analyzer. (b) Formula of XCI ratio. XCI ratio was estimated by the area of the smaller base size (XC_1) and larger base size (XC_2) peaks. The AR profiling and XCI ratio of the patient showed the maternal chrX allele (in blue) had skewed X chromosome inactivation (XCI = 88% ± 2% *SD*). Patient's mother also showed unbalanced skewed X chromosome inactivation (XCI = 80% ± 2% *SD*). Paternal chrX allele was labeled as star (*). XCI were calculated in three independent experiments. *SD*, standard deviation

Because every person in the world carries thousands of gene mutations, a diagnosis can only be made when a mutation is consistent with both the genetic mode of inheritance and the functional changes leading to the disorder. In these "experiments of one," reliance of previously published cases of similar clinical features and gene mutations makes it challenging to diagnose cases where mutations are found in genes the functions of which are little known. Similarly, it is difficult to make a diagnosis when a deleterious mutation is found in only one copy of a gene that causes an autosomal recessive disorder.

This study illustrates a way to establish a molecular diagnosis when there are issues with the mode of inheritance or functional consequences of a candidate gene mutation. By conducting additional experiments beyond whole exome or WGS, one can determine the functional consequence of a mutation and explain the inheritance pattern of the condition. In our case, while the *BCAP31* gene is well established as one associated with DDCH and our patient has clinical features of DDCH, the particular heterozygous de novo mutation in our patient has not been reported before. Furthermore, a female would not be affected by a heterozygous mutation in a gene on the X chromosome under normal circumstances.

We conducted three sets of experiments to characterize our case. First, we exhaustively searched for mutations in the maternally inherited *BCAP31* gene by linked-read WGS and optical mapping to determine that the maternally inherited *BCAP31* region harbors no deleterious mutations. Second, we conducted an XCI assay to confirm the hypothesis that preferential X inactivation of the maternally inherited X chromosome bearing the wild-type *BCAP31* gene created a setting where the patient expresses predominately the mutated *BCAP31* gene. Third, we conducted RNA studies to show that our patient expresses predominately the mutated gene, resulting in a frameshifted transcript that skipped over Exon 2.

XCI status has been used to explain the wide spectrum of phenotypes observed (from asymptomatic to severe) in some X-linked diseases in women, depending on the degree of silencing of the normal allele (Echevarria et al., 2016; Elstein, Schachamorov, Beeri, & Altarescu, 2012; Fahim et al., 2019; Juchniewicz et al., 2018). For example, the severity scores and progression phenomena in X-linked Fabry disease caused by α-Gal deficiency had significant correlation with XCI ratio in female patients (Echevarria et al., 2016; Elstein et al., 2012; Juchniewicz et al., 2018). Given the recent observation that skewed XCI is common in the general female population (Shvetsova et al., 2019), female patients with clinical features of an X-linked recessive disorder and one copy of the mutated gene should be screened for skewed XCI.

BCAP31 has two protein isoforms translated from four transcripts that differ in the 5′-untranslated region in Exon 1. Three transcripts (NM_001139441.1, NM_001256447.2, and NM_005745.7) utilize the start codon in Exon 2 and encode a canonical *BCAP31* Isoform 1 (UniPortKB ID: P51572-1). The NM_001139457.2 transcript utilizes the start codon in Exon 1 and encodes a *BCAP31* Isoform 2 (UniPortKB ID: P51572-2). Exon 2 is conserved in all four transcripts. In our patient, the de novo mutation at the splicing donor site disrupted the mRNA structure by splicing out the entire Exon 2, resulting in a frameshift transcript utilizing a new start codon site in Exon 3. Consequently, the mutant protein is shortened and has a completely different structure, essentially rendering our patient a loss-of-function *BCAP31* null. Full genome analysis that combines linked-read sequencing and optical mapping for comprehensive single nucleotide and SV identification with phasing allows one to characterize a de novo

heterozygous deleterious mutation in a female patient with *BCAP31*-related syndrome. After determining that the de novo mutation arose in the paternal X chromosome and that the maternal X chromosome is preferentially inactivated, we conclude that the *BCAP31* mutation is the cause of the patient's disorder because RNA studies confirm that her WBCs expresses only the mutant transcript. According to the ACMG guideline, this variant is deemed "pathogenic" (PS2, PS3, PM2, PP3, and PP4) and deleterious. Our study illustrates that the combination of full genome analysis (for comprehensive variant detection and phasing), RNA studies (for alternative splicing and transcript analysis), and XCI studies for female patients with features of X-linked recessive disorders can lead to molecular diagnosis of difficult cases of monogenic disorders.

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CONFLICT OF INTERESTS

The authors declare that there are no financial or other conflict of interests.

DATA AVAILABILITY STATEMENT

The authors submitted this variant to LOVD (https://databases.lovd.nl/shared/individuals/00305878). The data that support the findings of this study are available from the corresponding author upon reasonable request.

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